Molecular investigation of hormonal alterations in Oreochromis niloticus as a bio-marker for long-term exposure to zinc oxide nanoparticles

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ABSTRACT
This study aimed to investigate the potential effects of long-term zinc oxide nanoparticles’ (Zn-ONPs) exposure to the hormonal profile of $O$. niloticus and their application as a bio-marker for fresh water pollution by Zn-ONPs. Two hundred-fourty female $O$. niloticus were used. Growth hormone (GH), thyroid stimulating hormone (TSH), triiodothyronine (T3), tetraiodothyronine (T4), follicular stimulating hormone (FSH), luteinizing hormone (LH), estradiol (E2), glucagon, insulin, Adrenocorticosteroid hormone (ACTH) serum concentrations and the expression levels of GH, insulin-like growth factor gene (IGF-I), insulin and insulin receptor A (IRA) genes were determined. The levels of GH, TSH, FSH, LH, T3, T4, Estradiol, E2, insulin and GH, IGF-I, insulin and IRA genes expression decreased significantly in Zn-ONPs’ exposed fish in a concentration-dependent manner; meanwhile, serum ACTH and cortisol increased. The alterations in hormone levels and their gene expressions are considered as good bio-markers for the detection of fresh water pollution with Zn-ONPs.

Introduction
Despite their useful properties, nanoparticle hazards on the biological system are poorly understood up till now. The wide production and abundant use of nanoparticles facilitate their possibility to induce hazards, for example, their use of water waste treatment leads to their spread in the aquatic environment causing more hazards for both humans and aquatic beings. The hazards of nanoparticles (NPs) generally in the aquatic environment may be related to their ability to induce an oxidative stress [1].

Zinc oxide Nanoparticles (Zn-ONPs) are one of the most widely used nanomaterials in agriculture, industry, medicine and water treatment [2]. They are widely produced and applied in many products including sunscreen, waste water treatment and environmental remediation. Direct and indirect releases of Zn-ONPs into aquatic environments via engineering applications and sewage effluent increase the exposure of humans and ecosystems to NPs’ impacts [3]. The potential effects of Zn-ONPs on aquatic ecosystems have attracted special attention and investigated in various aspects, including biochemistry and toxicology and have been confirmed to be one of the most harmful NPs in the aquatic environment [2].

Fahmy and Sayed [4] examined the Zn-ONPs’ cytotoxicity and genotoxicity of fresh water molluscan Coelatura aegyptiaca and confirmed the antioxidant

disruptions caused by Zn-ONPs, also the embryotoxicity to marine medaka, Oryzias melastigma, was examined by Cong et al. [5], suggesting the sensitivity of embryonic stage of marine medaka to Zn-ONPs’ toxicity more than aqueous Zn ions; whereas Kim et al. [6] examined the molecular alterations in Larval Zebra fish subjected to Zn-ONPs and SO4NPs and suggested that Zn-ONPs slightly induced cell differentiation and pathways associated with the immune system and activated several key genes involved in cancer cell signalling.

Subashkumar and Selvanayagam [7] showed the acute toxicity of Zn-ONPs for common carp (C. carpio) at 4.897 mg/L and this is in good in accordance with the acute toxicity of ZnONPs in zebra fish (96 h LC50 of 4.92 mg/L). Previously we recorded that, the 96 h LC10, LC50 and LC90 of ZnONPs on $O$. niloticus were 2.3 ± 0.5, 3.1 ± 0.4 and 4.4 ± 0.2 mg/L, respectively [2]. In other study for our group, the 69 h LC50 was 5.5 ± 0.6 and 5.6 ± 0.4 for $O$. nilotica and T. zillii, respectively [8].

Fish is considered the major sensitive organism affected by ecosystem toxicities caused especially by heavy metals [9]. The use of bio-markers is an effective strategy for monitoring the aquatic environment and diagnosing the negative impact [2]. $O$. niloticus is considered one of the topmost candidate species for fresh water aquaculture due to its meat quality, market demand and well-established rearing protocol;
however, stressful environmental conditions are expected to deteriorate the performance and health conditions of fish; these changes on the cellular and molecular levels could be used as bio-markers for pollution [10].

We previously investigated the impacts of Zn-ONPs on *O. niloticus* and proved the negative impacts of Zn-ONPs’ sub-lethal concentration on *O. niloticus* haematology manifested by the study revealed the presence of normocytic normochromic anaemia, leucocytosis, heterophilia, lymphopenia and monocytopenia and a significant increase in the serum levels of alkaline phosphatase, aminotransferases, urea, creatinine and erythrocytic nuclear and morphological abnormalities along the experimental periods in all treated groups [2]. In other works, the ultrastructure impacts of Zn-ONPs’ sub-lethal concentration on *O. niloticus* gills and the liver were proved [3], and the pathological alterations in gills the liver [11] and the brain [12] were investigated. On the molecular levels, our groups proved the potential impacts of Zn-ONPs’ sub-lethal concentration on *O. niloticus* antioxidant enzymes’ activity and gene expression in the gills and liver [13], muscle [1] and brain [8] manifested by the increase of malondialdehyde, and reduction in glutathione levels, and inhibition of superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GR), glutathione peroxidase (GPx), and glutathione-S-transferase (GST) activities and gene expression.

Hormones, chemicals secreted from the endocrine glands, affect the vital functions of the body. Hormone levels are affected by environmental factors surrounding the organism and, therefore, are considered one of the fastest and best bio-markers for environmental pollutants [14]. Very few reports detected the toxic effects of Zn-ONPs at hormonal and molecular levels in *O. niloticus*. Our previous work detected LC₅₀ and the acute toxicity of Zn-ONPs on hormonal and molecular levels [15]. Within the current study, we examined the effects of Zn-ONPs’ long-term exposure on *O. niloticus* growth performance, hormonal disturbances and molecular alterations, suggesting the use of hormonal and molecular alterations as early bio-markers for monitoring the nanoparticle pollution of fresh water.

### Material and methods

#### ZnONPs’ preparation and characterization

Zn-ONPs’ preparation and characterization was done as mentioned in Reference [15]. Zn-ONPs were supplied from Sigma-Aldrich, Steinheim, Germany (CAS Number 1314-13-2) of concentration 50 wt % in H₂O, the average particle size (APS) was < 35 nm. The particle size distribution (hydrodynamic diameter) was < 100 nm using the dynamic light scattering (DLS) technique, pH 7 ± 0.1 (for aqueous systems) and density 1.7 ± 0.1 g/mL at 25°C. Suspensions of Zn-ONPs in concentrations of 0.41, 0.20 and 0.14 mg/l were daily prepared in distilled water using a sonicator (JL-360, Shanghai, USA). To characterize the Zn-ONPs’ shape and size, a small drop of aqueous Zn-ONPs solution was air-dried by directly placing it onto a 300-mesh carbon-coated copper grid then examined under the transmission electron microscope (TEM) (JEM- 1011, JEOL, Japan) data previously published [1,15]. Inductive coupled plasma mass spectrometry (ICP-MS) was applied for the quantification of Zn-ONPs’ concentrations in the aquariums at zero, 12 and 24 h of exposure, to ensure that there is no change in ZnONPs’ concentrations (Table 1). The final concentrations of Zn-ONPs were consistent with their initial concentrations, indicating that they were constant throughout the exposure period.

### Fish preparation

Two hundred and forty female *O. niloticus*, weighing 11.2 ± 0.25 g, were purchased from El-Abbassa fish hatchery, Egypt (AL-Sharkia Province). The fish were held in twelve glass aquaria (*n* = 20 individuals/aquarium) measures 80 × 60 × 50 cm containing 100 L fresh dechlorinated water, (pH7.16 ± 0.3, 0.52 mM Ca2⁺, and 0.24 mM Mg2⁺) that was changed daily. A continuous system of water aeration (Eheim Liberty 150 Bio-Espumador cartridges) and a 10:14-h light:dark photoperiod was maintained. Temperature was maintained at 28 ± 2°C and dissolved oxygen, at 7.0 ± 0.5 mg/L. Fish were fed with a commercial fish diet [1]. The daily feed amount was 3% of body weight and the fish were fed three times daily. Fish were acclimatized for 14 days before the beginning of the experiments.

### Ethical statement

The study was performed following the main guidelines for care and of the laboratory animals that was approved by National Institutes of Health (NIH) and authorized by local authorities of Cairo University, Egypt.

### Fish grouping and induction of Zn-ONPs’ toxicity

After the period of acclimatization, 180 fish were randomly divided into four groups, 45 fish in each. Fish in each group were held in three aquarium (*n* = 15 individuals/aquarium) containing 100 L water. The first

| Concentrations (mg L⁻¹) | Zero | 12 | 24 |
|-------------------------|------|----|----|
| Control                 | Nd   | Nd | Nd |
| 0.41                    | 0.41 ± 0.003 | 0.40 ± 0.003 | 0.38 ± 0.001 |
| 0.20                    | 0.20 ± 0.005 | 0.19 ± 0.006 | 0.17 ± 0.004 |
| 0.14                    | 0.14 ± 0.003 | 0.14 ± 0.003 | 0.12 ± 0.001 |

Note: nd = not detected.

Table 1. The actual ZnONPs’ concentrations (mg L⁻¹) in the exposure water.
group was left as control; the second, third and fourth groups were exposed to Zn-ONPs of 0.41 mg/l (1/10 of LC50 Zn-ONPs), 0.2 mg/l (1/20 of LC50 Zn-ONPs) and 0.14 mg/l (1/30 of LC50 Zn-ONPs), respectively for 1 month. Water was changed daily for the control and treated groups. The different concentrations of Zn-ONPs were prepared daily as mentioned above. The desired concentrations of Zn-ONPs were added daily to the changed water and three samples were taken daily from the different aquaria for monitoring if there is any change in ZnONPs’ concentration as mentioned above.

**Determination of growth performance and mortality rate**

Fish of each group (triplicate) were counted and weighted individually using an electrical balance (FA-T Series model FA3004T) and body gain, body gain % and mortality rate were determined according to Siddiqui et al. [16].

**Blood and tissues sampling**

Thirty fish (10 from each aquarium) were used for obtaining six blood samples from each group by pooling at the thirty days of the experiment. Fish were anaesthetized with buffered tricaine methane sulphonate (20 mg/L) and blood was collected from the caudal vertebral vessels according to Christine et al. [17]. Blood was collected in tubes without anticoagulant and left at room temperature for coagulation then centrifuged at 3500 rpm for 5 min for the separation of serum using Sorvall Legend X1 Centrifuge (cat. number 75004220, Thermo Fisher Scientific United States), serum was kept under −80°C for hormonal investigations. Then fish were killed by the transaction of the spinal cord. Pituitary gland, liver, pancreas and muscles were quickly removed from six fish in each group (two fish from each aquarium), weighed, rinsed with ice-cold saline, frozen in liquid nitrogen, and kept at −80°C until used for RNA isolation to detect the expression levels of GH, IGF-I, Insulin, IRA and β-actin genes, respectively.

**Biochemical determination**

Hormones of fish were measured using serum ELISA kits purchased from MyBioSource.com. The catalogue numbers of kits were (MBS2701414) for growth hormone, (MBS282744) for thyroid-stimulating hormone, (MBS705669) for adrenocorticotropic hormone, (MBS03576) for follicular stimulating hormone, (MBS283097) for Luteinizing hormone, (MBS2700145 and MBS701162) for T3 and T4, (MBS283228) for E2, (MBS017490) and (MBS034316) for insulin and Glucagon. But, cortisol ELISA kit from DioMed (Cairo, Egypt) was used for the determination of fish Serum cortisol levels.

**Molecular determination**

 Stored liquid nitrogen tissues of the pituitary gland, liver, pancreas and muscle were used for total RNA isolation using RNeasy Mini Kit (Cat. No. 74104) from Qiagen. Quantification of obtained total RNAs was then performed using a Nano-Drop® ND-1000 Spectrophotometer (Wilmington, Delaware, USA). Samples with purities larger than 1.8 were used for the following steps. QuantiTect SYBR Green master mix kit with Cat. No. 204143 from Qiagen was also used for the detection of the expression levels of GH, insulin, IGF-I and IR genes using the corresponding designed primer, as listed in Table 2 and manufactured with the aid of Sigma-Aldrich using β-actin gene as a housekeeping gene and determining the relative fold changes using the 2−ΔΔCt formula of Livak and Schmittgen [18].

**Statistical analysis**

SPSS 21 version was used to analyse the obtained data using one-way ANOVA test followed by Duncan’s test for significant differences and expressing the results as Mean ± SE. P-values < 0.05 were accepted as statistically significant.

**Results**

**Effect of Zn-ONPs (1/10, 1/20 and 1/30 of LC50) chronic exposure on O. niloticus growth performance**

There was a significant reduction in body gain, final B.wt., and body gain percentage within the treated groups in a way related to Zn-ONPs’ concentrations (Table 3).

**Effect of Zn-ONPs’ (1/10, 1/20 and 1/30 of LC50) chronic exposure on O. niloticus mortality rate**

The results in (Table 4) revealed that, the mortality rate of groups 2, 3 and 4 was 28.3%, 15% and 8.3%, respectively by increasing Zn-ONP’s concentration.

**Table 2. Primer sequences of GH, IGF-I, Insulin, IRA and β-actin genes.**

| Gene   | Primer Sequence (5’→3’) | Gene accession number | Annealing temp °C/cycles | References |
|--------|-------------------------|-----------------------|--------------------------|------------|
| GH     | CTGTCGTGTGCTGTGTCACTGTCACTGTCAGTCGTAGAGGAGACGCCCAAACAC | M26916.1 | 60 °C/40 cycle | [19] |
| IGF-1  | CCGAATCTCCTGACCTGCTGCTGCTGCTGTCAGTCGTAGAGGAGACGCCCAAACAC | EU272149 | 60 °C/40 cycle | [20] |
| Insulin| ACACCAACAGGAGAGATGGTCTGGGTTTGATTGACAGATTCC | XM_003458679.5 | 56 °C/50 cycle | [21] |
| IRA    | AGACGGTAGAAACGAGTCGGCCTGGCTTACCTCAACGCCAAG | KCS17071.1 | 65 °C/50 cycle | [21] |
| β-actin| ACCCACACAGTGCCCATCCAGGTCCAGACGCAGGAT | EU887951 | 60 °C/40 cycle | [22] |
In the present study, we evaluated the possible effect of Zn-ONPs on the hormone profile in Oreochromis niloticus. We also validated the use of hormones as bioindicators for NP exposure. The high exposure of humans and animals to NPs is the main subject that motivates us to do this work. There is great importance for such a study. First, to the best of our knowledge, this is the first record about the chronic effect of Zn-ONPs in O. niloticus. Second, it is important to assess the impacts resulted from Zn-ONP exposure, not only for aquatic organisms but also for human and allconsummated beings. Third, O. niloticus is a vital indicator of the aquatic ecosystem and has been used as a well-established model to detect the aquatic toxicity in many toxicological research studies [23]. The hazards of Zn-ONPs by increasing its environmental concentration may lead to higher toxicity and pollution for aquatic ecosystem [24].

**Effect of Zn-ONPs’ (1/10, 1/20 and 1/30 of LC50) chronic exposure on Oreochromis niloticus growth performance.**

The current study has shown a great effect of Zn-ONPs on the O. niloticus weight gain, final body weight, body gain percentage and final body length (Table 3), serum GH levels (Table 5) and mRNA levels of pituitary hormone genes (Figure 1); all parameters were decreased significantly in a dose-dependent manner (Figure 1).

**Effect of Zn-ONPs’ (1/10, 1/20 and 1/30 of LC50) chronic exposure on Oreochromis niloticus hormone concentrations.**

Table 5. Effect of Zn-ONPs’ chronic exposure on Oreochromis niloticus hormone concentrations.

| Treatments                  | Control          | 1/30 LC50 Zn-ONPs | 1/20 LC50 Zn-ONPs | 1/10 LC50 Zn-ONPs |
|-----------------------------|------------------|-------------------|-------------------|-------------------|
| GH (pg/ml)                  | 658.4 ± 26.48    | 569 ± 28.6         | 516 ± 24.8        | 486 ± 22.2        |
| TSH (μIU/ml)                | 2.42 ± 0.11      | 2.18 ± 0.1         | 1.91 ± 0.06       | 1.83 ± 0.04       |
| ACTH (pg/ml)                | 171.6 ± 17.6     | 235.4 ± 9.3        | 2513 ± 7.2        | 8213 ± 6.3        |
| FSH (mIU/ml)                | 4.4 ± 0.92       | 3.91 ± 0.43        | 3.6 ± 0.83        | 2.4 ± 0.75        |
| LH (mIU/ml)                 | 20.1 ± 2.4       | 17.2 ± 2.8         | 13.6 ± 2.2        | 10.4 ± 1.62       |
| T3 (pg/ml)                  | 374.8 ± 29.6     | 326 ± 26.4         | 296.4 ± 28.4      | 264 ± 22.8        |
| T4 (ng/mL)                  | 166.4 ± 16.4     | 139.8 ± 14.8       | 112.8 ± 12.5      | 92.6 ± 6.4        |
| Cortisol (μg/ml)            | 5.36 ± 1.1       | 7.4 ± 0.82         | 8.6 ± 1.2         | 11.4 ± 3.3        |
| Estradiol (pg/ml)           | 33.6 ± 3.4       | 29.7 ± 1.25        | 26.7 ± 1.85       | 21.4 ± 2.2        |
| Insulin (μIU/ml)            | 5.82 ± 0.94      | 4.96 ± 0.46        | 4.1 ± 0.36        | 3.2 ± 0.62        |
| Glucagon (mU/L)             | 4.46 ± 0.63      | 4.48 ± 0.64        | 4.51 ± 0.64       | 4.53 ± 0.44       |

Note: Means ± SE in the same row with different superscripts are significantly different at P < 0.05.

**Discussion**

In the present study, we evaluated the possible effect of Zn-ONPs on the hormone profile in O. niloticus. We also validated the use of hormones as bioindicators for NP exposure. The high exposure of humans and animals to NPs is the main subject that motivates us to do this work. There is great importance for such a study. First, to the best of our knowledge, this is the first record about the chronic effect of Zn-ONPs in O. niloticus. Second, it is important to assess the impacts resulted from Zn-ONP exposure, not only for aquatic organisms but also for human and all consummated beings. Third, O. niloticus is a vital indicator of the aquatic ecosystem and has been used as a well-established model to detect the aquatic toxicity in many toxicological research studies [23]. The hazards of Zn-ONPs by increasing its environmental concentration may lead to higher toxicity and pollution for ecosystem [24].
or the decrease in hormones’ controlling metabolism as thyroxin and insulin or also the increase of stress hormones as cortisol. GH controls the growth, reproduction and regeneration in both animal and human cells with diverse effects and multiple targets in vertebrates usually as a growth promoter [25]. At the molecular levels, the mRNA transcriptional levels of GH were clearly also down-regulated due to chronic Zn-ONPs’ exposure which reflected and confirmed the hormonal and molecular alterations. Indeed, there are no reports about the possible mechanisms of negative impact of NPs especially Zn-ONPs on pituitary gland function in fish or aquatics, except our previous work [15] that proved the negative impact of sub-lethal concentration of Zn-ONPs on *O. niloticus* hormone profile. This impact on pituitary gland may be explained by the ability of these molecules to penetrate blood–brain barriers and accumulation in different parts of the brain as hypothalamus and pituitary [26], induces a disturbance in brain antioxidant systems, increasing reactive oxygen species (ROS) that could affect pituitary function and hypothalamus-pituitary axis, and deteriorate DNA and gene expression in fish [1].

**Effect of Zn-ONPs’ chronic exposure on *O. niloticus* mortality rate**

The mortality rate of groups 2, 3 and 4 was 28.3%, 15% and 8.3%, respectively. Regarding the mortality of the fish exposed to chronic Zn-ONPs’ toxicities that will flow to the accumulative toxic effect of Zn-ONPs during the long exposure [27]. Hormonal disturbances, sensory, neurological and metabolic alterations are the major physiological effects of fish exposed to toxic substances and pollutants; these effects usually affect mortality rates [28].

The study of Bai et al. [29] on zebrafish (*Danio rerio*) embryos after 96 h of exposure to 50 and 100 mg/l of Zn-ONPs showed the mortality of zebrafish at a level of about 30 and 65%. No increased mortality was observed at concentrations 0.5–6.3 mg/l of Zn-ONPs. It is believed that it is caused by the creation of nano-aggregates that block the access of oxygen through channels in the pores of choriocarcinoma or by the formation of ROS.

**Effect of Zn-ONPs’ chronic exposure on *O. niloticus* TSH, T3 and T4 hormones**

Thyroid stimulating hormone (TSH), as glycoprotein from adenohypophysis, is secreted and released in response to thyroid releasing hormone (TRH) to enhance the secretion of thyroid hormones (T3 and T4) from thyroid glands, modulating different multiple metabolic processes that inhibit TSH production in a negative feedback mechanism [30]. The first role of T3 and T4 in mammals as fish is playing a task in metamorphosis and early development. Fish T3 and T4 play a vital role in adapting to environmental changes such as salinity and thermal changes as well as the maintenance of tissue and cellular activities, reproduction, development and embryogenesis [31]. Zn-ONPs’ chronic exposure results in a significant decrease in T3 and T4 serums in relation to the exposure concentration. Despite the decrease of T3 and T4, the levels of TSH
also decreased (Table 5), which refers to the failure in hypothalamic-pituitary-thyroid axis. The decrease in T3 and T4 levels could be explained by the inability of pituitary gland to respond and compensate that by increasing the production of TSH. The adverse effect of Zn-ONPs on pituitary gland may be due to the accumulation of Zn in gland tissue resulting in Zn toxicity. Regarding fish there is no study discussed the adverse chronic effects of Zn-ONPs on T3, T4 and TSH except our previous work Alkaladi et al. [15] that proved the deleterious effect of sub-lethal toxicity of Zn-ONPs on O. niloticus hormones. There are some reports in rats as studied by Luab et al. [32] who proved the adverse effects of Zn-ONPs on T3 and T4 in rats received intraperitoneal dose (30 and 60 mg/kg) for 7, 14, and 28 days, and showed a high significant decrease in the level of T3 and T4 at a high and low dose for 7, 14 and 28 days, while the level of TSH showed no significant change in all doses and durations of time.

Effect of Zn-ONPs’ chronic exposure on O. niloticus FSH, LH and Estradiol hormones

FSH and LH, the fish gonadotropins from pituitary glands play a major role in gametogenesis and steriodogenesis like all other vertebrates [33]. No exact description of actual function of both hormones in fish in spite of several previous studies because of the little genetic studies in adult fish [34]. FSH especially in O. niloticus has the main role in vitellogenesis and earliest development of gonads by enhancing gonadal testosterone and Estradiol (E2) [35]. The role of LH and FSH in fish is more complex than being only stimulating androgen secretion from Leydig cells or regulating Sertoli’s cell functions as in mammals; this is because of their prominent steroidogenic potency [36]. The decrease in the serum FSH and LH levels due to Zn-ONPs chronic exposure suggests gonadal dysfunction in the form of E2 depletion in all treated groups which may affect the reproductive function of fish. E2 has an important role in the growth and differentiation of reproductive tissues, maintaining follicular development and fertility [37]. Zn-ONPs and other metal nanoparticles’ toxicities altered the serum Estradiol levels [15,38]. In male rats received 5, 10, 20 and 40 mg/kg-B.W ZnONPs for 56 days the level of LH was decreased [26], rats injected intraperitoneally with a high dose of ZnONPs (40 mg/kg) indicated a reduction in FSH concentration compared with the controls [39].

Effect of Zn-ONPs’ chronic exposure on O. niloticus ACTH and cortisol hormones

Adrenocorticotrophin (ACTH) from hypophysis and cortisol hormone from ductless glands are the most hormones contributing to fish stress [40]. Cortisol secretion is stimulated by ACTH production from hypophysis through multiple various mechanisms using cAMP/PKA as the main pathway [41]. In the present study, Zn-ONPs chronic toxicity in all exposure concentrations results in the increase of cortisol and ACTH levels in relation to exposure concentrations (Table 5). The increase of cortisol in this study could be explained in three ways: the first one is the induction of its production by the action of ACTH from pituitary gland as indicated in our results, the second one possibly Zn-ONPs transport cholesterol into the inner mitochondrial membrane by increasing the Steroidogenic Acute Regulatory protein expression and eventually converts the cholesterol to cortisol increasing its levels [42]. The third explanation is that Zn-ONPs are considered strong stressors on fish, especially as they accumulate on the gills and lead to a decrease in the fish’s ability to extract oxygen [3] and also stimulate the production of free radicals in various parts of the body; these stresses stimulate the production of cortisol. Ellis et al. [43] reported that cortisol hormone is the main indicator of stress response in fish and has relevancy to fish welfare, so its increases directly modifying fish behaviour by affecting the brain function. The induction of ACTH production in this study despite the increase of cortisol may be interpreted by the failure of hypothalamic-pituitary-adrenal axis, resulting from the deleterious effect of reactive oxygen species (ROS) on hypothalamus or pituitary gland. ROS attenuate the glucocorticoid-induced down-regulation of pro-opiomelanocortin in pituitary corticotrophs, thereby promoting an increase in hypothalamus-pituitary-adrenal axis (HPA) activity via reduced negative feedback [44]. The HPA axis is the main neuroendocrine system that regulates responses to stress. The production of high levels of ROS into the glands that comprise the HPA axis is associated with the activation of a stress-response system in several models of stress, including social isolation, and inflammatory and infectious diseases. HPA axis hyperactivity induced by redox imbalance may occur by a reduction in negative feedback through a decrease in glucocorticoid receptor translocation to the cellular nucleus in corticotroph cells of the pituitary [44].

Effect of Zn-ONPs’ chronic exposure on O. niloticus insulin, glucagon levels and gene expression levels of insulin, hepatic IGF-1 and muscular IRA

Zinc balance in the body is particularly important for the operation of the pancreas. The zinc ions are involved in regulating the signals of insulin, glucagon, and activation of digestive exoenzymes. Zinc promotes hepatic glycogenesis through its actions on the insulin pathways and thus improves glucose utilization. Zinc is also known to keep the structure of insulin and has a role in insulin biosynthesis, storage and secretion [45]. Despite the importance of Zn for production, secretion and storage of insulin the present results
indicated that, Zn-ONPs’ exposure accompanied with a decrease of insulin level (Table 5) and insulin gene expression (Figure 1); the impact of Zn-ONPs on biological functions depends on its morphology, particle size, exposure time, concentration, pH, and biocompatibility. Zn-ONPs can penetrate the different cell organelles and induce ROS that inhibit gene expression, decreasing mRNA of insulin gene and directly decreased insulin level. ROS may cause Zn-ONPs-induced cytotoxicity; they are more toxic than other metallic oxide NPs because of their ion-shedding ability. They are not very soluble in neutral solutions and in acid environments. Zn-ONPs-induced significant cytotoxicity in a size-dependent manner [46]. The inhibition of muscular IRA gene expression may be related to the decreased insulin levels. Despite this study, there is a lack of reports contributing the effect of Zn-ONPs on hormones levels and their gene expression in fish, but there are some reports in other animals as rats. In rats Zn-ONPs’ supplementation could induce a hyperglycaemic response by the inhibition of insulin secretion. A report in β-cell islets showed that zinc inhibits insulin secretion concentration-dependent [47]. Alterations in insulin (decrease) and cortisol (increase) induced by the exposure to Zn-ONPs reported in the present study induce metabolic disturbances such as hyperglycaemia that has recently been reported in other species of animals [48].

The liver is the master organ of synthesis and secretes IGF-1. We found decreased hepatic gene expression levels of IGF-1 gene in fish exposed to the different concentrations of Zn-ONPs in a concentration-dependant manner. Zn-ONPs accumulate in the liver and dissociate Zn that accumulates in hepatocytes deteriorating it. Xu et al. [49] found decreased serum levels of IGF-1 in rats administrated 203 and 610 mg/kg Zn-ONP. In accordance with a previous finding that liver function is injured by Zn-ONPs, we speculate that decreased IGF-1 levels were due to Zn-ONP damage to liver function.

**Conclusion**

Long-term exposure of *O. niloticus* to Zn-ONPs resulted in the alterations in growth performance, hormones’ concentrations and gene expression that could be considered as good bio-markers for the detection of fresh water pollution with Zn-ONPs.

**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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