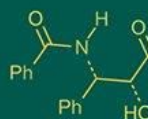


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Effects of zinc oxide nanoparticles on haematological parameters of *Cirrhinus mrigala*

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Abstract

Zinc is a trace element crucial for growth, immunity, antioxidant defence mechanisms, and the co-factor of over 300 enzymes. It should be included in fish diets. This study aimed to assess the effectiveness of nano zinc on *Cirrhinus mrigala*'s haematological system. For eight weeks, this experiment was conducted in a lab setting. Five experimental setups in triplicate were used to stock uniform-size advanced mrigal fry, and each tank was assigned fifteen fish. Each tank was filled with 162L of tap water and an aeration system. The fish were given two different diets: a basic diet (control) and a treatment diet enriched with zinc oxide at 30 mg/kg (T₁) and nanoparticles of zinc oxide at 30, 15, and 7.5 mg/kg (T₂, T₃, and T₄), respectively. The fish were fed twice a day at satiation level. Once the experiment is over, the haematological parameters are examined. Following a 56-day feeding study, *Cirrhinus mrigala*'s haematological alterations revealed substantial ($p < 0.05$) differences between the control and other treatments. Blood parameter analysis, such as haemoglobin, RBC, WBC, PCV, MCV, MCH, and MCHC counts, is also used to evaluate haematological performance. WBC count dropped in treatment compared to control, whereas Hb, RBC, PCV, and platelet counts increased. From the result analysis, it is possible to conclude that the mrigal haematological parameter performed better and significantly ($p < 0.05$) when fed 15 mg/kg of nano ZnO supplemental feed, which can replace the control diet to improve mrigal fish health and production performance.

Keywords: Mrigal, *Cirrhinus mrigala*, ZnO, nanoparticles, haematology

Introduction

Zinc is the second most prevalent trace element found in the animal body, following iron. The body can't store it, but it can be regularly consumed as food to meet the body's physiological needs (Zalewski *et al.*, 2005) [38]. Numerous functions in animals depend on it, including growth, metabolism, immunological response, wound healing, fertilization, and the maintenance of oxidative stress (Kietzmann and Braun, 2006; Feng *et al.*, 2010; Liu *et al.*, 2011; Zhao *et al.*, 2014) [18, 12, 21, 39]. In addition, zinc is an essential trace element for fish and is required for numerous biological and physiological functions, such as the production of hormones, the activity of enzymes, and the metabolism of proteins, carbohydrates, and fats (Muralisankar *et al.*, 2014) [24]. It is essential for fish growth, metabolism, and immune system function, and to stop free oxygen radicals (ROS) or reactive oxygen species (ROS) from activating (NRC, 2011) [25]. It is necessary for almost a thousand structural, catalytic, and regulatory proteins, all of which are essential for the physiology, growth, and development of animals (Eide, 2006; Maret and Krezel, 2007) [10, 22]. The majority of zinc absorption in freshwater fish comes from dietary sources, while they can also absorb zinc from water. Despite this, the gills showed their importance in the uptake of this mineral in controlled environments with high water-borne or low dietary zinc levels (Spry *et al.*, 1988) [34]. According to Hogstrand *et al.* (1998) [14], a calcium-mediated process in freshwater fish mainly employs the gills to absorb zinc from the water, and over 300 enzymes and vitamins require zinc as a co-factor (Sau *et al.*, 2004; Sau and Paul, 2006; Karjee *et al.*, 2023) [31, 17].

Nanotechnology, whose name comes from the Latin word "nanus," which means "dwarf," is the study of materials and structures whose sizes are measured in nanometers. The word "nano" more accurately refers to 10^{-9} or one billionth of a meter. Materials between 1 and 100 nm in size are referred as nanomaterials (Rai and Ingle, 2012) [28]. Nanotechnology is a very viable technology that has various scientific and technological applications.

Recent advances in nanotechnology and nanoscience have opened up new avenues for a variety of consumer and industrial sectors, including agriculture and allied fields that are considered to be the cradle of a new revolution. One of the most recent scientific discoveries, nanoparticles are quickly emerging as the scientific and technological foundation for the next stage of the development and transformation of the agri-food system as well as for improving the lives of the poor.

According to Birntha and Ajitha (2015) [5], nanoparticles (NP) hold great potential for lowering infection rates, boosting immunity, and encouraging aquaculture growth. Zinc oxide, one of the many metal oxide nanoparticles, is essential to the field of nanotechnology because it is more specific than other metal oxide micro and nanoparticles. Zinc oxide is an inexpensive material that has numerous applications, including the production of nanostructured thin films. It is also given to water and food as a vitamin to support plankton production and as a source of zinc for fish growth. One potential tactic to replace both organic and inorganic Zn sources is to use nanotechnology to produce nanoscale Zn as ZnO, or nano ZnO (nZnO). According to Sahoo *et al.* (2014) [30], use nZnO has been shown better yield results and be less hazardous than utilizing conventional Zn sources.

The most widely used nanomaterials (NMs) are zinc oxide nanoparticles (ZnO NPs). Due to its many beneficial applications, such as photocatalysis, anticancer, and antibacterial properties, as well as their ecologically favourable qualities, zinc oxide nanoparticles (NPs) have garnered significant attention (Jiang *et al.*, 2018) [16]. According to Piccinno *et al.* (2012) [27], nanozinc oxide (nZnO) is the third most produced metal nanoparticle (NP) in the world, after SiO₂ and TiO₂. Zinc oxide nanoparticles (ZnO NP) have better antibacterial capabilities than regular zinc oxide, which is primarily responsible for the unexpected increase in demand for them (Padmavathy and Vijayaraghavan, 2008) [26]. It has been demonstrated that ZnO NP affects livestock and poultry growth performance in a dose-dependent manner (Hongfu, 2008; Yang and Sun, 2006; Lina *et al.*, 2009; Mishra *et al.*, 2014; Sahoo *et al.*, 2014) [30, 15, 37, 20, 23], it also suppresses piglet diarrhea rates, acting as an immune-modulating and antibacterial agent (Hongfu, 2008) [15]. But there is less information about the effect of zinc on haematology of mrigal. Thus, the goal of this study was to evaluate the effects of nano ZnO and graded levels of dietary ZnO on the haematological parameters of mrigal (*Cirrhinus mrigala*).

Materials and Methods

The research was carried out with 15 tanks of 243 litre capacity. The tanks were cleaned thoroughly using scrub and then sun-dried for three days. Tanks are placed in triplicate and then they were filled with tap water up to 162 liters maintaining 30 cm height and providing sufficient aeration throughout the experiment.

Stocking of experimental fish

Healthy and uniform mrigal (*Cirrhinus mrigala*) fingerlings irrespective of sex were used for all the experiments. Three hundred (300) fish were collected from Naihati fish seed hatchery, Kolkata, West Bengal. Fish were brought to the laboratory with proper oxygen packaging and on reaching the laboratory, the experimental fishes were disinfected by

immersion in 0.02 ppm potassium permanganate (KMnO₄) solution for 10 min, then released into the glass tanks. Keeping them unfed for 24 hours, then they are fed with sinking pelleted control feed (28.37% protein) for 7 days @ 3% body weight with proper aeration facilities where they got acclimatized fully before experimentation. Chlorine-free aerated tap water was used throughout the experiment. Faecal matters were siphoned out and one third of the water exchange was done with fresh water on every alternative day. During the acclimatization period, the fish showed no abnormalities such as lethargy, opercular flaring, abrupt swimming, etc.

Experimental design

Two hundred twenty-five (225) fish with average body weight (3.63±0.01 g) were allotted into five experimental groups. Each group is subdivided into three replicates and each replicate comprises fifteen (15) fish selected at random. Each fish's body weight was measured, and the mean body weight of the experimental groups was maintained identically throughout the allocation process.

Fish and Rearing Conditions

Following acclimatization, 225 fish with an average body weight of (3.63±0.01 g) were stocked in 15 aquaria tanks in triplicate, at a density of 15 fish per tank. Each fish's body weight was measured, and the mean body weight of the experimental groups remained constant ($p>0.05$) during the allocation period. Fish were kept from jumping out of the tanks by covering them with nylon netting as well as to avoid insects and dust introduction. Feeding was done twice a day (9 AM and 4 PM) at satiation level. The excess feed and faecal matters were siphoned daily before and after feeding. The physicochemical characteristics of the water were measured periodically to maintain the optimal level throughout the experiment.

Preparation of feed

The raw materials are chosen according to their capacity to provide nutrients. As a protein source, groundnut oil cake and soybean meal were utilized; using wheat flour as a source of carbohydrates; as fat sources, fish oil and sunflower oils were employed. The different supplements were also added to make the feed balanced. The ingredients used for feed preparation were dried, powdered, and sieved through a 425-micron sieve. After that, the main ingredients' protein contents were determined using the Micro-Kjeldahl method, and then the ingredients were weighed and carefully combined with 130–150 ml of distilled water. The mixed feedstuff was autoclaved for 15 minutes at 100 °C and afterward cooled.

After cooling, fish oil, sunflower oil, lecithin, vitamin mixtures and binders were mixed with the ingredients, and different concentrations of zinc oxide nanoparticles were added as required in the treatment feed. The control feed was prepared without any zinc source. The treatment feeds were prepared with standard ingredients along with normal zinc of 30 mg/kg (T₁), nano zinc 30 mg/kg (T₂), nano zinc 15 mg/kg (T₃), and nano zinc 7.5 mg/kg (T₄). With the help of a hand pelletizer machine, the pellets were prepared and dried at room temperature. The prepared feed was packed in an airtight container at -20 °C until used to prevent contamination.

Haematological analysis

Blood smear preparation, fixation, and staining

Preparation of blood smear

One drop of blood was placed on one end of a chemically cleaned slide. A second slide was positioned roughly in the middle of the first slide, at a 45° angle. The second slide was brought back against the blood sample until it spread by capillary action accompanied by the interface between the slides. After moving the slide in the opposite direction, a thin, even layer of blood formed on it, and it was left to air dry.

Fixation

When the smear was dry, it was placed into a Coplin jar filled with 95% methanol for at least 5 min for fixation of the cells on the slide and stored in a dust-free condition until staining.

Staining

The methanol-fixed slides were then stained by the May Grunwald Giemsa method. The undiluted Giemsa stain was poured on the slide and allowed to stain for 5 min. After that, phosphate buffer was used to wash the slides, and they were let to dry.

Erythrocytes Count

A hemocytometer was used to count the erythrocytes. Neubauer's counting chamber was used to count the cells (Behera *et al.*, 2014) [12]. The following formula was then used to determine the total number of erythrocytes:

$$\text{No of RBCs} = \frac{\text{No. of erythrocytes} \times \text{dilution factor}}{\text{Area counted} \times \text{depth of fluid}}$$

(million/cu mm of blood)

$$\text{Dilution} = 200 \text{ area counted} = 5 \times 0.04 = 0.2 \text{ mm}^2$$

Estimation of Haemoglobin (Hb)

Using Drabkin's fluid and the cyano-methaemoglobin method, the haemoglobin level in blood was determined (Drabkin, 1950) [19]. The following formula was then used to determine the haemoglobin content:

$$\text{Haemoglobin (g/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of standard}} \times \text{concentration of standard}$$

Haematocrit

Using a RM 12 °C micro-centrifuge and a micro-hematocrit reader, the hematocrit was calculated using the micro-hematocrit (capillary) technique as described by Dacie and Lewis (Dacie and Lewis, 1991) [17].

Mean Corpuscular Volume

The average volume of red blood cells, or mean cell volume (MCV), is determined from the haematocrit (HCT) and the red blood cell count (RBC), (Stoskopf, 1993) [35].

$$\text{MCV} = \frac{\text{HCT}(\%)}{\text{RBC count} \left(\frac{\text{millions}}{\text{mm}^3} \right)} \times 10$$

Mean Corpuscular Haemoglobin

Mean corpuscular haemoglobin (MCH) measures the average amount of haemoglobin in each red blood cell.

According to Drabkin (1950) [19], it is computed using the red blood cell count and haemoglobin concentration.

$$\text{MCH (pg)} = \frac{\text{hb} \left(\frac{\text{g}}{\text{dL}} \right)}{\text{RBC count} \left(\frac{\text{million}}{\text{mm}^3} \right)} \times 10$$

Mean Corpuscular Haemoglobin Concentration

The average amount of haemoglobin in a specific volume of packed red blood cells is known as the mean cell haemoglobin concentration, or MCHC. The hematocrit and haemoglobin concentration are used to estimate it (Stoskopf, 1993) [35].

$$\text{MCHC (g/dL)} = \frac{\text{Hb} \left(\frac{\text{g}}{\text{dL}} \right)}{\text{HCT}(\%)} \times 100$$

Leucocyte Count

The hemocytometer was used to count the leucocytes. A Neubauer's counting chamber was utilized to conduct cell counts (Behera *et al.*, 2014) [12]. We next used the following formula to get the total number of leucocytes:

$$\text{No of WBCs} = \frac{\text{Number of leucocytes} \times \text{dilution factor}}{\text{Area counted} \times \text{depth of fluid}}$$

(1000/cu mm of blood) Dilution = 20,

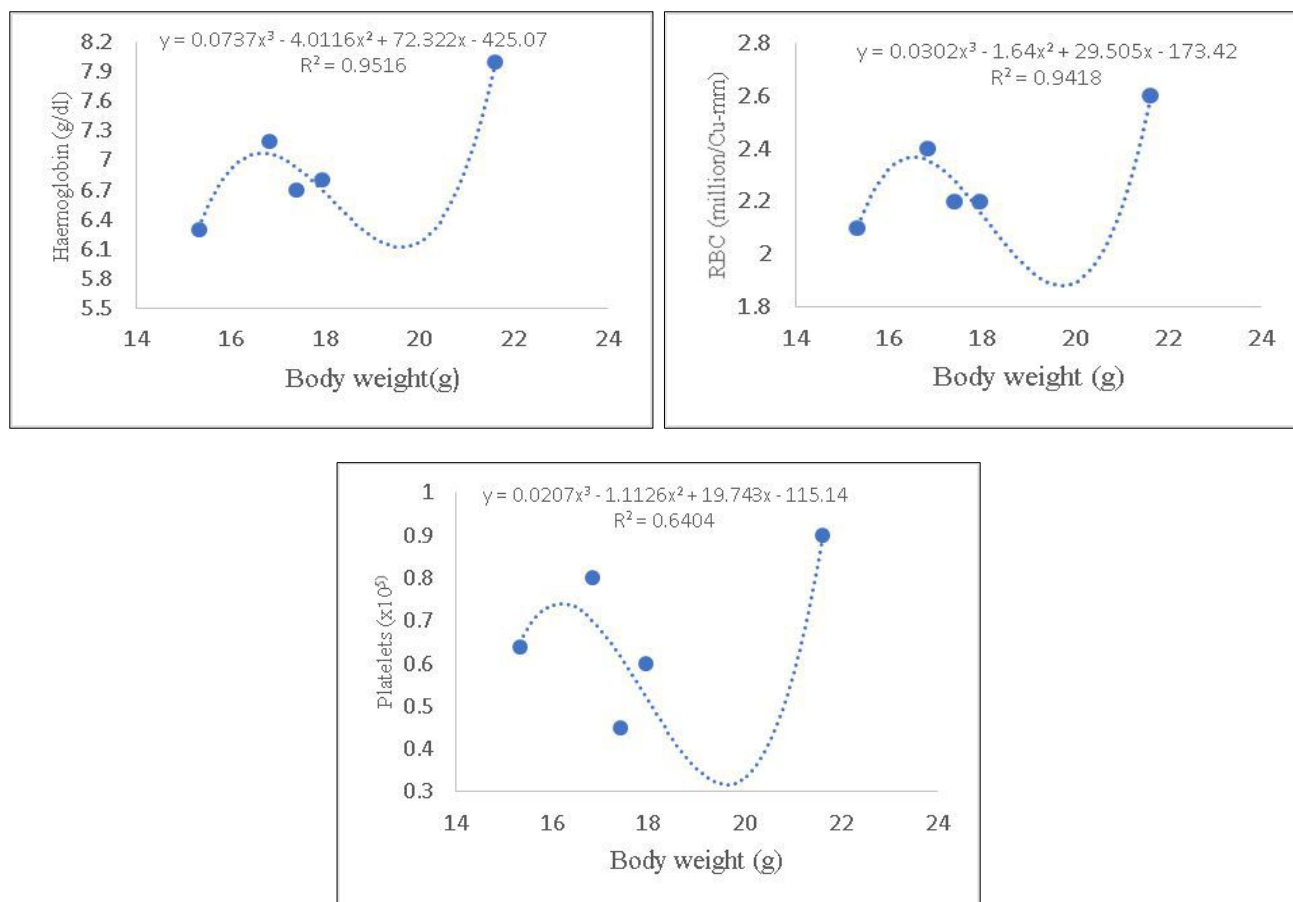
Area counted = $4 \times 1 = 4 \text{ mm}^2$ and Depth of fluid = 0.1 mm

Results and Discussion

In the present study, blood parameters are shown with both increased and decreased value in effect of nZnO against control. In comparison to the control, haemoglobin, RBC, platelets count, and PCV all exhibited higher values and these parameters showed the highest value in T₃ (8.0±0.11g/dl). A significant difference in haemoglobin content was found in treatments than control groups. Although there was no significant difference found between T₁ and T₂ for haemoglobin. But WBC was decreased in the treatment tank than control tank. The lowest WBC content was found in T₂. However, there was no apparent difference between T₁ and control. Some of the parameters were remain minor changes like monocytes, eosinophils, MCV, MCH, MCHC counts. The T₃ treatment (nZnO 15 mg/kg) exhibited progressively better results, compared to all other treatments and the control tank (Table 1). From the figure 1, it is clear that there is strong relationship of RBC and haemoglobin content with body weight as R² is nearer to 1. But there is moderate relationship of platelets count with body weight (R² =0.6404). The haematological analysis is a quick and affordable way to evaluate fish metal oxide toxicity (Rajan and Rohini, 2021; Bera *et al.*, 2022) [29, 3, 4]. According to Shah and Altindag (2005), haematological indicators including haemoglobin, haematocrit, red blood cells, and white blood cells are utilized as an indicator of fish health and to evaluate the functioning state of the bloodstream's ability to deliver oxygen. According to Kori-Siakpere *et al.* (2008) [19], this study is highly useful in assessing the health conditions of fish and is currently utilized as an efficient measure for tracking physiological and pathological changes in fish.

Table 1: Different haematological parameters treatment-wise of *Cirrhinus mrigala*

	C	T ₁	T ₂	T ₃	T ₄
Haemoglobin (g/dl)	6.3±0.37 ^a	6.7±0.20 ^b	6.8±0.14 ^b	8.0±0.11 ^d	7.2±0.12 ^c
RBC (Million/Cu-mm)	2.1±0.01 ^a	2.2±0.03 ^a	2.2±0.06 ^a	2.6±0.04 ^b	2.4±0.02 ^b
WBC (no/Cu-mm)	3900±25 ^d	3600±23 ^d	1300±24 ^a	3200±25 ^c	1900±28 ^b
Platelets count (x10 ⁵)	0.64±0.02	0.45±0.01	0.6±0.02	0.9±0.03	0.8±0.01
P.C.V (%)	18.9±0.11	20.1±0.12	20.4±0.14	24±0.14	21.6±0.13
M.C.V (fL)	90±0.02	91.3±0.03	92.7±0.06	92.3±0.05	90±0.02
M.C.H (Pg)	30±0.01	30.4±0.02	30.9±0.05	30.7±0.01	30±0.03
M.C.H.C (g/dl)	33.3±0.15	33.3±0.20	33.3±0.30	33.3±0.40	33.3±0.22

**Fig 1:** Relationship of Haemoglobin, RBC and platelets with body weight(g).

Rajan and Rohini (2021) ^[29] reported that the haematology performance was higher in nZnO 15 mg/kg diet of mrigal than concentrations of 5, 10, 20 and 25 mg per kg supplementary diet of nZnO. When the amount of zinc oxide nanoparticles rose, haemoglobin, red blood cells, haematocrit, MCV, MCH, and MCHC of mrigal gradually increased while WBC and platelets decreased. As similar to Present study, (Firat, 2007) ^[13] experienced that a drop in WBC count may be linked to the cortisol hormone, which is crucial for the mitigation and repair of inflammation in fish. Similar to present study higher concentrations of nZnO (60 mg/kg) in grass carp dramatically reduced RBC and WBC counts compared to 30 mg/kg of nZnO diet, according to Faiz *et al.* (2015) ^[11]. Another study where the fish given a diet supplemented with 10 mg/kg ZnONP exhibits progressively increased haematological changes (Thangapandian and Monika, 2019) ^[36]. According to Akbary *et al.* (2018) ^[1], grey mullet fish exposed to sublethal concentrations of copper oxide nanoparticles showed a substantial rise in WBC count and a decrease in Hb, Hct, and RBC counts when compared to the

control, which is totally against my findings. Chupani *et al.* (2018) ^[6] found that diet-born ZnO nanoparticle exposure did not affect the haematological parameters of common carp (*Cyprinus carpio*), but current research showed that nZnO affects haematology. From this experiment, it can be concluded that 15 mg nZnO may incorporated during the preparation of feed for *Cirrhinus mrigala* to improve the overall haematological performance.

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