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Effect of supplementation of zinc nanoparticles on disease resistance and haematological parameters in Nile tilapia (*Oreochromis niloticus*) fry

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Abstract
Present study was taken to investigate effect of supplementation of zinc nanoparticles on disease resistance and haematological parameters in Nile tilapia (*Oreochromis niloticus*) fry. The experiment was carried out for 60 days. Four experimental animals were prepared namely T_0 (control), T_1 (10 mg/kg), T_2 (20 mg/kg) and T_3 (30 mg/kg). Zinc was selected as a nanoparticle ingredient. The antioxidant chemicals sulfoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and malondialdehyde (MDA) were measured at the end of the experiment along with haematological parameters. Highest SOD, CAT and GPX activity were found in the T_3 treatment (30 mg/kg). Lowest activity of SOD, CAT and GPX were found in T_0 treatment (0 mg/kg). While no significant difference was observed in MDA activity. In blood parameters, highest WBC, haematocrit, platelets were found in T_3 treatment (30 mg/kg) while lowest were found in T_0 treatment (0 mg/kg). RBC count and haemoglobin value did not show any significant difference within treatment. Results indicated that increase in zinc supplementation has shown to increase activity of SOD, CAT, GPX along with showing decreasing MDA level. In the case of haematological parameters, WBC, haematocrit, haemoglobin and platelet counts were increased with increase zinc nanoparticle supplementation. While RBC count did not show significant difference. It can be concluded that overall zinc supplementation increase disease resistance though antioxidant chemicals and also increase haematological parameters. Therefore, to increase the disease resistance T_3 treatment (30 mg/kg) is recommended to increase disease resistance in Nile tilapia (*Oreochromis niloticus*) fry.

Keywords: *Oreochromis niloticus*, Nile tilapia, zinc, nanoparticle, disease resistance, hematology

Introduction
In recent years, aquaculture has emerged as an increasingly prominent industry, driven by its pivotal role in providing a sustainable solution to address the escalating global demand for seafood while simultaneously alleviating the intensifying pressure on wild fish stocks (Sharifinia *et al*., 2023; Yeganeh *et al*., 2020) [44, 51]. The sector has garnered significant attention, attributable to its substantial potential for generating high yields and the efficient recycling of feed protein resources (Khanjani *et al*., 2022; 2023) [22-23]. Among the species under aquaculture, tilapia, belonging to the family Cichlidae, stands out as an exceptionally suitable choice for cultivation due to its remarkable tolerance and growth characteristics (Hargreaves, 2013; Khanjani *et al*., 2023) [19, 23].
Notably, in the year 2020, Nile tilapia (*Oreochromis niloticus*) secured a noteworthy third position in global aquaculture production, contributing a substantial 4407.2 thousand tons, which translates to approximately 9% of the inland water aquaculture production worldwide (Anon, 2021) [2]. Nile tilapia exhibits a spectrum of advantageous traits, including rapid growth performance, robust resistance to stress and diseases, adaptability, and the capacity to subsist on lower trophic levels of the food web (Durigon *et al*., 2020; Haridas *et al*., 2017) [10, 20]. The foundational role of nutrition in animal growth and maintenance cannot be overstated, as organisms with superior nutritional profiles not only yield higher-quality products but also exhibit enhanced growth potential. Consequently, feed costs represent a substantial proportion, typically ranging from 30% to 70%, of the operational expenses associated with aquaculture, contingent on the species (Rumsey, 1993) [42]. As the aquaculture industry continues to expand, the demand for aquatic feeds is poised to increase concomitantly, further underscoring the imperative for consistent, efficient, and cost-effective feed solutions.
The term "tilapia" encompasses a diverse array of commercially significant food fish species within the Cichlidae family, and these species are cultivated in approximately 100 countries,

positioning them as one of the most critical aquatic species for aquaculture in the 21st century (Fitzsimmons, 2000) [13]. Notably, tilapia's omnivorous dietary habits make them exceptionally suitable for aquaculture, as they efficiently feed on lower trophic levels. Within the genus *Oreochromis*, which includes Nile Tilapia (*O. niloticus*), Mozambique Tilapia (*O. mossambicus*), and Blue Tilapia (*O. aureus* and *O. urolepis hornorum*), Nile tilapia, in particular, emerges as an exemplary candidate species for intensive and super-intensive farming systems, owing to its remarkable growth rate and resilience to stressful environmental conditions (Dawood *et al*., 2021) [9]. It is predominantly reared in freshwater systems across numerous countries globally, which often contend with a myriad of pollutants and toxins, making Nile tilapia's adaptability and hardiness all the more significant (Dawood *et al*., 2021) [9].

Nanotechnology has huge application in aquaculture fields like nanomaterials, nanosensors, DNA nanovaccines, Gene delivery and smart drug deliver. Currently over 300 nanoparticle products are available. Nanotechnology involves the application of materials at nano scale to new products or processes. It is a rapidly growing industry currently. There are several definitions of nano materials, but it is generally agreed that they have at least one dimension <100 nm (Masciangioli and Zhang, 2003, Roco, 2003) [31, 41] or have a primary size in the 1–100 nm range (Schenir, 2007). For fish health in aquaculture, nano technological applications include antibacterial surfaces in the aquaculture system, nano delivery of veterinary products in fish food using porous nanostructures, and nano sensors for detecting pathogens in the water.

Zinc (Zn) is a vital micro-mineral that serves diverse physiological functions, including supporting growth, development, immune system regulation, and scavenging oxygen-free radicals (Watanabe *et al*. 1997) [48]. This essential trace element acts as a necessary co-factor in various enzymes involved in the synthesis and breakdown of proteins, lipids, carbohydrates, and nucleic acids (Vallee & Falchuk, 1993) [47]. Furthermore, zinc is a key component of the superoxide dismutase (SOD) enzyme, which plays a potent role in the body's defense against oxidative stress (Feng *et al*., 2011) [12]. In addition, zinc plays a role in maintaining bone structure and mineralization by promoting osteoblastic cell activity and inhibiting osteoclastic bone resorption (Yamaguchi, 1998) [50]. When fish are fed a zinc-deficient diet, they may exhibit symptoms such as stunted growth, increased mortality, body dwarfism, cataracts, and reduced zinc levels in their tissues (Jobling, 2012) [33]. Zinc (Zn) is also an essential trace mineral that is required for growth and metabolism of all vertebrates including fish. It is needed in more than 1000 structural, catalytic and regulatory proteins, which are important for growth, development and physiology of animals (Eide, 2006; Maret and Krezel, 2007) [11, 30]. Among the nanoscale metal oxides, ZnO nanoparticles have the third highest global production after TiO_2 and SiO_2 nanoparticles. The retardation of bone growth due to deficiency of Zn also proves its importance in the growth and mineralization of bone tissues (Liang *et al.*, 2012) [28]. Zinc nanoparticles have shown effects on the embryo development as well as in oxidative stress. The Major reason for that is zinc is an essential key component in several metabolic pathways and is essential for the regulation of protein synthesis, energy consumption, and as well as vitamin A and lipid metabolism (Muralisankar *et al.*, 2014) [32]. Because of all the given statement, it is evident that zinc play an important role into the fish physiology and immunity. Thereby zinc nano particles have been chosen in the experiment to check its effect on growth and disease resistance.

Antioxidant enzymes are crucial in the effort to counteract oxidative stress caused by toxicants once the supply of other antioxidant compounds are depleted (Radi and Matkovics, 1988). The definition of an antioxidant as any substance that, when present at low concentrations compared to those of an oxidizable substrate (Halliwell, 1989) [17] indicates that almost everything found in foods and in living tissues including proteins, lipids, carbohydrates, and DNA is able to act as an antioxidant (Halliwell *et al.*, 1995) [18].

Sulfoxide dismutase (SOD)

Superoxide dismutase (SOD) consists of a family of metalloenzymes containing copper and zinc or manganese or iron that breaks down the superoxide radical into H_2O_2, which can then be further broken down by catalase and glutathione peroxidase (Beyer & Fridovich, 1987) [5]. It is an important defence in almost all living being. It catalyses superoxide anion into ordinary molecule of O_2 and H_2O_2. It is a by-product of oxygen metabolism and it is necessary to keep it within the range, because it could harm the cell otherwise. It follows the overall metabolic oxygen consumption and oxidative activity of each major fish taxonomic group (Haard, 2000) [15].

Catalase (CAT)

It is a metal containing enzyme. It is the most effective enzyme that promote the redox reaction. It has the highest turnover number means it can convert millions of hydrogen peroxide into water and oxygen every second. It is a tetramer of four polypeptide chain and is almost 500 amino acid long. It also has four iron containing heme group which react with hydrogen peroxide.

CAT works through the reactions:

$CAT + H_2O_2 \rightarrow CAT\text{-}H_2O_2$ and

$CAT\text{-}H_2O_2 + H_2O_2 \rightarrow CAT + 2H_2O + O_2$ (Wheeler *et al.*, 1990) [49].

Although CAT seems to be more important in catalyzing the decomposition of H_2O_2, it shares this role with glutathione peroxidase (GPx) and GPx may be more sensitive to H_2O_2 in some cases (Grant *et al.*, 1998; Barim-Oz and Yilmaz, 2016) [14, 3].

Glutathione peroxidase (GPx)

It is a common name of the enzyme family having peroxidase activity in tissue, their main role is preventing the body from the oxidative damage. It is the enzyme of capacity to scavenge free radicals. Their main function is lipid hydroperoxides into alcohol and also reduces hydrogen peroxide to water. Unlike catalase, GPx requires co-factors, including glutathione reductase (GR) and NADPH. It also works through two intermediates, glutathione (GSH) and glutathione disulfide (GSSG) (Cullen and Weydert, 2010) [8].

Considering various factors related to Nile tilapia, such as the presence of zinc nanoparticle in the diet, we have undertaken a study to investigate the effects of supplementing with zinc nanoparticles on disease resistance and haematological parameters. This research seeks to provide a comprehensive understanding of how introducing zinc nanoparticles can impact the fish's ability to resist diseases and any changes in their blood characteristics.

Materials and Methods

Experimental setup

The experiment took place at the Inland Fisheries Research Station, JAU, Junagadh, and spanned a duration of 60 days. For the experiment, rectangular plastic aquariums measuring 2 × 1 × 1 feet were used. A total of 12 such aquariums were filled with 30 liters of filtered and disinfected freshwater. The experimental fish chosen for the study were Nile tilapia (*Oreochromis niloticus*) fry, which were obtained from a commercial hatchery. They were carefully transported in oxygenated polythene bags and introduced to the Inland Fisheries Research Station (IFRS), College of Fisheries Science, Junagadh. Here, the fry were acclimatized in circular FRP tanks with proper aeration and feeding. The experiment was conducted during the year 2020-21, and the experimental design involved randomly selecting the *O. niloticus* fry and distributing them across three different experimental groups with ten treatments in three replications. This distribution followed a completely randomized design (CRD), and the overall experiment lasted for a duration of 60 days.

Experimental Feed

The fry of O. niloticus exhibit a protein requirement of 40% in their diet, as indicated by Rathore and Yusufzai in 2018. Maintaining this consistent protein requirement of 40%, we prepared a total of four experimental diets for zinc nanoparticle (in the form of ZnO) supplementation, each offering three different treatments (10 mg/kg, 20 mg/kg, and 30 mg/kg), along with a shared control diet for all three treatments, as detailed in Table-1. These experimental diets were formulated using the Pearson Square Method, following the methodology outlined by Pearson and Tauber in 1984.

Each tank was equipped with a plastic cover to prevent fish from leaping out. Prior to commencing the experiment, the fish were fed a control diet for a period of 10 days. No deliberate efforts were made to manipulate or regulate the environmental conditions, which remained consistent throughout the entire experiment. Water quality parameters were assessed on a weekly basis throughout the experimental duration.

To maintain a hygienic environment, the experimental tanks were manually cleaned, and siphoning was performed daily to eliminate excess feed pellets and residual fecal matter. The removed water was replaced with an equal volume of clean water. This maintenance routine was diligently carried out over the 60-day experimental period.

Table 1: Composition of experimental diet prepared by using ZnO as zinc nanoparticle

	Diet prepared with 40 % protein			
	T_0 (control)	**T_1 (10 mg/kg zinc)**	**T_2 (20 mg/kg zinc)**	**T_3 (30 mg/kg zinc)**
Fish meal	74	74	74	74
Tapioca Powder	10	10	10	10
Wheat Flour	10	10	10	10
Plant oil	2	2	2	2
Fish oil	2	2	2	2
Vitamin and Mineral	2	2	2	2
Zinc nanoparticles	0	10 mg/kg	20 mg/kg	30 mg/kg

Each tank was covered with a plastic cover to prevent fish from jumping out. The fishes were fed with a control diet for 10 days before the commencement of the experiment. No attempts were made to stimulate or control the environmental condition. The experimental conditions were kept same throughout the experiment. The weight was measured at an interval of 15 days to assess the growth. The fishes were starved overnight before taking body weight. While the water quality parameters were analysed on weekly basis during the whole experimental period. The experimental tanks were cleaned manually and siphoning was done every day in order to remove excess feed pellets and the remaining faecal matter. An equal volume of clean water replaced the siphoned water. This was carried out throughout the experimental period of 60 days.

Analysis of Antioxidant Chemicals

On the 60th day, 10 fishes were randomly collected from each treatment set and sacrificed to perform the antioxidant assay to get the disease resistance.

Sample preparation

The fish muscle tissues were dissected, thoroughly washed with ice-cold 0.9% NaCl, weighed, minced, and homogenized (10% w/v) using phosphate buffer (pH 7.0). The homogenate that was centrifuged at 1000×g for 20 min at 4 °C was used for the estimation of catalase (CAT), superoxide dismutase (SOD), Malondialdehyde (MDA), and glutathione (GSH) of fish tissue.

Sulfoxide dismutase (SOD)

Two different types of sample were run. One is blank and others are with sample. For blank sample 193.3 µL Tris-EDTA and 6.7 µL pyrogallol were used. Whereas for the sample 192.64 µL of Tris-EDTA buffer (pH-8.5) + 6.7 µL of pyrogallol + 0.66 µL of tissue homogenate were mixed. The samples were run in the spectrophotometer at the wavelength of 420 nm (Shimatzu, UV-Viz. 2600). The procedure was carried out as per Cortés-Ríos *et al.* (2017) [7].

Catalase (CAT)

For catalase activity, above prepared samples were taken and 2 ml of dichromate/acetic acid was added and blue colour appears. The samples were put in the water bath for 10 min for heating. Sample colour changes into green. The samples were then cooled at room temperature and run in spectrophotometers at the wavelength of 570 nm. The procedure was carried out as per Hadwan, M. H. (2016) [16].

Glutathione peroxidase (GPX)

Prepared samples were taken and 5 % 5 – sulfosalicylic acid solution was added and centrifuged. Samples turned yellow in colour. The sample was run in spectrophotometer at 412 nm. The procudeure was carried out as per Wheeler *et al.* (1990) [49].

Malondialdehyde (MDA)

Samples were taken and 600 µL of the TBA solution was added into each vial containing standard and samples (200

µL). Mix well. Vials were then incubated at 95° C for 60 min then tubes were cooled at room temperature for 10 min. 300 µl of n-butanol was added and mixed. Then the samples were transferred into 96 well plate for analysis. Samples were then run in spectrophotometer at the wavelength of 532 nm. The procedure was carried out as per Lykkesfeldt, J. (2001) [29].

Hematological Parameters

Hematological parameters like hemoglobin, red blood cells (RBCs), white blood cells (WBC), and hematocrit value were measured. The hematological parameter measurement was carried out at the end of the experiment in *O. niloticus* fry. For measurement of hematological parameter, blood was drawn by cardiac puncture using 1ml syringes that rinsed first with 2.7% EDTA solution. The point of insertion for heart puncture was ventral, midway between the anterior bases of the pectoral fins. Blood was then collected in Eppendorf tubes coated with 20µl of 2.7% EDTA solution. After blood collection in Eppendorf tubes, it was kept in a plastic container filled with ice to avoid blood clotting (Li *et al*., 2006) [27]. The amount of haemoglobin, red blood cells (RBCs), white blood cells (WBCs), and hematocrit value present in the blood was measured from 20µl of blood using a complete blood count machine i.e. fully automated cell counter. The procedure was carried out as per Bojarski *et al.* (2018) [6] and Svobodova *et al.* (1991) [45].

Statistical Analysis

All data were subjected to a one-way analysis of variance (ANOVA) considering zinc nanoparticle incorporations as variables. All means were compared by the Duncan's Multiple Range Test using SPSS version 22.

Results and Discussion

Antioxidant chemicals

Sulfoxide dismutase (SOD)

The data related to the Superoxide Dismutase (SOD) enzyme activity within *O. niloticus*, as influenced by various treatment conditions, are exhibited in Table 2 and visually depicted in the graphical representation in Figure 1. Notably, highest SOD enzyme activity was obtained in treatment T_3, where it reached a substantial value of 8.55±0.300 µmol/min/mg tissue. However, lowest SOD enzyme activity was conspicuously witnessed in the T_0 treatment, with a value of 6.40±0.008 µmol/min/mg tissue, indicating a relatively lower enzymatic response. Furthermore, treatments T_1 and T_2 exhibited intermediary SOD enzyme activities, with values of 7.23±0.230 µmol/min/mg tissue and 7.88±0.150 µmol/min/mg tissue, respectively, as shown in Table 2. Overall, there was a significant difference among the treatments.

These findings collectively signify a noteworthy trend, wherein the augmentation of zinc nanoparticles is positively correlated with a significant enhancement in SOD enzyme activity. However, it is intriguing to note that the SOD activity of T_2 treatment mirrors that of treatments T_1 and T_3, indicating that the difference between treatment T_1 and T_2 and T_2 and T_3 were at par. This implies that while zinc nanoparticle supplementation leads to increased SOD enzyme activity, there is a point at which further augmentation may not yield additional benefits in terms of SOD activity, as observed in the T_1, T_2, and T_3 treatments where the difference in SOD activity was not significant.

The probable reason behind the observed increase in superoxide dismutase (SOD) activity can be attributed to zinc, which serves as a significant source of antioxidants with a profound impact on the overall antioxidant capacity, of which SOD is a crucial component (Onderci *et al*., 2003) [34]. It is well-established that the inclusion of probiotics and nanoparticles in fish diets has demonstrated a notable enhancement in SOD activity (Ai *et al*., 2011) [1]. This suggests that these dietary additives play a role in bolstering the fish's antioxidant defense mechanisms. Furthermore, similar findings were reported by Kishawy *et al.* (2020) [24], who conducted research on Nile tilapia. Their study revealed that supplementation with zinc led to a considerable increase in SOD activity. This underscores the importance of zinc as a dietary component for promoting enhanced antioxidant capabilities within fish, which can have positive implications for their overall health and well-being.

Catalase (CAT)

Catalase is a crucial enzyme that plays a significant role in determining the antioxidant status of fish. We collected data on the activity of this enzyme, known as catalase (CAT), in *O. niloticus* under various treatment conditions. You can find the detailed results in Table 2, and we have presented them graphically in Figure 2.

In our study, we found that the highest catalase enzyme activity was observed in treatment T_3, with a notable value of 8.77±0.320 µg/mg tissue. On the contrary, the lowest catalase activity was observed in treatment T_0, with a value of 5.15±0.120 µg/mg tissue, indicating a relatively weaker enzymatic response. For treatments T_1 and T_2, the catalase activities were intermediary, measuring at 6.88±0.150 µg/mg tissue and 7.23±0.120 µg/mg tissue, as outlined in Table 4.43.

Our results indicate that as the supplementation of zinc nanoparticles increased, the activity of the catalase enzyme also increased. The likely reason behind the observed increase in catalase activity can be attributed to the essential role played by antioxidant enzymes like catalase, particularly when fish are subjected to stressful conditions (Raza, 2012; Johansen, 2005) [40, 21]. However, it's important to note that there was no significant difference between treatment T_1 and T_2 (with a p-value greater than 0.05), meaning that the catalase activity in these two treatments was statistically similar. In summary, our findings reveal a clear trend where higher levels of zinc nanoparticles positively correlate with an enhancement in catalase enzyme activity, indicating improved antioxidant status in the fish. Nevertheless, it's interesting to observe that the catalase activity in treatments T_1 and T_2 is not significantly different, suggesting a point at which further increases in zinc nanoparticles may not yield additional benefits in terms of catalase activity, similar to what we observed in the SOD enzyme activity in treatments T_1, T_2, and T_3.

Kishawy *et al.* (2020) [24] conducted a study yielding similar results to those observed with the increase in zinc supplementation, which revealed a noteworthy rise in catalase (CAT) activity in Nile tilapia. This finding indicates that the presence of increasing concentrations of zinc nanoparticles in the fish's diet results in the effective elimination of free radicals. This phenomenon has been substantiated by previous research demonstrating that the supplementation of selenium has a similar effect, leading to an increase in catalase activity and the subsequent activation of the antioxidant defense system (Patching & Gardiner, 1999; Kumar *et al*., 2018) [26, 36]. In summary, the rise in catalase activity in response to

increased zinc supplementation suggests a strengthening of the fish's antioxidant defense mechanisms, which can be crucial in mitigating oxidative stress and ensuring their overall well-being.

Glutathione peroxidase (GPX)

Glutathione peroxidase (GPX) is a crucial enzyme that plays a vital role in removing harmful peroxides from the fish's body. We've conducted a study to investigate the activity of the Glutathione Peroxidase (GPX) enzyme in *O. niloticus* under different treatment conditions. You can find the comprehensive results in Table 2, and these findings are visually represented in Figure 3.

In present research, we observed that the highest GPX enzyme activity was recorded in treatment T_3, with a substantial value of 3.54±0.020 µg/mg tissue. In contrast, the lowest GPX activity was noted in treatment T_0, where it registered at 1.90±0.008 µg/mg tissue, indicating a relatively weaker enzymatic response. For treatments T_1 and T_2, the GPX activities fell between these extremes, with values of 2.25±0.050 µg/mg tissue and 3.05±0.020 µg/mg tissue, as detailed in Table 4.44. A clear trend was seen where increase in zinc incorporation in Nile tilapia diet has shown clear increasing pattern.

Our results clearly indicate that as the supplementation of zinc nanoparticles increased, the activity of the glutathione peroxidase enzyme also significantly increased, revealing a positive correlation between the two. The greater the zinc nanoparticle supplementation, the more effective the GPX enzyme becomes in removing peroxides from the fish's body.

The observed increase in glutathione peroxidase (GPX) activity is likely due to the role of zinc as a significant source of antioxidants (Onderci *et al*., 2003) [34]. Zinc, being an essential micronutrient, is known to play a crucial part in reinforcing the body's antioxidant defense mechanisms. Zinc, when present in sufficient quantities in the diet, contributes to the overall antioxidant capacity by providing the essential resources required for enzymes like GPX to effectively counteract oxidative stress. GPX, as a fundamental element of the body's antioxidant defense system, plays a pivotal role in neutralizing detrimental reactive oxygen species, thus preserving the integrity of cellular structures and supporting overall health (Onderci *et al*., 2003) [34]. Similar results were obtained by Zhou *et al*. (2009) [52] when selenium nanoparticle s (Se-np) was incorporated in the diet of crucian carp, *Carassius auratus gibelio*. Selenium has shown to increase the glutathione activity in crucian carp. Similar result was also observed by (Bell *et al*., 1986) [4]. Similar results were also obtained by Kishawy *et al.* (2020) [24], where increase in the zinc supplement has shown increase in the enzyme activity of glutathione peroxidase in Nile tilapia. Which shows that peroxidase radicals are eliminated from the body.

Malondialdehyde (MDA)

Elevated levels of malondialdehyde within the fish organism are indicative of heightened oxidative stress. We conducted a study to investigate malondialdehyde levels in O. niloticus under various treatment conditions, and the results are comprehensively detailed in Table 2, with graphical representation provided in Figure 4.

In our research, we identified the highest malondialdehyde activity in treatment T_0, measuring at 1.25±0.010 µmol/mg protein, while the lowest activity was observed in treatment T_3, with a value of 1.03±0.030 µmol/mg protein. Treatments T_1 and T_2 exhibited intermediary malondialdehyde levels, recording values of 1.13±0.040 µmol/mg protein and 1.06±0.008 µmol/mg protein, as documented in Table 2. Treatment T_1, T_2 and T_3 treatment did not show significant difference.

Our results indicate that as the supplementation of zinc nanoparticles increased, the malondialdehyde activity decreased. This suggests that zinc nanoparticle supplementation has a mitigating effect on oxidative stress within the fish's body, resulting in reduced malondialdehyde activity. Importantly, it's worth noting that there were no significant differences in malondialdehyde activity among treatments T_1, T_2, and T_3, emphasizing the consistent impact of zinc nanoparticles on these treatments. Which shows that incorporation of zinc nanoparticle in Nile tilapia fry at higher amount will not provide higher disease resistance.

Zinc, being an essential micronutrient, is known to play a crucial part in reinforcing the body's antioxidant defense mechanisms, due to that malondialdehyde (MDA) has shown decrease although not significant but it has shown to decrease the oxidative stress in Nile tilapia (*Oreochromis niloticus*) fry (Onderci *et al*., 2003) [34]. Results of the present study clearly indicate that incorporation of zinc nanoparticle reduces the oxidative stress in the Nile tilapia. Which will indirectly inhibit the growth and reproduction of pathogen in the fish body. Similar results were also observed by Kishawy *et al.* (2020) [24], it also showed decreasing trend in the activity of malondialdehyde (MDA). In that research, they incorporated zinc in different forms and malondialdehyde has shown decreasing trend with increase of zinc incorporation.

Table 2: Effect of supplementation of zinc nanoparticles on antioxidant chemicals in Nile tilapia (*O. niloticus*) fry

Treatments	SOD (µmol/min/mg tissue)	CAT (µg /mg tissue)	GPX (µg/mg tissue)	MDA (µmol/mg protein)
T_0 (control)	6.40±0.008^{a}	5.15±0.120^{a}	1.90±0.008^{a}	1.25±0.010^{b}
T_1 (10 mg/kg)	7.23±0.230^{b}	6.88±0.150^{b}	2.25±0.050^{b}	1.13±0.040^{a}
T_2 (20 mg/kg)	7.88±0.150^{bc}	7.23±0.120^{b}	3.05±0.020^{c}	1.06±0.008^{a}
T_3 (30 mg/kg)	8.55±0.300^{c}	8.77±0.320^{c}	3.54±0.020^{d}	1.03±0.030^{a}

Values are expressed as mean ± SE. a, b, c values in a column with different superscript differ significantly (p<0.05).

Haematological parameters

White blood cells (WBC)

The outcomes of our investigation concerning the white blood cell (WBC) count in *O. niloticus*, conducted under various treatment conditions, are presented in Table 3 and graphically depicted in Figure 5. These results shows the effect of different treatments on the WBC count, which serves as a critical indicator of the fish's immune response.

Notably, among the treatments assessed, the highest WBC count was observed in treatment T_3, with a value of 47.54±2.16 $\times 10^3$/µL. This marked the highest level of white blood cells recorded among the treatments. Whereas, the treatment T_0, which served as our control group, exhibited the lowest WBC count, which was 35.84±0.05 $\times 10^3$/µL.

However, the treatments T_1 and T_2 were at par (p>0.05), with values of 39.30±0.80 and 43.08±0.63 $\times 10^3$/µL, respectively. The white blood cells count observed in the experiment was within range of healthy tilapia (Osman *et al.*, 2018) [35]. Which was 35 – 50 $\times 10^3$/µL. These results signify variations in the fish's immune response under the influence of different levels of supplementation with zinc nanoparticles. A trend between the WBC count was observed, where as the supplementation of zinc nanoparticles increased, the WBC count also demonstrated a significant increase. This suggests a potential relationship between zinc nanoparticle supplementation and the enhancement of the fish's immune response, as reflected in the elevated WBC counts.

In an investigation involving the administration of silver nanoparticles (Ag-NPs) to common carp (*Cyprinus carpio*) in order to assess their influence on hematological parameters, the outcomes demonstrated a notable augmentation in the white blood cell (WBC) count, as reported by Vali *et al*. in 2020. These findings are consistent with the findings of the present study. Similar results were also obtained by the Kishawy *et al.* (2020) [24], where WBC increases, with increase in the zinc supplementation in Nile tilapia. Another researcher have also reported similar results of WBC in *Mugil* species (Rajan and Rohini, 2021) [38].

Higher zinc nanoparticle supplementation correlates with increased WBC count, potentially enhancing the immune system of fish, particularly *O. niloticus*. Nevertheless, additional research is warranted to substantiate this hypothesis.

Red blood cells (RBC)

The results of our investigation regarding the red blood cell (RBC) count in *O. niloticus*, conducted under various treatment conditions, are presented in Table 3 and visually represented in Figure 6. These findings provide insights into the influence of different treatments on the RBC count, a crucial parameter related to the fish's physiological well-being.

It's worth noting that the highest RBC count was identified in treatment T_0, with a measurement of 1.61±0.005 $\times 10^6$/µL. This marked the highest RBC count among the treatments. While, both treatments T_1 and T_2 exhibited the lowest RBC counts, with values of 1.57±0.004 $\times 10^6$/µL and 1.57±0.008 $\times 10^6$/µL, respectively. Importantly, statistical analysis indicated that there were no significant differences in RBC counts between the treatments (p>0.05). The zinc supplement in diet did not have effect on the RBC count (p>0.05). However, it was in the range of healthy tilapia which is 1.5 – 2.0 $\times 10^6$/µL (Osman *et al.*, 2018) [35].

In summary, our investigation revealed that the RBC counts across the treatments did not exhibit significant differences, implying that the treatments had a consistent effect on RBC counts with no difference. While these findings contribute to our understanding of the fish's physiological response, further research may be necessary to explore potential treatment-related variations in RBC count in *O. niloticus*.

Furthermore, a study by Kishawy *et al* (2020) [24] found that giving Nile tilapia zinc nanoparticle supplement didn't change their red blood cell counts, and this is in agreement with the findings of present study. Another study by Rajan and Rohini in 2021 discovered the same trend when using zinc oxide nanoparticles for mrigal fish, supporting the idea that zinc nanoparticle supplementation doesn't really affect red blood cell counts in different types of fish. These combined findings help us understand how zinc affects various aspects of fish blood, showing that the response can vary depending on the specific blood measurement and fish type.

Hematocrit (HCT)

The outcomes of our examination of hematocrit levels in *O. niloticus* under distinct treatment conditions are detailed in Table 3 and visually depicted in Figure 7. These results offer valuable insights into how different treatments influence the hematocrit percentage, which is a vital indicator of the fish's physiological status.

It's essential to highlight that the highest hematocrit percentage was observed in treatment T_3, reaching 36.77±1.81%. Conversely, the lowest hematocrit percentage was recorded in treatment T_0, measuring 21.46±0.19%. These findings demonstrate a significant difference in hematocrit levels across the treatments (p<0.05). Treatment T_0 and T_1 were significantly at par. Similar case was also observed in treatment T_2 and T_3. The haematocrit (%) in all the treatments were observed in the range of healthy Nile tilapia (Osman *et al.*, 2018) [35]. Which was 21 – 38 %. Additionally, results of the present study revealed a notable trend – an increase in hematocrit percentage with an increase in zinc nanoparticle supplementation. This suggests a positive relationship between the amount of zinc nanoparticles administered and the enhancement of hematocrit levels in *O. niloticus*, indicating potential benefits to the fish's overall physiological condition.

Similar results were obtained by the Kishawy *et al.* (2020) [24]. They observed that, zinc supplementation has shown to increase haematocrit (%) in Nile tilapia compared to control. Rajan and Rohini (2021) [38] have also concluded same in *Mugil* species.

In summary, our investigation showcases the considerable impact of different treatments on hematocrit levels, with an evident correlation between increased zinc nanoparticle supplementation and elevated hematocrit percentages. These findings contribute to our understanding of how such supplementation can positively affect the physiological well-being of *O. niloticus*, although further research is needed into the treatment-related variations in hematocrit levels.

Hemoglobin

The results from our assessment of hemoglobin levels in *O. niloticus* under diverse treatment conditions are comprehensively presented in Table 3, and they are visually represented in figure 8. These findings provide valuable insights into the influence of various treatments on hemoglobin concentrations, a critical marker of the fish's physiological condition.

It is crucial to emphasize that the highest hemoglobin concentration was observed in treatment T_2, measuring 7.83±0.99 g/dl. In contrast, the lowest hemoglobin concentration was detected in treatment T_0, with a measurement of 7.22±0.05 g/dl. Meanwhile, treatments T_1 and T_3 displayed hemoglobin concentrations of 7.54±0.59 g/dl and 7.82±0.30 g/dl, respectively). The haemoglobin was found to be in the range of healthy tilapia (Osman *et al.*, 2018) [35]. Which was 7 – 11 g/dL. Moreover, results indicated that the increase in zinc nanoparticle supplementation did not yield any significant differences in hemoglobin levels (p>0.05). This suggests that the variations in zinc nanoparticle supplementation across the treatments did not have a significant impact on hemoglobin concentrations in *O.*

niloticus.

In summary, our investigation highlights the influence of different treatments on hemoglobin levels, with no significant variations noted with increased zinc nanoparticle supplementation. These findings contribute to our understanding of how these treatments impact the physiological well-being of *O. niloticus*, although further research may be needed to explore potential treatment-related differences in hemoglobin concentrations.

In this experiment, as the level of zinc nanoparticle supplementation increased in diet, the hemoglobin did not show significant difference (p>0.05). Similar results were also obtained by the Kishawy *et al.* (2020) [24], when they incorporated zinc in the diet of koi carp (*Cyprinus carpio*).

Platelets

The results from our experiment of platelet counts in *O. niloticus* under various treatment conditions are comprehensively detailed in Table 3 and graphically depicted in figure 9. These findings offer valuable insights into the impact of different treatments on platelet counts, an important marker of the fish's physiological state.

It is important to highlight that the highest platelet count was observed in treatment T_3, measuring 371.56±3.58 × 10^3/µL. In contrast, the lowest platelet count was found in treatment T_0, with a measurement of 328.28±1.39 × 10^3/µL. Meanwhile, treatments T_1 and T_2 exhibited platelet counts of 334.08±1.87 and 353.10±2.10 × 10^3/µL, respectively. The platelets count observed in the experiment is in the range of healthy tilapia (Osman *et al.*, 2018) [35]. Which is 320-475 ×10^3/µL. Notably, the introduction of zinc nanoparticle supplementation resulted in a significant increase in platelet counts in Nile tilapia. However, it is worth noting that treatment T_2 was significantly at par to treatments T_1 and T_3.

In summary, our investigation sheds light on how various treatments affect platelet counts, with a notable increase in platelet counts attributed to zinc nanoparticle supplementation. These findings enhance our understanding of how these treatments influence the physiological well-being of *O. niloticus*, although further research may be necessary to explore potential treatment-related variations in platelet counts.

Additionally, a study conducted by Kishawy *et al.* in 2020 [24] revealed that the administration of zinc nanoparticle supplements to Nile tilapia did not result in any significant alterations in their red blood cell counts, aligning closely with the findings of our present study. An investigation carried out by Rajan and Rohini in 2021, a similar trend emerged when they used zinc oxide nanoparticles in mrigal fish. This corroborates the notion that zinc nanoparticle supplementation does not exert a substantial influence on red blood cell counts across different fish species. All these findings help us better understand how zinc can affect different aspects of fish blood. It shows that the impact of zinc can vary depending on what we're measuring in the blood and the type of fish we're studying.

Table 3: Effect of supplementation of zinc nanoparticles on haematological parameters in Nile tilapia (*O. niloticus*) fry

Treatments	WBC (×10^3/µL)	RBC (×10^6/µL)	Hematocrit (%)	Hemoglobin (g/dl)	Platelets (×10^3/µL)
T_0 (control)	35.84±0.05^a	1.61±0.005^a	21.46±0.19^a	7.22±0.05^a	328.28±1.39^a
T_1 (10 mg/kg)	39.30±0.80^{ab}	1.57±0.004^a	25.44±1.19^a	7.54±0.59^a	334.08±1.87^a
T_2 (20 mg/kg)	43.08±0.63^b	1.57±0.008^a	33.07±1.13^b	7.83±0.99^a	353.10±2.10^{ab}
T_3 (30 mg/kg)	47.54±2.16^c	1.59±0.010^a	36.77±1.81^b	7.82±0.30^a	371.56±3.58^b

Values are expressed as mean ± SE. a, b, c values in a column with different superscript differ significantly (p<0.05).

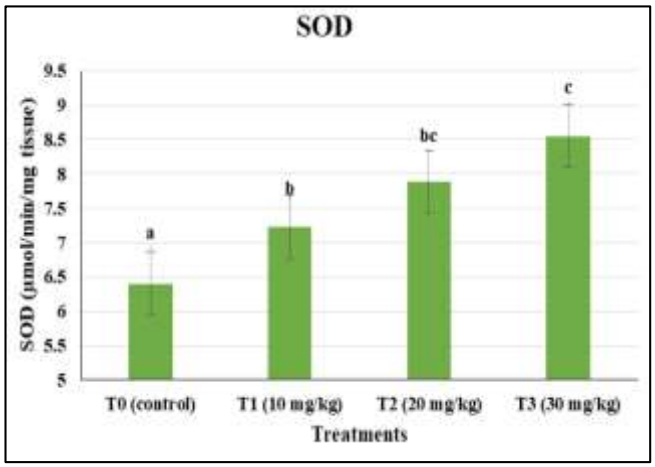

Fig 1: Effect of different treatments of zinc nanoparticles diets on sulfoxide dismutase (SOD) enzyme activity (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period

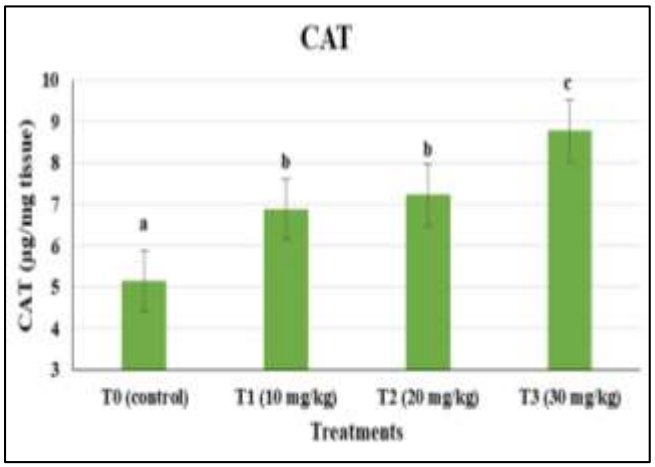

Fig 2: Effect of different treatments of zinc nanoparticles diets on catalase (CAT) enzyme activity (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period

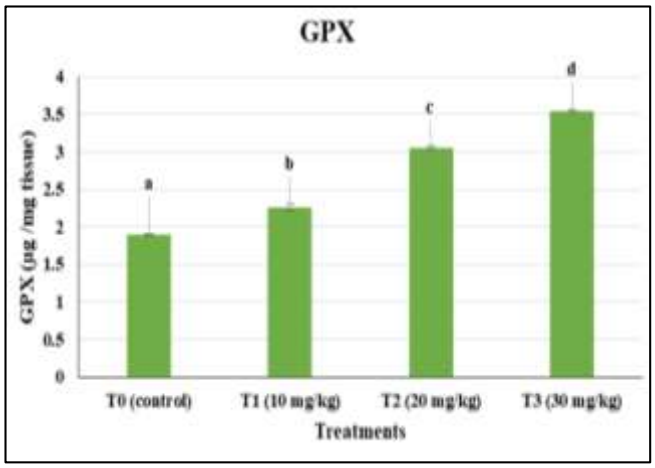

Fig 3: Effect of different treatments of zinc nanoparticles diets on glutathione peroxidase (GPX) enzyme activity (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period

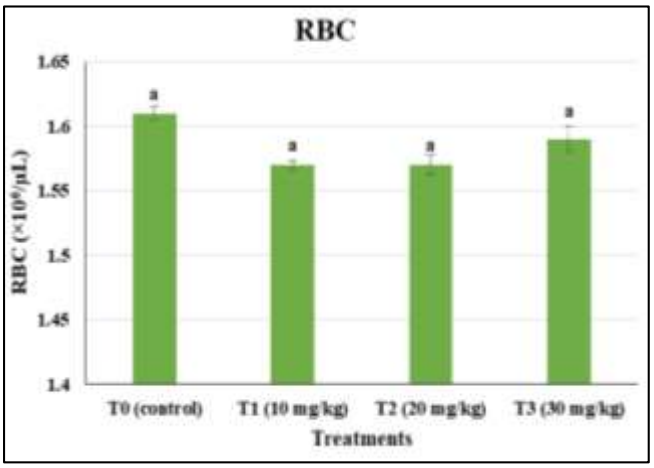

Fig 6: Effect of different treatments of zinc nanoparticles diets on red blood cells (RBC) count (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period

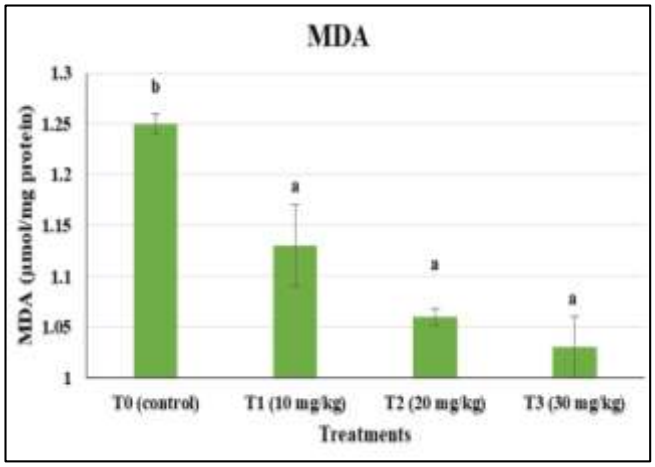

Fig 4: Effect of different treatments of zinc nanoparticles diets on malondialdehyde (MDA) enzyme activity (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period.

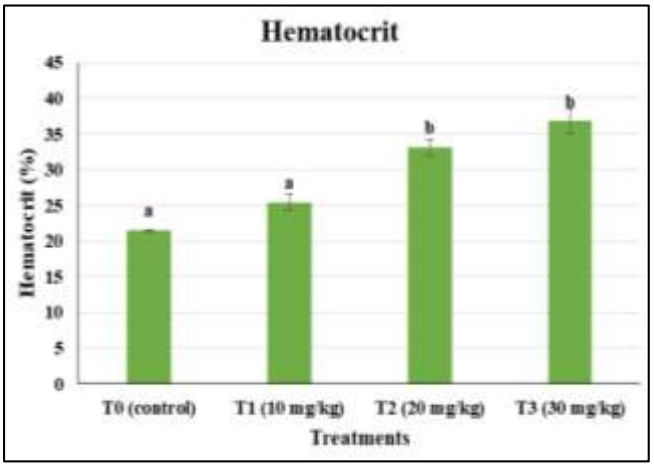

Fig 7: Effect of different treatments of zinc nanoparticles diets on haematocrit (%) count (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period.

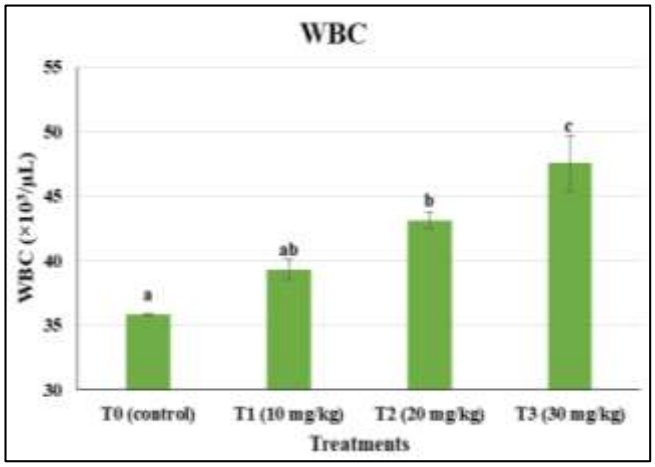

Fig 5: Effect of different treatments of zinc nanoparticles diets on white blood cells (WBC) count (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period.

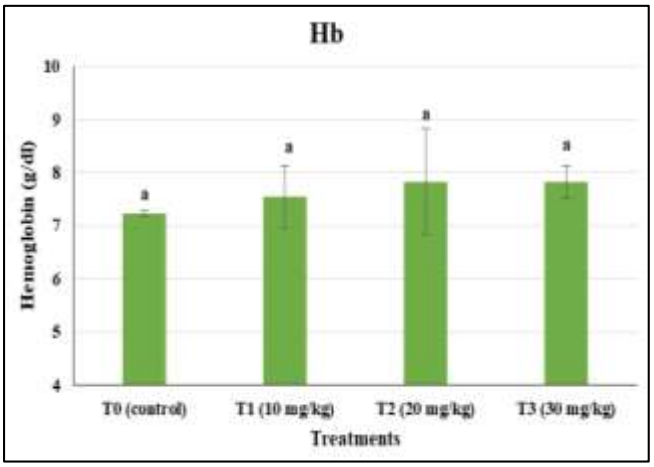

Fig 8: Effect of different treatments of zinc nanoparticles diets on hemoglobin (g/dl) count (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period

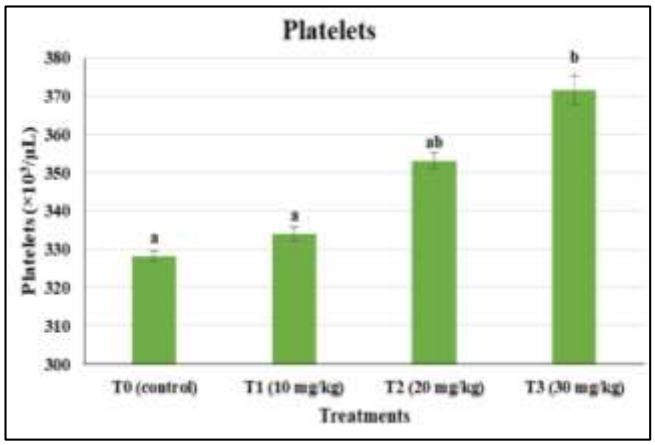

Fig 9: Effect of different treatments of zinc nanoparticles diets on platelet count (Mean ± SE) in *O. niloticus* fry at the end of 60 days of culture period.

Conclusion

This study was conducted with the primary objective of investigating the effects of supplementing zinc nanoparticles on disease resistance and hematological parameters in Nile tilapia (*Oreochromis niloticus*) fry. The research spanned a period of 60 days, during which four distinct experimental groups were established: T_0 (the control group), T_1 (administered 10 mg/kg of zinc nanoparticles), T_2 (administered 20 mg/kg), and T_3 (administered 30 mg/kg). Zinc nanoparticles were specifically chosen as the nanoparticle ingredient of interest. To assess the impact of this supplementation, the activity of essential antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and the level of malondialdehyde (MDA), were evaluated at the conclusion of the 60-day experiment. Furthermore, hematological parameters were examined to provide a comprehensive understanding of how zinc nanoparticle supplementation influenced the health and disease resistance of the Nile tilapia fry.

The results of this study revealed several noteworthy findings. As zinc supplementation levels increased, there was a significant increase in the activity of SOD, CAT, and GPX enzymes, which are crucial in the body's defence against oxidative stress and damage. Simultaneously, MDA levels decreased, indicating a reduction in lipid peroxidation and oxidative stress. This trend suggested that the zinc nanoparticle supplementation had a positive impact on the fish's antioxidant defence system, helping to mitigate oxidative damage.

In the case of hematological parameters, the results indicated a significant increase in various key blood parameters. White blood cell (WBC) counts, hematocrit levels, hemoglobin count, and platelet counts all exhibited an upward trend with increasing zinc nanoparticle supplementation. These findings suggested an improvement in the fish's immune response and overall health status. However, red blood cell (RBC) counts did not show any significant variations across the experimental groups.

In conclusion, the outcomes of this study underscore the potential benefits of zinc nanoparticle supplementation for enhancing disease resistance and overall health in Nile tilapia fry. The increase in antioxidant enzyme activity and the improvement in hematological parameters provide strong support for the idea that zinc nanoparticle supplementation can contribute to better disease resilience and overall well-being in these fish. As a result, we recommend the T_3 treatment group, which received 30 mg/kg of zinc nanoparticles, as an effective strategy to boost disease resistance in Nile tilapia (*Oreochromis niloticus*) fry, leading to improved overall health and well-being in these aquatic organisms.

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