PUBELES PUBLISHER

ISSN:2454-3225
Journal of Advanced Applied Scientific Research (JOAASR)

Haematological studies on freshwater Tilapia treated with ZnO nanoparticles

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Abstract

Our study was framed to evaluate the acute toxicity, behavioural and heametalogical effects of ZnO NPs on freshwater fish *Oreochromis mossambicus.* ZnO NPs characterized by FTIR and UV Spectroscopy. Various behaviour patterns were studied in 30, 50 and 70ppm ZnO NPs concentration. The actue toxicity (96hrs LC_{50}) of ZnO NPs was observed between 100-110ppm. Significantly decreased number of Total Red cell count (RBC), haemaglobin (Hb), Haematocrit (Ht) reflects changes in red blood indicies such as MCV, MCH, MCHC and oxygen carrying capacity. Decreased leucocytes and differential count differences observred in ZnO NPs exposed groups. As a novel attempt, our study showed the impact of ZnO nanoparticles in Acute toxicity and Haematological parameters of freshwater fish *O. mossambicus.*

Keywords

ZnO, XRD, LC50, RBC, WBC

1. Introduction

Nanoparticles (NPs) are particles with one or more dimesnsions in the nanoscale range of 1-100nm (Farre et al., 2009). Today, nanoscale materials used in a variety of different areas such as electronic, biomedical, pharmaceutical, cosmetic, energy, environmental, catalytic and material applications (**Guzman et al., 2006**). ZnO NPs were widely used in industrial, cosmetic and medical applications (Nohynek et al., 2007; **Nowack and Bucheli, 2007**) and act as a effective photocatalyst (Hoffmann et al., 1995). ZnO nanopowder is currently used in products including plastics, ceramics, glass, cement, rubber, lubricants, fire retardants, etc. (Ma et al., 2013) and used as a promising material for numerous applications such as gas sensors, transparent electrodes, pH sensors, biosensors, acoustic wave devices and UV photodiodes (Ahmad and Zhu, 2011).

ZnO NPs partially but relatively quickly dissolved in water, and released free zinc ions were the primary source of toxicity (Blinova et al., 2010; Buerki-Thurnherr et al., 2013; Franklin et al., 2007) or induced additional effects (Poynton et al., 2011). The solubility of nano-ZnO may play a more important role in its toxicity.The toxic action of metal and metal oxide NPs can potentially involve at least three distinct mechanisms (Brunner et al., 2006). First, particles may release toxic substances into exposure media, e.g. free Zn^{+} ions from zinc particles. Second, surface interatctions with media may produce toxic substances, e.g. chemical radiacals or reactive oxygen species. Third, particle or their surfaces may interact directly with, and disrupt biological targets, e.g. carbon nanotube interaction with membranes or intercalation with DNA (Ma et al., 2013).

Ferry et al. (2009) first reported that NPs could pass from the water column to the aquatic food web and acumulated by the organisms through internal or external exposure routes and it is neccessary to understand the biological behavior of nZnO in the aquatic environment (Lee et al., 2010). Because ZnO NPs partially dissolve in water exposures in aquatic systems are expected to involve both soluble and particulate species, suggesting that these three mechanisms of toxic action were tenable for ZnO. Solubilized Zn^{+} from ZnO NPs has proven to contribute substantially to the cytotoxicity of these NPs (Brunner et al., 2006; Heinlaan et al., 2008).

Fishes acts as the ideal sentinels for toxic chemical exposure due to their constant and direct contact with the aquatic environment (**Little et al., 1993a**) and susceptible to any alteration in the physico-chemical characteristics of the habitat (**Sadiq Bukhari et al., 2012**). Haematological studies furnishes an index of physiological changes in fish (Adhikari et al., 2004; Suvetha et al., 2010) and the fish blood acts an impressive tool for detection of alterations in the tested organism (**Rambhaskar and Rao, 1987**; **Sancho et al., 2000; Adhikari et al., 2004).** The most common hematological variables measured during stress included Red and White blood cells count, hemoglobin content, and hematocrit value and red blood cells indices (Ololade and Oginni, 2010).

The changes in hematological (RBC, Hb, Hct, MCV, MCH and MCHC) parameters were greatly used to evaluate the toxic stress of the fishes (**Romani et al., 2003**; **Barcellos et al., 2004**; **Kavitha et al., 2010**). Total leucocyte count (TLC) and differential leucocyte counts (DLC) which occupies an important role in fish studies (**Blaxhall and Daisley, 1973**). DLC were classified into granular and agranular cells. Lymphocytes were regarded as immunocompetent cells (**Ellis, 1981**). Increased leucocytes (leucocytosis) count is a normal reaction of the fish

body, against infections of foreign substances, which can alter the normal physiological processes in fish (**Gail et al., 1995**).

A limited number of nanoparticle studies have been done with fish as a model organism (**Karthikeyeni et al., 2013**). Particularly, the toxicity tests on adult fish have mainly focused on carbon-based NPs (**Oberdörster, 2004**; **Smith et al., 2007**; **Zhu et al., 2006**). Moreover, the studies about toxicity of metal oxide NPs (TiO_2, ZnO NPs) on fishes have concentrated the early developmental stages (**Zhu et al., 2008, 2009**) and enzymatic studies. Being a novel attempt, this study was framed to evaluate the acute toxicity and haematological effects of ZnO NPs on adult tilapia (*O. mossambicus*) fish.

2. Materials and methods

2.1. Experimental animals and ZnO NPs suspension preparation

The freshwater fish *Oreochromis mossambicus* of both sexes (♂:♀) were collected from Cauvery River (lat. 10º 51´ and long. 70º 30´) in Tiruchirappalli district, Tamil Nadu (South India). Fishes were acclimatized in Environmental Research laboratory (Jamal Mohamed College, Tiruchirappalli) and the water parameters were maintained (**APHA, 1998)**. Fish food pellets were provided ad libitum (Affonso et al. 2002). ZnO nanoparticles were characterized and sonicated for 30 min in a bathtype sonicator (100W, 40KHz) to disperse the particles **(Wang et al., 2011; Karthigarani and Navaraj, 2012**). The sonicated nanoparticles exposed to the fish groups in respective concentrations and control group maintained separately.

2.2. Acute lethal assay

Acclimatized fishes were separated into groups and exposed to different concentrations of sonicated ZnO nanoparticles for acute lethal concentration studies. Feeding was stopped 24

hours before the commencement of the test (Punitha et al., 2014) and during experimental period to avoid absorption of NPs by food or fecal materials (**Karthigarani and Navaraj, 2012**). The number of dead fish was recorded every 12h, and removed immediately to avoid contamination of the exposure solutions.

2.3. Haematological analysis

After 96 hours, blood were collected from fishes (from each group) by cardinal vein puncture technique using an insulin syringe containing 0.1ml of 0.2% EDTA (**Remya 2010**). RBC and WBC were counted with a Neubauer haemacytometer with RBC and WBC diluting fluids (**Rusia and Sood, 1992**) respectively. The red blood indices such as haemoglobin (Hb), haemtocrit (Hct or Ht) or packed cell volume (PCV), mean cellular volume (MCV), mean cell haemoglobin (MCH) and mean cellular haemoglobin concentration (MCHC) were calculated by standard formulas (**Kang et al., 2005**). Oxygen carrying capacity was calculated by multiplying the haemoglobin content with 1.25 oxygen combining power of Hb/g (**Johansen, 1970; Sampath et al., 1998**). Differential Leukocytes (Neutrophils, Lymphocytes, Monocytes) were counted using Leishman stain method (**Mukherjee and Ghosh, 2012**).

$$MCV = \frac{\text{Ht \%}}{\text{Total RBC count}} \times 10$$

$$MCH = \frac{\text{Hb g\%}}{\text{Total RBC count}} \times 10$$

$$MCHC = \frac{MCH}{MCV} \times 100$$

2.4. Statistical analysis

All exposure experiments were repeated three times independently, and data were recorded as the mean with standard deviation (SD), probit analysis, bar and line graph diagrams using the SPSS software package for windows (version 17.0)

3. Results

3.1. Acute lethal assay

The acute toxicity of ZnO NPs to *O. mossambicus* increased with particle concentration, demonstrating a dose dependency. The observations were done manually and recorded. By using probit analysis, the 50% of the fish mortality were observed between 100 – 110ppm ZnO nanoparticles (Figure 1) in three replicates.

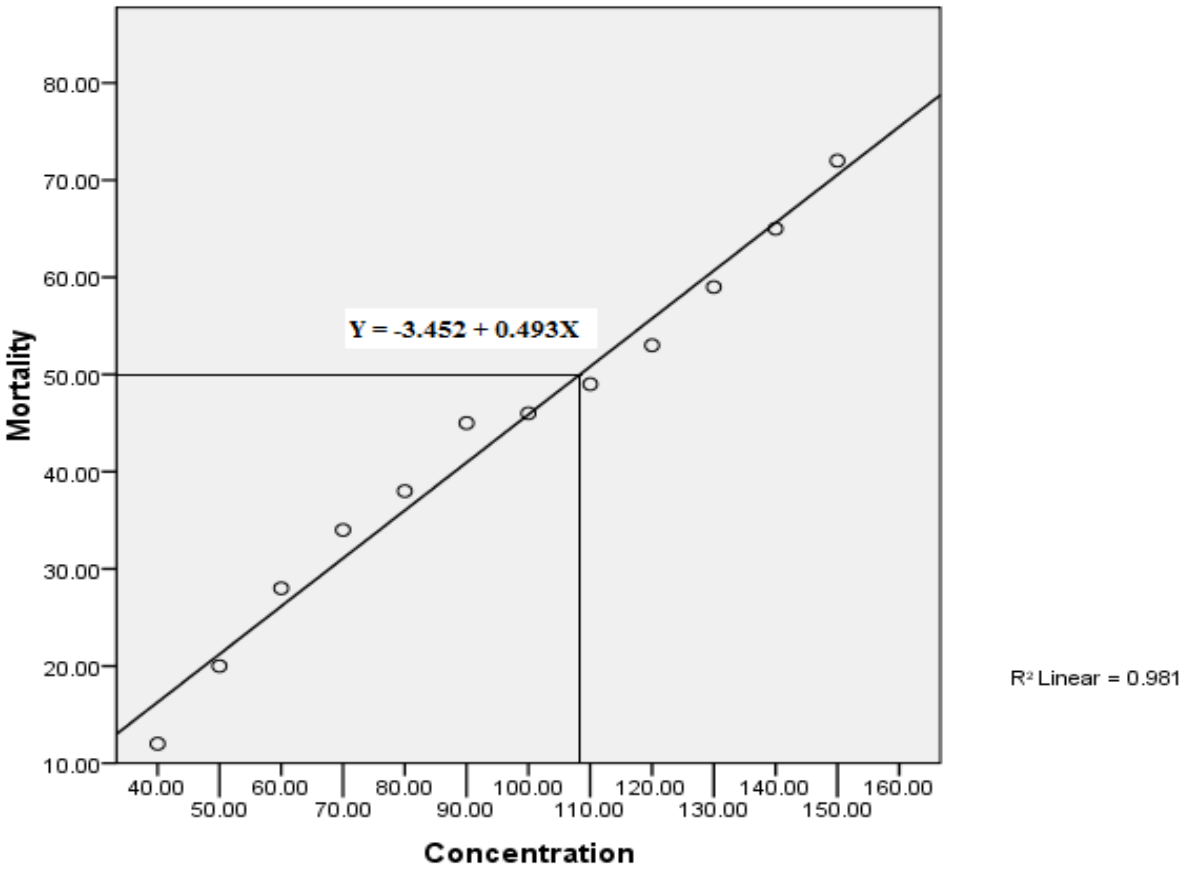

Figure 1. Probit analysis Graph showing LC50 of ZnO NPs in *Oreochromis mossambicus*

3.2. FTIR

The FT-IR spectra of the prepared ZnO NPs are shown in Figure 2. The FT-IR measurements of ZnO samples are performed using the KBr pallet method in the wave number range 400-4000 cm^{-1}. The broad absorption in the frequency band 3750-3000 cm^{-1} were assigned to O-H stretching from residual alcohols, water and Zn-OH. The absorption peaks observed at 3446 cm^{-1} for ZnO NPs. The CO_2 peaks observed at 2374 cm^{-1} for ZnO NPs. These CO_2 band may arise due to some trapped CO_2 in air ambience. The H-OH bending vibration bands around 1517cm^{-1} for ZnO samples, which assigned to a small amount of H_2O in the both samples. The most intense broad absorption band at ~ 438 cm^{-1} is attributed to the stretching of vibration in ZnO. The Zn-O stretching bands at 422 cm^{-1} for ZnO NPs.

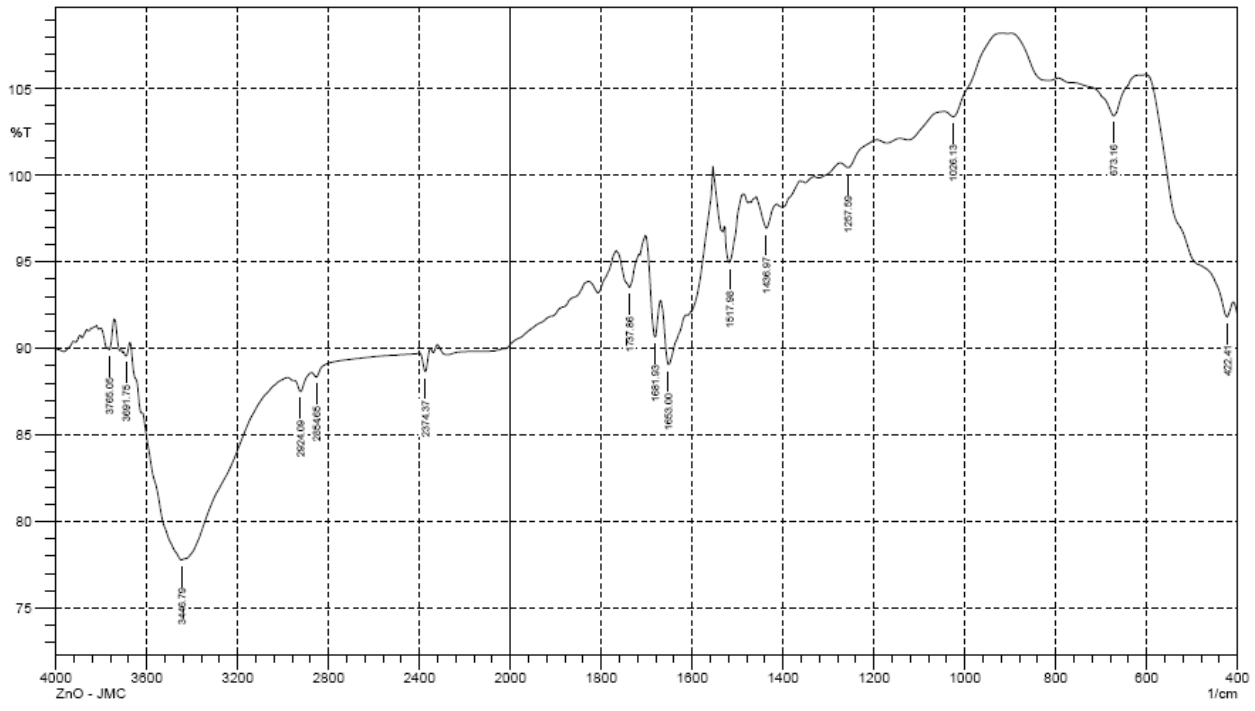

Figure 2. FTIR Spectrum of ZnO nanoparticles

3.3. UV-visible Spectroscopy studies

Figure 3 shows the UV-visible absorption spectrum of ZnO nanoparticles. The ZnO samples absorption spectrum sharp peaks at 382 nm were observed, which believe to arise from the near band edge free excitons. The ZnO NPs are expected to show a small red-shift in comparison to bulk ZnO. The band gap energies E_g, of ZnO NPs were found 3.2 eV. Showed the small 'red shift' of 0.17 eV from standard bulk band gap at room temperature (E_g = 3.37 eV).

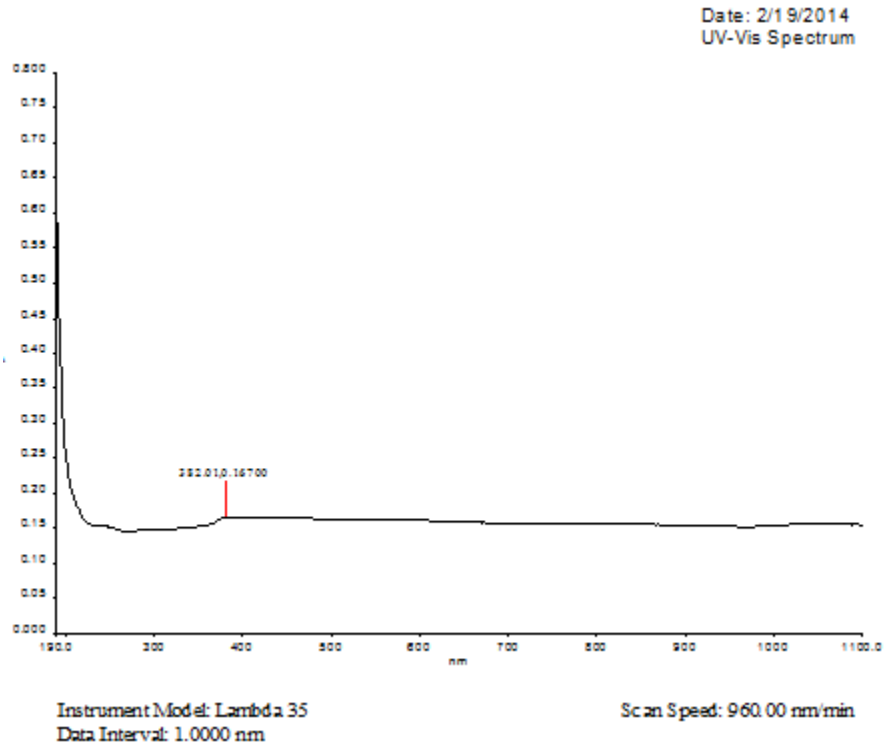

Figure 3. UV-Vis spectrum of ZnO nanoparticles

3.4. Haematological parameters

The total red blood cell (RBC) count in all treated groups (30, 50 and 70ppm) were reduced due to the disruption of blood cells i.e. haemolysis (Table 1) which reflected in the changes of red blood indices such as Hb, Hct, PCV, MCV, MCH, MCHC when compared to control group fishes (Figure 4 & 5). Changes in the red blood indices clearly indicated the abnormalities of blood tissue composition. Oxygen carrying capacity of treated fishes were gradually reduced which intrudes the oxygen supply to various organs which cause dreadful condition.

Agranular leucocytes included lymphocytes and monocytes which display cytoplasm without a granular appearance and Monocytes were round cells, larger than the other leucocytes in the peripheral blood were counted. Granular leucocyte - neutrophils were round or oval in shapes in blood smear (Table 2) were observed. Decreased leucocyte (leucopenia) and differences in neutrophils, lymphocytes and monocytes were observed in treated groups than control group (Figure 6 & 7).

Parameters	Control	30 ppm	50 ppm	70 ppm
Haemoglobin level (g/dL)	10.0±0.50	8.6±0.30	7.6±0.31	6.3±0.26
Total RBC count (x10^{6}cells/µl)	7.25±0.23	6.05±0.34	5.65±0.24	5.35±0.15
Haematocrit (%)	22.0±2.4	18.3±2.2	16.4±1.4	12.0±1.8
MCV (fl)	30.30±0.15	30.24±0.25	29.02±0.19	22.42±0.18
MCH (pg)	13.79±0.34	14.21±0.25	13.45±0.21	11.7±0.30
MCHC (g/dL)	219.72±11.2	212.80±14.6	215.76±16.3	191.62±13.5
Oxygen Carrying Capacity of blood (mlO_2 g^{-1}Hb)	12.5±0.02	10.75±0.08	9.50±0.07	7.87±0.04

Table 1 .Total Red Blood Count and the red blood indices in control and ZnO exposed groups (n=10).

Experimental groups	Total WBC count (cells/ cu. mm)	Differential leukocyte count (%)		
		Neutrophils	Lymphocytes	Monocytes
Control	4205	40±0.06	52±0.03	7±0.05
30 ppm	2316	46±0.08	50±0.06	3±0.06
50 ppm	1943	56±0.05	38±0.04	5±0.02
70 ppm	1380	67±0.09	25±0.06	6±0.05

Table 2. Total White Blood Count and Differential leukocyte count in control and ZnO exposed groups (n=10).

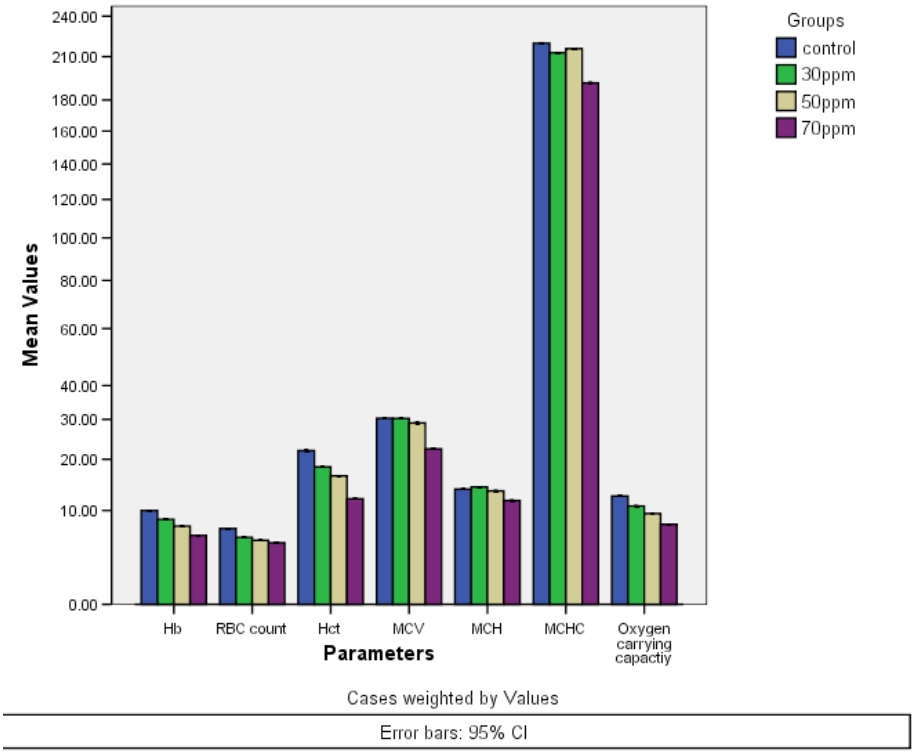

Figure 4. Total RBC count and Red blood indices - Hematocrit (Hct), hemoglobin concentration (Hb), mean cell volume (MCV), cell hemoglobin (MCH), cell hemoglobin concentration (MCHC) of control and ZnO NP exposed groups

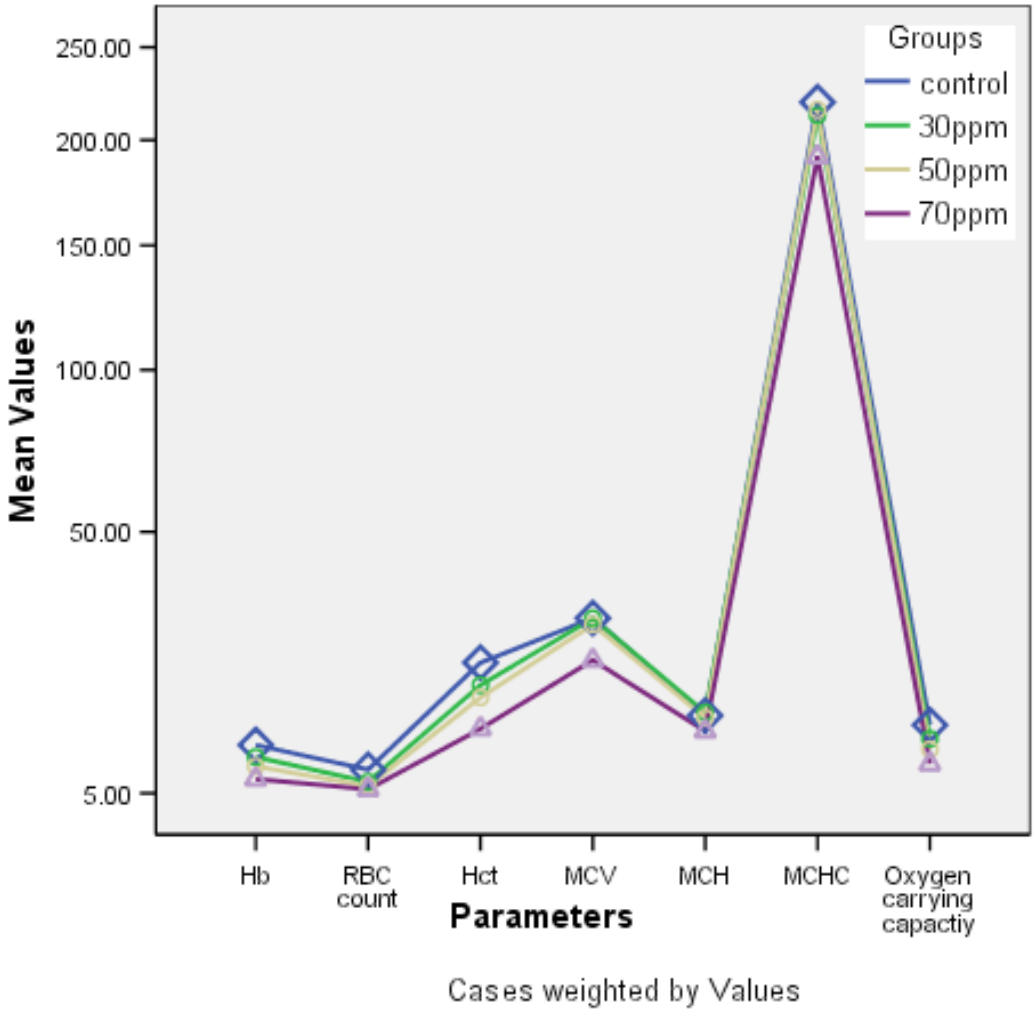

Figure 5. Line graph of Total RBC count and Red blood indices - Hematocrit (Hct), hemoglobin concentration (Hb), mean cell volume (MCV), cell hemoglobin (MCH), cell hemoglobin concentration (MCHC) of control and ZnO NP exposed groups

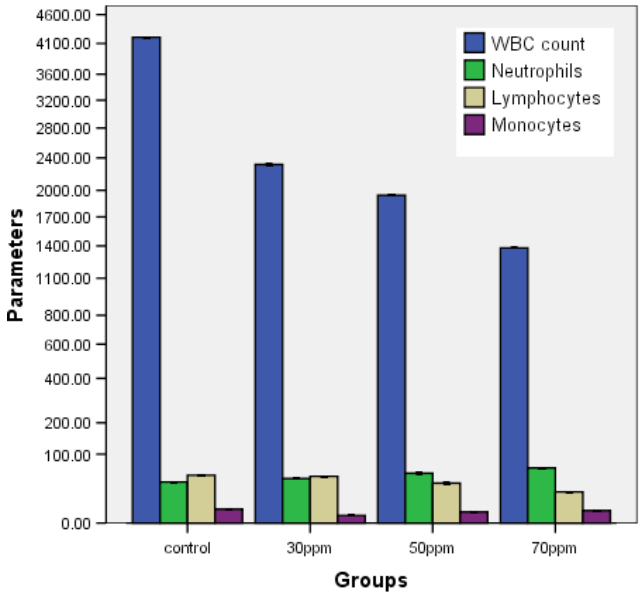

Figure 6. Total White Blood Count and Differential count (neutrophils, lymphocytes and monocytes) in control and ZnO NP exposed groups

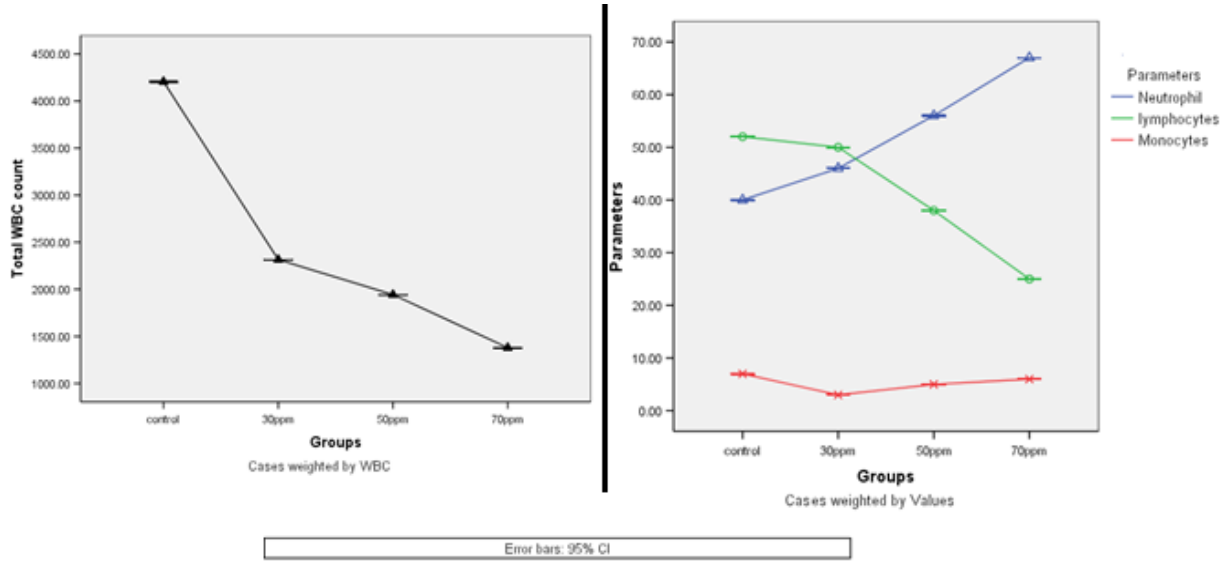

Figure 7. Line graph of Total White Blood Count and Differential count (neutrophils, lymphocytes and monocytes) in control and ZnO NPs exposed groups

4. Discussion

Similar characterization studies results observed by various researchers (Oo et al., 2005, Xinog et al. 2007, Yogamalar et al., 2008). The mean lethality of *O. mossambicus* were 30% in 100ppm concentration, 10% in 90ppm concentration in oral administration of ZnO NPs (Amutha and Subramanian, 2009). Acute toxicity (96h LC50) of ZnO NPs to adult zebrafish reported as 3.97mg/l with primary particle size of 30nm (Yu et al. 2011). In carp (*Cyprinus carpio*), upto 50mg/l of ZnO NP was not lethal to the fish, but caused significant oxidative stress (Hao and Chen, 2012). AL-Taee and AL-Hamdani (2013) identified the acute toxicity (LC_{50}) of N-ZnO in *Cyprinus carpo* was 30ppm for 24 hrs.

In toxicological studies, the most common hematological observations were reduced RBC count, haemoglobin concentration and haematocrit (Ht) (**Aliakbar and Zahra, 2013**). **Panigrahi and Misra (1987)** evidenced reduced hemoglobin and red blood cell count in fish *Anabas scandens* treated with mercury. Reduced RBC, Ht and Hb were reported in *Tinca tinca* exposed to lead and mercuric chloride (Shah and Altindag 2004). The increased MCH and MCHC values of the *Cyprinus carpio* blood, conforms the disturbances in erythrocyte count, due to the destruction of red blood cells and reduced Hb content in each cell (Sakthivel and Sampath, 1990).

Fish exposed to mercury (96h) exhibited a significant change in haematological parameters (RBC, Hb, Ht, MCH, MCHC, MCV) than control group ($p<0.05$), whereas among significant indices MCV, RBC, Ht, Hb in fish exposed were significantly lower ($p<0.05$) and MCH, MCHC were significantly ($p< 0.05$) greater compared to the control groups (**Aliakbar and Zahra, 2013**). The decreased haemoglobin concentration represents the reduced supply of adequate oxygen to the tissues and resulted in decline of physical activities (**Nussey et al., 1995**).

James and Sampath (1995) found that the oxygen carrying capacity of blood of Heteropneustes fossilis declined due to the reduction of RBC count and Hb content which reflected on tissue respiration.

Oxygen carrying capacity of blood was declined in metal-exposed *O. mossambicus* due to the reduction of RBC count and Hb content (**Sampath et al., 1998**). The pollutants were entering into fish via gills which were continuously exposed to ambient waters. This causes hindrance to O_2 absorption throuh gill surface (Christine and Gokhale, 2000) which was reflected in the reduced O_2 carrying capacity of blood. Similar results were observed in our work as decreased RBC & Hb concentration and as a result there was a reduced O_2 carrying capacity in ZnO nanoparticle exposed fishes.

White blood cells play a major role in the defence mechanism of the fish and consist of granulocytes, monocytes, lymphocytes and thrombocytes. Granulocytes and monocytes functions as phagocytes to salvage debris from injured tissue and lymphocytes produce antibodies (Ellis et al. 1978; Wedmeyer and Mcleay, 1981; Maheswaran et al. 2008). Leucocyte subpopulations were known to fluctuate in response to a variety of environmental stimuli (**Johansson-Sjobeck and Larsson, 1978; 1979**). In *O. mossambicus*, the monocytes and neutrophils were reduced in circulation for the elevation of phagocytic activity in affected tissues such as gills, liver and kidneys which were damaged by copper (**Wepener, 1990; Gey van Pittius, 1991; Van der Merwe, 1992**) while ZnO NP treated fishes showed increased moncytes and neutrophilic conditions. Our studies showed reduced leucocyte count in all treated groups. The white blood cells leave the circulating blood, to protect the body, by moving (ameboid movements) to the infected tissue (**Wepener, 1990)** resulted in reduced cell number in blood which was similar to our results. Decreased Hct and Hb values coupled with decreased

erythrocytes count were evidenced in *Clarias batradrus* exposed to mercuric chloride (Maheswaran et al. 2008).

Reduced erythrocyte count and lower Hct and Hb levels were observed due to severe anemia. Decrease of Hct due to the destruction of erythroblast (Zorriehzahra et al., 2010). Similar trends in erythrocytes in fishes exposed to various toxicants and pathogens have been observed by various researchers (Mc Leay and Brown, 1975; Smit et al., 1979; Koyama and Ozaki, 1984; Srivastava and Narain, 1985; Van der Merwe, 1992). Decreased Hb and MCV provides an indication of the status or size of the erythrocytes and reflects an abnormal or normal cell division during erythropoiesis (Zorriehzahra et al., 2010) indicates that the erythrocytes have shrunk, either due to hypoxia or a microcytic anamia.

4. CONCLUSION

Our study provens that ZnO NP exposure (even in ppm conc.) ultimately disturbs the survival of the freshwater fish, *Oreochromis mossambicus*. Due to the invasion of ZnO NP through gill or external body surface (by diffusion), the number of RBC cells distrubed/reduced by hemolysis, which leads to significant reduction in red blood indices i.e. involved in various physiological activities of the fish. Fish immune system gets triggered, as a result the WBC and differential leucocyte cells leave blood and entered into the affected regions, which reflects in the reduced number of cells in blood. Presence of Micronuclei indicates that ZnO NP cause chromosomal damages during cell division in fish *O. mossambicus*.

5. ACKNOWLEDGEMENT

Authors were thankful to Dr. A.K. Khaja Nazeemudeen Sahib, Secretary and Correspondent, Dr. S. Mohamed Salique, Principal and Dr. Mohamed Shamsudin, Dean of Science & Head, P.G. and Research Department of Zoology, Jamal Mohamed College (Autonomous), Tiruchirappalli for Institutional support. We also thank Bhabha Atomic Research Centre, Department of Atomic Energy, Mumbai for financial support.

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