ACUTE EFFECT OF ZINC OXIDE NANOPARTICLES ON TOTAL PROTEINS OF *Clarias gariepinus* EMBRYOS

BY

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SEPTEMBER, 2023

CERTIFICATION

This is to certify that this research titled "Acute effect of zinc oxide nanoparticles on total proteins of *Clarias gariepinus* embryos" was carried out by "EUNICE NGOZI IKHUOHON-EBOREIME" and presented to the Department of Environmental Management and Toxicology, Faculty of Life Sciences, University of Benin, Benin City; in partial fulfillment of the requirements for the award of Bachelor of Science (B.Sc) in Environmental Management and Toxicology. It was conducted under suitable conditions, was carefully supervised and subsequently approved as having met the requirements for the award of Bachelor of Science degree in Environmental Management and Toxicology.

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Dr. M. O. Akharame **Date**

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DECLARATION

I "EUNICE NGOZI IKHUOHON-EBOREIME" declare that "Acute effect of zinc oxide nanoparticles on total proteins of *Clarias gariepinus* embryos" is my own work and that all sources that I have used or quoted have been acknowledged by means of complete references and that this work has not been submitted before for any other degree at any other University.

Eunice Ngozi Ikhuohon-Eboreime

….…….

DATE

DEDICATION

This project is dedicated to God for all His goodness and grace which has brought me thus far in my academic journey. Also to my loving parents and siblings for all their love and great support.

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ABSTRACT

This dissertation focused on the effects of zinc oxide nanoparticles (ZnO NPs) on the African catfish (*Clarias gariepinus*) embryotoxicity. The total protein levels in the embryos of *Clarias gariepinus* were determined following a standard method after exposure to different concentrations (0.0, 0.5, 1.0, 5.0, and 10.0 µg/L) of ZnO NP for 96 hours. The results showed that there was a minor increment in the total protein levels at the exposure of *Clarias gariepinus* embryos to 0.5 µg/L ZnO NPs and a significant increase in NP concentration at 1 µg/L. The highest level of the total protein was recorded at the 10.0 µg/L concentration exposure of the ZnO NP to the embryos. The effect of ZnO NP increases with increasing concentration of the ZnO NP in a dose-dependent manner. Elevated total protein levels imply that there is a possible inflammation or infection, and this could lead to adverse developmental challenges for the fish embryos.

CHAPTER ONE

1.0 INTRODUCTION

Concerns concerning the possible toxicity of metal oxide-based nanoparticles (NPs) to human and environmental health have been raised due to their widespread use in many commercial goods (Aschberger et al., 2011). Because of this growing use of metal oxide nanoparticles, they are released into the aquatic environment and have negative impacts on creatures that are the subject of significant interest (Blaise et al., 2008; Ferré et al., 2009). Zinc oxide nanoparticles (ZnO-NPs) are utilized in a variety of products, including sunscreen, cosmetics, paint, papers, plastic, ceramics, and building materials (Osmond and McCall, 2010). They have great stability, anticorrosion, and photocatalytic capabilities. After TiO2 and SiO2, ZnO NPs are the third most produced metal oxide on a global scale (Piccinno et al., 2012). Three main sources—anthropogenic sources, unintentional sources, manufacture, and use of designed nanoparticles—are responsible for the entry of nanoparticles into the environment (Ferré et al., 2009).

ZnO NP exposure to aquatic species has been documented in a number of literature articles, with various levels of responses being recorded. For instance, comparing ZnO nanoparticles to ZnO bulk salt, Hao et al. (2013) studied the bioaccumulation and sub-acute toxicity of ZnO NPs in juvenile carp (Cyprinus carpio). The findings demonstrated that ZnO NPs were more harmful than their bulk salt counterparts because they may build up in tissues and cause oxidative cellular reactions. Similar research by Kaya et al. (2015) demonstrated the bioaccumulation and oxidative stress caused by ZnO NPs in many organs of Nile tilapia (Oreochromis niloticus). In their study, Xiong et al. (2011) examined the effects of TiO2, ZnO, and their bulk counterparts on zebrafish, finding that ZnO caused more oxidative damage than its counterparts in the bulk. In the goldfish (Carassius auratus) liver, Fan et al. (2013) studied the subcellular distribution of zinc

from ZnO NPs. Aquatic species' organs may have histopathological changes as a result of the ZnO NPs. While exposing tilapia (Oreochromis niloticus) subchronically to zinc ZnO NPs, Kaya et al. (2016) detected tissue accumulation, serum biochemistry, and histological alterations. After being exposed to ZnO NPs, Suganthi et al. (2015) detected behavioral and histological changes in Oreochromis mossambicu. Similar to this, Kaya et al. (2016) reported finding organ diseases connected to both tiny and large-size ZNO NPs. Xiong et al. (2011) found that zebrafish exposed to nanoscale TiO2, ZnO, and their counterbulk components suffered oxidative damage. Zinc oxide nanoparticles were found to have a genotoxic effect on the freshwater snail Lymnaea luteola in a different study by Ali et al. (2012). Zhao et al. (2013) reported oxidative stress and DNA damage in zebrafish larvae exposed to ZnO NPs in another investigation. In the brains of Oreochromis niloticus and Tilapia zillii, zinc oxide nanoparticles (ZnO NPs) also affected the activity of antioxidant enzymes and genes related to oxidative stress (Saddick et al., 2015). In response to ZnO NP exposure, Hao and Chen (2012) hypothesized that the common carp (Cyprinus carpio) exhibits oxidative stress responses in many organs. Abdel Khalek et al. (2015) have discovered oxidative stress caused by zinc metal as opposed to its nanoparticles in Nile tilapia (Oreochromis niloticus). Similarly, Xiong et al. (2011) came to the same conclusions after studying the effects of nanoparticle exposure on zebrafish. According to the findings of Hackenberg et al. (2011), ZnO NPs may cause cytotoxic, genotoxic, and pro-inflammatory effects in human nasal mucosa cells. As a result, this study will look at how ZnO NPs affect the total protein levels in the African catfish (Clarias gariepinus) embryos. The purpose of the inquiry is to provide light on how ZnO NPs affect fish development and growth in their early stages.

1.1 AIMS

This project aims to assess the total protein of oxidative stress in African catfish (*Clarias gariepinus*) embryos exposed to ZnO NPs.

1.2 OBJECTIVES

The objectives of the study are to:

1. Assess the acute toxicity level of ZnO NPs in Clarias gariepinus.

2. Determine the assess the total protein levels in African catfish (*Clarias gariepinus*) embryos exposed to ZnO NPs.

CHAPTER TWO

2.0 Literature review

2.1 Zinc oxide

An inorganic substance with the formula ZnO is zinc oxide. It comes in the form of an insoluble white powder. According to Battez et al. (2008), ZnO is used as an additive in a wide range of materials and goods, including cosmetics, food supplements, rubber, plastics, ceramics, glass, cement, lubricants, paints, sunscreens, ointments, adhesives, sealants, pigments, foods, batteries, ferrites, fire retardants, semiconductors, and first-aid tapes. The majority of zinc oxide is created synthetically, even though it can be found naturally as the mineral zincite (De Liedekerke, 2006). Zinc oxide is a versatile material thanks to its special physical and chemical characteristics, including its high chemical stability, high electrochemical coupling coefficient, wide spectrum of radiation absorption, and high photostability (Segets et al., 2009).

2.2 Properties of zinc

2.2.1 Physical properties

The two primary crystal forms of zinc oxide are hexagonal wurtzite and cubic zincblende (Fierro, 2006). The wurtzite structure is the most typical and most stable at ambient settings. By allowing ZnO to grow on substrates with a cubic lattice structure, the zincblende form can be stabilized. Tetrahedral zinc and oxide centers—Zn(II's most distinctive geometry—are present in both instances. At relatively high pressures of around 10 GPa, ZnO transforms into the rock salt motif (Ozgur et al., 2005). ZnO's elastic softness, which is typical of tetrahedral coordinated binary compounds at the transition to octahedral structures, can be used to explain the numerous outstanding medicinal qualities of creams containing ZnO (Phillips, 1970).

2.2.2 Mechanical properties

A wide-bandgap semiconductor belonging to the II-VI semiconductor family is ZnO. According to Ozgur et al. (2005), oxygen vacancies or zinc interstitials cause n-type native doping of the semiconductor. ZnO has a Mohs hardness of about 4.5, making it a comparatively soft substance (Battez, 2008). In comparison to comparable III-V semiconductors like GaN, its elastic constants are lower. Ceramics benefit from ZnO's high melting temperature, low thermal expansion, high heat capacity, and high heat conductivity (Porter, 1991). According to Millot (2010), the E2 optical phonon in ZnO has an extraordinarily long lifespan of 133 ps at 10 K. It has been claimed that ZnO possesses the highest piezoelectric tensor among the tetrahedrally linked semiconductors, or at least one that is comparable to that of GaN and AlN. Due to its large electromechanical coupling need in many piezoelectrical applications, this feature makes it a technologically significant material. Therefore, one of the most researched resonator materials for thin-film bulk acoustic resonators is ZnO in the form of thin films.

2.2.3 Electrical and optical properties

Good transparency, high electron mobility, a broad band gap, and potent room-temperature luminescence are some of zinc oxide's advantageous characteristics. Because of these characteristics, ZnO is useful for a number of new applications, including transparent electrodes in liquid crystal displays, energy-efficient or heat-protecting windows, and electronics like thin-film transistors and light-emitting diodes.

2.2.4 Chemical properties

Although natural ZnO is a rare mineral that typically contains manganese and other impurities that give it a yellow-to-red tint, pure ZnO is a white powder. When heated in air, crystalline zinc oxide undergoes a thermochromic change from white to yellow, returning to white upon cooling (klingshim, 2007). At high temperatures, a little amount of oxygen is lost to the air, resulting in the formation of the non-stoichiometric Zn1+xO, where x = 0.00007 at 800 °C (Wiberg, 2001). An amphoteric oxide is zinc oxide. While most acids, including hydrochloric acid, may dissolve it, it is very insoluble in water (Eaenshaw, 1997).

$$ZnO + 2HCl \rightarrow ZnCl2 + H2O$$

Additionally, soluble zincates are produced when solid zinc oxide dissolves in alkalis:

$$ZnO + 2NaOH + H2O \rightarrow Na2[Zn(OH)4]$$

Oil fatty acids and zinc oxide slowly react to form the matching carboxylates, such as oleate or stearate. ZnO reacts with a strong aqueous solution of zinc chloride to produce compounds that resemble cement and are more accurately referred to as zinc hydroxy chlorides. Dental work involved the use of this cement (Ferracane, 2001). When treated with phosphoric acid, ZnO also transforms into a substance like cement; comparable materials are employed in dentistry (Ferracane, 2001). Hopeite, Zn3 (PO4)2•4H2O, is a key component of the zinc phosphate cement created by this reaction (Park et al., 1998).

2.3 Zinc oxide nanoparticles (ZnO NPs)

ZnO nanoparticles with a diameter of under 100 nm are referred to be zinc oxide nanoparticles. They have a high catalytic activity and a huge surface area in relation to their size. The various

methods of synthesizing zinc oxide nanoparticles affect the precise physical and chemical characteristics of those particles. Laser ablation, hydrothermal methods, electrochemical depositions, sol-gel methods, chemical vapor deposition, thermal decomposition, combustion methods, ultrasound, microwave-assisted combustion method, two-step mechanochemical-thermal synthesis, anodization, co-precipitation, electrophoretic deposition, and precipitation processes using solution concentration, pH, and washing medium are some potential methods for producing ZnO nanoparticles. At ambient temperature, the energy gap of the wide-bandgap semiconductor ZnO is 3.37 eV (Haynes, 2016). Along with silicon dioxide and titanium dioxide nanoparticles, ZnO nanoparticles are thought to be among the top three most manufactured nanomaterials (Haynes, 2016). Sunscreen is where ZnO nanoparticles are most frequently used. They are utilized because they have a large enough band gap to be entirely transparent to visible light while still effectively absorbing UV light (Haynes, 2016). Additionally, its ability to eradicate dangerous germs from UV-protective materials including textiles and packaging is being studied (Takahashi, 2007). It is challenging to make claims concerning production and pervasiveness in consumer items because many companies do not label products that include nanoparticles (Haynes, 2016).

ZnO nanoparticles are a relatively new substance, thus there are questions about the dangers they might present. Due to their small size, nanoparticles may often move throughout the body and have been demonstrated in animal tests to pass through the blood-brain barrier, the placenta, individual cells, and their nuclei. They are hard to detect since tissues can quickly absorb them due to their size. However, unless there are abrasions, human skin serves as an excellent barrier to ZnO nanoparticles, such as when sunscreen is applied. When applying sunscreen, ZnO nanoparticles may be accidentally ingested in trace amounts and enter the body. The ZnO

nanoparticles in sunscreen can wash off into runoff water and move up the food chain. According to Haynes (2016), no synthetic nanoparticle-related ailments in humans were known to exist as of 2011.

2.4 Clarias gariepinus

A species of catfish belonging to the family Clariidae, which includes air-breathing catfishes, is the African sharp-tooth catfish, or Clarias *gariepinus.*

2.4.1 Distribution

They can be found in freshwater lakes, rivers, and swamps all over Africa and the Middle East, as well as in man-made environments like oxidation ponds or even urban sewage systems. Early in the 1980s, the African sharp-tooth catfish was distributed globally for aquaculture purposes, and today it may be found in nations like Brazil, Vietnam, Indonesia, and India that are a long way from its native continent.

2.4.2 Description

The African sharp-tooth catfish is a sizable, eel-like fish with a back that is often colored dark gray or black and fades to a white belly. Only the vundu of the Zambesian seas are larger than this catfish in Africa, according to reports (Froese, 2014). However, FishBase claims that the African sharp-tooth catfish is larger and heavier than the vundu (Froese, 2014). Adult length for the C. gariepinus ranges from 3 feet 3 inches to 4 feet 11 inches, or 1-1.5 m. Its maximum length is 1.7 meters (5 feet 7 inches) TL, and its maximum weight is 60 kilograms (130 pounds). 2014's Froese. The body of these fish are thin, and their flat, bony heads are noticeably flatter than those of the Silurus genus. They also have big, terminal mouths with four pairs of barbels. Large supplementary breathing organs made of modified gill arches are also present in them. The only fins with spines are the pectoral fins.

2.4.3 Habitat

Like many catfish, it is a nocturnal fish. It consumes both living and dead animal stuff, including fruit and seeds from plants as well as plankton, snails, crabs, shrimp, other crustaceans, birds, reptiles, amphibians, small mammals, and other fish (Hal, 2004). It can swallow relatively large prey whole because to its huge jaws. Large waterbirds like the common moorhen have been known to be taken by it (Anoop, 2009). In order to get out of drying ponds, it may also crawl on dry ground. In addition, it can endure prolonged periods of shallow muck between wet seasons. African catfish occasionally make loud croaking noises resembling a crow's voice.

2.4.4 Natural spawning

The majority of spawning occurs at night in the shallow, submerged sections of the rivers, lakes, and streams. Males engage in very aggressive interactions prior to courtship. In shallow waters, solitary male and female pairs engage in courtship and mating. For several seconds, the male holds the position of the U-shape around the female's head. The female releases a batch of milt and eggs and then vigorously swishes her tail to spread the eggs out over a large region. After mating, the pair often takes a short break (a few seconds to several minutes) before starting over. Except for the careful selection of a suitable place, parental care is not provided to ensure the survival of the catfish progeny. Within 48–72 hours of fertilization, the larvae can swim and the development of the eggs and larvae is quick.

2.4.5 Rearing

Early in the 1970s, in Central and Western Africa, the African catfish was first raised since it was found to be a particularly good species for aquaculture:

• It feeds on a wide range of agricultural byproducts and grows quickly.

• It can withstand poor water quality situations.

• It can produce large net yields (6–16 t/ha/year) when grown in high densities.

• Since it can be purchased live at the market, it sells for more money than tilapia in the majority of nations.

• When kept in captivity, it develops and reproduces rather easily.

• It can endure challenging aquaculture conditions.

2.4.6 Parasites and Diseases

In addition to various endo- and ectoparasites, C. *gariepinus* may host a number of digenean species (Jansen, 2013).

2.5 Life cycle of the African Catfish

The life cycle of the African catfish, Clarias gariepinus, includes the following stages:

1.Egg stage: The life cycle of a catfish begins with the female laying eggs, which the male fertilizes. Typically, the eggs are fastened to a substrate or a safe place.

2. Embryonic stages: The eggs develop into embryos inside the protective egg sac after they hatch. The embryos are now able to take up nourishment from the yolk.

3. Larval stage: The catfish larvae emerge from the eggs after hatching. They don't have fully developed features at first and are quite little. They still eat from their yolk sacs at this stage.

4.Adolescent stage: As the larvae mature, they take on increasingly distinct features and begin to eat small aquatic creatures. They progressively start to rely more on outside food sources and less on their yolk sacs.

5.Sub-Adult and Adult Stages: As the catfish develops, they finally reach the sub-adult and adult stages. They develop into more active predators and start eating a wider range of food, including aquatic invertebrates, small fish, and insects.

Clarias gariepinus may adapt to a variety of environmental conditions during their life cycle, and elements like water temperature and the availability of suitable breeding locations may have an impact on how they behave during reproduction. Remember that depending on the habitat and local conditions, several aspects of their life cycle may differ.

2.6 Toxicity of ZnO NPs

2.6.1 Effects of airway exposure in experimental animals

According to theoretical modeling, 10-100 nm NPs preferentially deposit in the alveolar and tracheobronchial areas (Witschger, 2005). Alveolar macrophages typically phagocytose particles in the case of alveolar deposition, and these cells migrate to the tracheobronchial region through the mucociliary escalator where they are coughed up and sometimes ingested. Particles may enter the interstitium, where they are no longer eligible for clearance, if these systems malfunction. As a result, there may be fibrosis and inflammation in the cells that are chronically stimulated (Osmond, 2010). The lung lining fluid's acidic environment may cause ZnO to dissolve, which might then temporarily raise the concentration of Zn2+ ions and cause local toxicity (Osmond, 2010). Rats exposed to 3 m ZnO microparticles (25 and 50 mg/m3) and instilled with 300 nm ZnO NPs (1 and 5 mg/kg body weight [bw]) experienced transient

inflammation as indicated by elevated lactate dehydrogenase (LDH) release, protein content, and neutrophil content in the bronchoalveolar lavage (BAL) (Warheit, 2009). Strong but reversible inflammation was produced in rats after a single intratracheal instillation of 50-70 nm ZnO NPs and 1000 nm ZnO microparticles (1 and 5 mg/kg bw), as indicated by increased LDH release, cell number, and neutrophil concentration in the BAL. One month after the instillation, this inflammation subsided (Sayes, 2007). Rats were given a single intratracheal instillation of 10 nm ZnO NPs, which resulted in the recruitment of eosinophils and neutrophils to the BAL and an eosinophilic/fibrotic/granulomatous inflammation (Cho et al., 2012 2012).

The primary mechanism of ZnO NP-induced lung damage is rapid pH-dependent breakdown of ZnO NPs inside phagosomes. (2011) Cho et al.Due to the low pH and the presence of lysosomal enzymes inside of this organelle, a recently published model proposed that for high-solubility NPs, such as ZnO NPs, the protein corona is digested inside lysosomes. Following this, the NPs quickly dissolve and cause lysosomal destabilization by Zn2+. In comparison to non-doped NPs, iron-doped 20 nm ZnO NPs in mice demonstrated reduced lung toxicity (neutrophil counts and interleukin [IL]-6 expression), as well as lower heme oxygenase (HO) expression. Following exposure to iron-doped NPs, there was also a decrease in pulmonary inflammation in rats.

Due to the compound's high solubility, a recent study has demonstrated that ZnO (and CuO) NPs form a distinct group within the family of metal oxide NPs (Zhang et al., 2012). ZnO (and CuO) NP toxicity is governed by its solubility, whereas for the majority of metal oxide NPs examined in vivo and in vitro, toxicity could be anticipated by their band-gap energy. The proposed technique for toxicity prediction makes two decisions: if the band-gap energy overlaps with the cellular redox potential, and whether the solubility of the metal oxide NP is over a particular threshold (as it is for ZnO NPs).

Rats exposed to 20 nm ZnO NPs (2.5 mg/kg bw) twice daily for three days showed increased Zn concentration in the kidneys and liver after 36 hours. According to histopathology, the liver and lungs have been damaged (Wang et al., 2011).

Zn2+, which is present because ZnO NPs are soluble, is thought to be the cause of inflammation and necrosis (Landsiedel et al., 2010). After inhaling 38 nm ZnO NP, it was discovered that rat BAL cells and white blood cells had a greater cytosolic [Zn2+] (Kao et al., 2012). According to Ayres et al. (2008), the ability of ZnO NPs to produce reactive oxygen species (ROS) in vitro appears to be correlated with their capability to cause cellular inflammation in vivo.

A tiny fraction of 50 nm ZnO NPs might permeate the airway mucus, even though metal oxide nanoparticles are typically held in the lung mucus by adhesive contact (Jachak et al., 2011). ZnO NPs cause systemic toxicity and pulmonary irritation when inhaled or injected. Inflammation caused by ZnO NPs is significantly influenced by their solubility. It is thought that phagosome dissolution is the primary initiator of inflammation. There are some signs, though, that ZnO NPs might also dissolve in the fluid that lines the lungs (surfactant). ZnO NPs cause an increase in intracellular [Zn2+] when inhaled. The inflammatory response is thought to be significantly influenced by ROS production. ZnO NPs cannot be (fully) trapped by lung mucus, although the majority of metal oxide NPs can.

2.6.2 Effects of Cutaneous Exposure in Experimental Animals and Humans

Since many people use sunscreens for many hours each day for weeks or months, the presence of ZnO (and TiO2) NPs in sunscreens implicates a significant source of exposure. Therefore, the ability of ZnO NPs used in sunscreens to penetrate the epidermal barrier is a topic of significant discussion. Due to their inherent UV-absorbing qualities, ZnO (and TiO2) are utilized as UV

blockers in sunscreens. These particles reflect and scatter light in the usual pigment size ranges (200–400 nm for ZnO), giving sunscreens their white appearance. However, sunscreens appear transparent on the skin due to 40-100 nm ZnO NPs, which absorb and scatter UV light while also absorbing the majority of visible wavelengths. Since ZnO (and TiO2) are semiconductors, some UV energy that is absorbed (about 10%) might produce free radicals on the surface of metal oxides when there is water present. With smaller NPs, this photocatalytic activity rises. When utilized in sunscreens, coating or doping are used to lower the metal oxide semiconductor activity (Osmond and McCall, 2010).

According to the majority of research conducted on healthy human skin, ZnO NPs used in sunscreens do not penetrate through the stratum corneum (Schilling, 2010). 68Zn was utilized to create ZnO particles in order to distinguish it from the body's own Zn (Gulsonet et al., 2010). 19 nm nanoparticles and microparticles smaller than 100 nm were the two types of particles used. Approximately 0.1% of the total Zn in the blood was 68Zn after applying sunscreen with one of these two types of particles to the skin of healthy human volunteers for five days. When NPs were applied, there was more 68Zn present in the blood and urine than there was with micro-sized particles.

2.6.3 Effects on The Nervous System in Test Animals

Few research have examined ZnO NPs' neurotoxicity and how it affects cognition. In rats, intraperitoneal injection of 20-80 nm ZnO NPs (4 mg/kg bw) twice weekly for 8 weeks impaired spatial learning and memory capacity through altering synaptic plasticity (Han et al., 2011). This work stands alone and needs to be supported by other research that considers more pertinent exposure routes.

CHAPTER THREE

3.0 Materials and Methods

3.1 Study Area

The study area is located in Benin metropolis, the main metropolis and capital of Southern Nigeria's Edo State. The capital and largest city of southern Nigeria's Edo State is Benin City. With a total size of 1,204 square kilometers and a population of 1,782,000 as of 2021, it is the fourth-largest metropolis in Nigeria. The average elevation of Benin City, which ranges from 6o19'00" to 6o21'00" N and 5o34'00" to 5044'00" E, is 77.8 meters above sea level. It is located 320 kilometers (200 miles) east of Lagos and about 40 kilometers (25 miles) north of the Benin River. Nigeria's oil production is a key business, and Benin City is home to the country's rubber industry. Some of Nigeria's universities, including the University of Benin, are located in Benin City, specifically at Ugbowo and Ekenwan. The tropical rain forest that surrounds Benin City, Edo State, has two different seasons (wet from April to October and dry from November to March) (Offodile, 2002). In Benin City, the average yearly temperature is 25.7 °C, with an average annual rainfall of 2,679 mm, precipitation primarily consists of rain, which adds a significant amount of water to the ecosystem.

3.1 Materials Used

Three female catfish (gravid) and two males were utilised for the research and were purchased from a commercial fish farm in Benin City, Edo State. Acute effect of zinc oxide nanoparticles on total proteins of *Clarias gariepinus* embryos.

• Nets: A substance that the fertilized eggs can cling to and stay floating on to increase their chances of survival.

- Clean syringes for injecting and withdrawing liquids;
- Petri plates; fish tanks made of chemically inert material (for example, glass) to house the specimen.
- An inverted microscope and/or binoculars with a minimum 80-fold magnification capability.
- Test chambers, such as common 24-well plates with a 20 mm depth.
- Pipettes with widened openings to collect eggs
- Glass vessels to prepare various test concentrations and dilution water (beakers, graduated flasks, graduated cylinders, and graduated pipettes); or Glass vessels to collect Fish eggs (e.g., beakers, crystallization dishes)
- Incubator or air-conditioned room with controlled temperature, allowing to maintain 26±1 °C in wells (or test chambers).

Plate 3.1: A slide showing the making of the nettings that will act as the substrate (Eunice, 2023).

Clean up

To make sure there were no contaminants present that would affect the experiment, I cleaned up the lab area and research space. Then, using a bar soap because of its gentleness, we cleaned all the instruments and materials we would use for the experiment, including tanks, syringes, nettings, Petri dishes, and pipettes. We then rinsed with a saline solution.

3.2 Artificial insemination and eggs collection

3.2.1 Inducing the gravid fishes

According to each fish's specific body weight (0.5 mL/kg), the hormone OVULIN was administered to induce pregnancy in the fish. The hormone is administered intramuscularly, and its main function is to induce the release of the eggs because captive animals and/or those outside of their native habitat do not readily release their eggs.

3.2.2 Precautions when inducing the female fish:

- The injections were given below the dorsal fin, above the lateral line towards the head at a 45 angle.
- The injected spot was massaged gently to avoid flow back
- Covering the eyes of the fishes with a cloth while injecting to keep them calm
- The process was repeated for two more gravid fishes, to increase sample size and aid viability.
- The fish were then kept in secured bowls and left for 8 hours.

Plate 3.2: ovulin-induced female fish.

3.2.3 Collection of the eggs and sperm sacs

After the fish were induced for 8 hours, the fishes were relieved of their eggs by "stripping". This process involved massaging the mid-region of the fish and collecting the eggs into a clean and sterile bowl. Saline water was then added to the bowl of eggs to provide a neutral environment for the eggs and also prevent agglomeration of the eggs.

For the sperm sac extraction from the male fish, a small incision was made on the ventral side of the fish and the sperm sac was taken. This was done for two fishes to aid viability. The sperm sac was incised and the sperm cells were then washed out of the sacs using saline water. This stops the sperm cells from being activated before being introduced to the egg cells. This method of sperm cell collection is called the "wet method".

Plate 3.3: Stripping of the eggs from the gravid fish.

Plate 3.4: Incision made on the ventral side of the male fish to remove the sperm sac

3.2.4 Artificial fertilisation and activation of the fertilised eggs

The sperm cells were added to the eggs and mixed with a plastic spoon. The mixture was not left to stand for long before it was introduced to the artificial breeding sites (sterile tanks).

Sterile nettings were placed in glass tanks containing deionized water with a pH of 5.2. The nettings act as a substrate for the fertilized eggs to latch onto, this is to mimic a natural environment where aquatic plants would be substrates for the fertilized eggs to get attached to and start their growth process where they have access to oxygen. The eggs were scooped with a spoon and gently scattered throughout the nettings.

Plate 3.5: Artificial fertilization and activation of the eggs

Plate 3.6: Nettings placed in sterile chambers with eggs gently scattered throughout it

3.2.5 Acute toxicity test set-up

Use glass or polystyrene test chambers (for instance, 24-well plates with a filling capacity of 2.5 to 5 Ll per well). For non-polar, planar chemicals with high KOW, for example, where adsorption to polystyrene is suspected, inert materials (glass) should be employed to minimize adsorption-related losses. The incubator's test chambers should be distributed at random.

Four distinct concentrations of each ZnO nanoparticle were measured and diluted; the control was with no NPs (i.e. 0.0 µg/L). The stock solution of the NPs was prepared and the dilution method to get the working concentrations. The concentrations that were tested were (0.0, 0.5, 1.0, 5.0, and 10.0 µg/L). Salts from the measurements were diluted and put in racks or wells.

Plate 3.7: Well arranged according to concentration

3.2.6 Placing the eggs in the wells

Twenty (20) fertilized eggs were carefully placed in each well. Each rack pair represented a different concentration of the introduced nanoparticles. There were five pairs of racks each of each nanoparticle (i.e. 0.0, 0.5, 1.0, 5.0, and 10.0 µg/L). They were clearly labelled to avoid any errors. The embryos were exposed to the different ZnO NP concentrations for 96 hours. Thereafter, samples were then collected for the biochemical tests.

Plate 3.8: Careful picking of embryos

Plate 3.9: Placing of twenty embryos in each well using a pipette.

3.3 Biochemical analysis (total protein)

3.3.1 Procedure for analysis

Centrifuge, atomic absorption spectrophotometer, microplate reader, appendix tubes, micropipettes, syringes, and gloves were a few of the lab tools and supplies used. The samples were homogenized, placed in sterile tubes, maintained in the refrigerator for the duration of the experiment, and centrifuged. Following that, a total protein serum analysis was performed.

Determination of serum total protein

Using a Randox kit, total protein was determined according to the procedure outlined by Weichselbaum (1946).

Principle of the assay

In an alkaline solution, interactions between cupric ions and peptide bonds result in the production of a colored complex. The absorbance, which is directly proportional to the amount of total protein in the sample, was measured at 546 nm.

Procedure

The test tubes have the names blank, standard, and sample on them. 1.0 mL of reagent 1 and 0.02 mL of distilled water were put into the blank. Reagent 1 and 0.02 mL of the standard reagent were added to the standard, and 0.02 mL of serum and 1.0 mL of reagent 1 were added to the sample. The mixes were incubated at 25 °C for 30 minutes. At 546 nm, the absorbance of the sample and the standard was measured in comparison to the reagent blank.

Calculation

The calculation was based on the use of a standard protein solution:

Total protein concentration (g/dL) = $\frac{\text{O.D of Test} \times \text{concentration of standard}}{\text{O.D of standard}}$

Plate 3.10: Homogenisation process

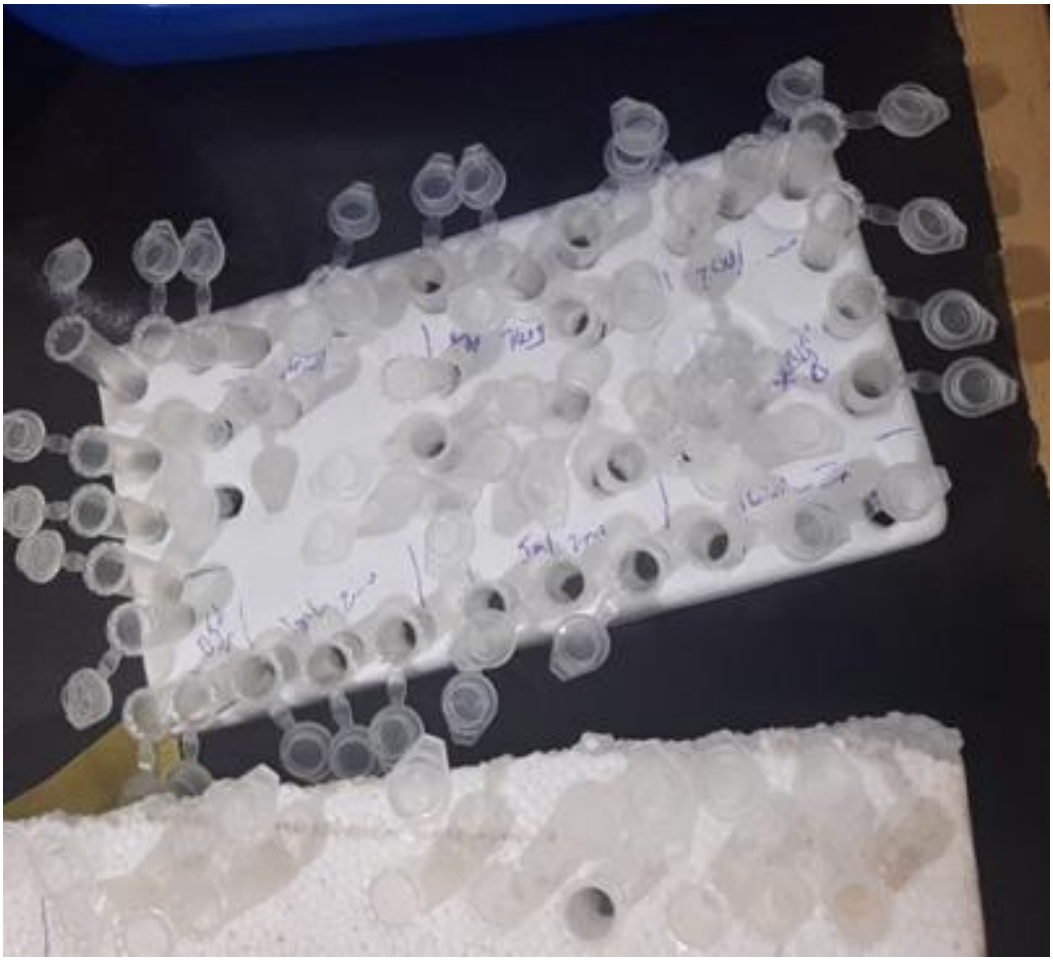

Plate 3.11: Arrangement of the Eppendorf tubes

Plate 3.12: Centrifugation of the homogenant.

CHAPTER FOUR

4.0 RESULTS

4.1 RESULTS PRESENTATION

The total protein at each concentration (0.5, 1.0, 5.0, and 10.0 µg/L) and the control levels are shown in Table 4.1.

Table 4.1: Total protein levels in the embryos of *Clarias gariepinus* for ZnO NP concentrations

	Total Protein				
	0mg/L	**0.5mg/L**	**1mg/L**	**5mg/L**	**10g/L**
Control	**4.297**	**4.297**	**4.297**	**4.297**	**4.278**
ZnO NPs	**4.297**	**4.302**	**4.342**	**4.344**	**4.414**

Zinc oxide concentrations are plotted against levels of total protein in Figure 4.1. ZnO NPs have no influence on the total proteins of the embryos at a concentration of 0 µg/L as expected. There is a minor increase in the total protein levels at the exposure of *Clarias gariepinus* embryos to 0.5 µg/L ZnO NPs and a significant increase in NP concentration at 1 µg/L. The highest level of the total protein was recorded at the 10.0 µg/L concentration exposure of the ZnO NP to the embryos. The effect of zinc oxide nanoparticles increases with increasing concentration of the ZnO NP in a dose-dependent manner. When the ZnO NP concentration, the recorded protein level also rises.

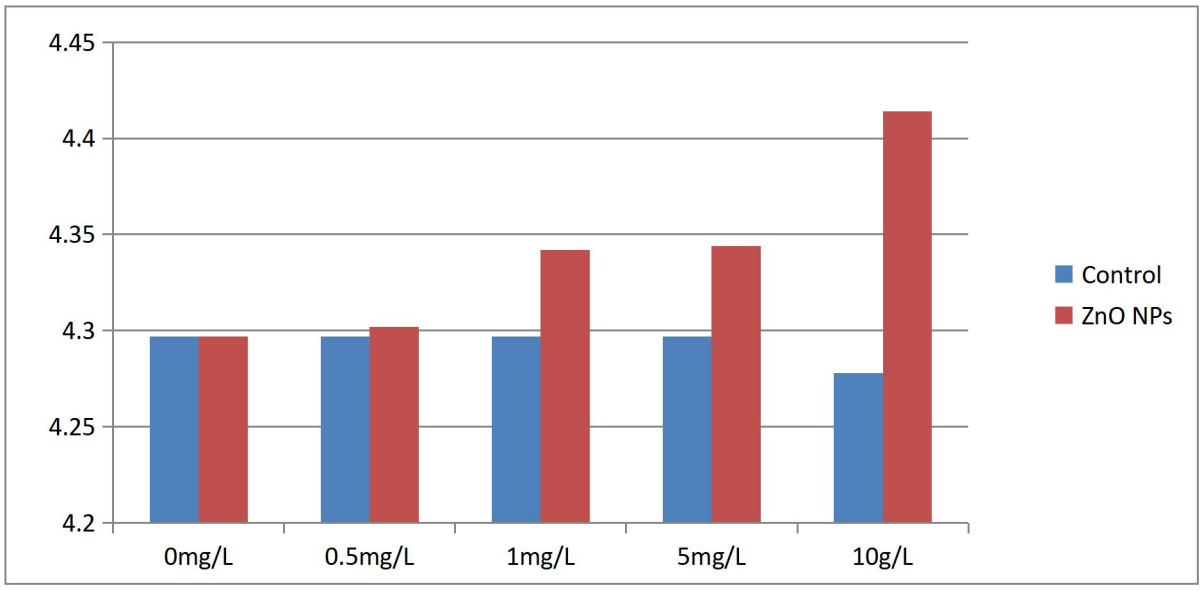

Figure 4.1: Concentration of ZnO NP (horizontal) against total protein levels (vertical)

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSION

5.1 Discussion

The quantity and variety of fish species have dramatically declined recently as a result of ecological changes and the destruction of their natural spawning grounds in most water bodies. To stop the decline of several priceless and endangered fish species, scientists are heavily focused on research to understand the effect of the numerous environmental stressors that may contribute to alterations in the growth and development of fish. The different stressors induced certain biochemical activities in the fish with adverse consequences. The total protein test determines the albumin albumin and globulin levels in cells, organs, or organisms. Its elevated levels imply that there is a possible inflammation or infection. The findings from this investigation show that there was a steady increment in the total protein levels with increasing concentration of the ZnO NP in a dose-dependent manner. The ZnO NP may entered the interstitium, where they are no longer eligible for clearance, which may have caused the cells to be chronically stimulated, leading to inflammation and fibrosis.

African catfish's liver and heart have previously shown signs of lipid peroxidation brought on by dietary nanoparticles (Baker et al., 1999). Zebrafish embryos treated with ZnO NPs showed an increase in intracellular reactive oxygen species (ROS), which resulted in certain harmful consequences (Zhu et al., 2009b). Emerging investigations revealed that nano-metals can pass through the chorion and hypothesized that ZnO NPs may be particularly hazardous to developing or juvenile organisms. The effects of ZnO NPs were investigated in an experiment involving medaka fish (*Oryzias latipe*) and their embryos. The results of these investigations demonstrated that the toxicity of the exposed medaka adults and embryos was dose-dependent. The adults'

liver and brain samples included considerably lower levels of SOD and GSH activity, but as exposure time extended, the adults seemed to recover by modifying the amounts of antioxidant enzymes (Gavaskar et al., 2005). The conclusion of some of the above research aligns with the result of this investigation.

5.2 Conclusion

Metal oxide-based nanoparticles (NPs) are often employed in a variety of commercial goods with their eventual deposition in aquatic ecosystems. This raises questions regarding their potential toxicity to aquatic organisms and the environment. This study assessed the acute effect of different concentrations of ZnO NP on the total protein levels of *Clarias gariepinus* embryos after exposure for 96 hours. The results showed that there was a minor increase in the total protein levels at the exposure of *Clarias gariepinus* embryos to 0.5 µg/L ZnO NPs and a significant increase in NP concentration at 1 µg/L. The highest level of the total protein was recorded at the 10.0 µg/L concentration exposure of the ZnO NP to the embryos. The effect of zinc oxide nanoparticles increases with increasing concentration of the ZnO NP in a dose-dependent manner. The toxicity of nanoparticles to aquatic organisms has been established by many research works, and it has become pertinent to seek measures and control in the form of regulations for the usage and disposal of nanoparticles.

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