Egyptian Journal of Veterinary Sciences
https://ejvs.journals.ekb.eg/

Ameliorative Effect of Zinc Oxide Nanoparticles against *Streptococcus parauberis* Experimental Infection in Nile Tilapia (*Oreochromis niloticus*)

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ZINC oxide nanoparticles (ZnO-NPs) exhibit antimicrobial properties against most bacteria in many in vitro studies. However, in vivo validations of their antimicrobial effects in Nile tilapia have not yet been investigated. This study aimed to investigate the impacts of ZnO-NPs and their therapeutic effectiveness in experimentally infected Nile tilapia with S. parauberis. For safety assessment, 120 fish were divided into four groups. They were fed a diet supplemented with 0, 125, 250 and 500 mg/kg dry feed of ZnO-NPs for 7 days, respectively. Blood and tissue samples were collected to evaluate haematological, biochemical and histopathological alterations. Another 120 fish were divided into four groups. Group 1 (G1) served as the negative control, while G2, G3, and G4 were challenged intraperitoneally with S. parauberis. G2 was fed on a basal diet without medication. G3 and G4 were treated with ZnO-NPs (125 mg/kg basal diet) or ampicillin (50mg/kg basal diet) supplemented diet for 7 days. Fish were carefully observed for any abnormalities in fish behaviour, clinical alterations and mortalities for 16 days post-challenge. Blood and tissue samples were collected from different groups. Survival rates were 100% (G1), 46.67% (G2), 53.34% (G3), and 53.34% (G4). Several adverse changes in haematological, biochemical, immunological parameters and tissue histopathology were recorded in the challenged groups. Treatment of Nile tilapia with dietary doses of ZnO-NPs or ampicillin ameliorated the effect of *S. parauberis* infection. Despite the nontoxic impact of high dietary doses of ZnO-NPs on Nile tilapia, further improvement is needed to achieve a higher survival rate.

Keywords: Antibiotic resistance, Tissue residue, CD 79b, Streptococoosis, S. parauberis, Nanoparticles.

Introduction

Tilapia farming plays a vital role in supporting rural communities by providing income and sustenance. Over the past two decades, tilapia production has experienced a significant increase, making it a popular choice in national and international markets. The most commonly farmed species is Nile tilapia (Oreochromis niloticus),

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(Received 15/01/2024, accepted 20/03/2024)
DOI: 10.21608/EJVS.2024.262933.1784

cultivated in earthen ponds and cage cultures across Asia, Africa, and the Americas. In 2019, the global production of Nile tilapia reached 4.6 million tons, marking a shift from subsistence to commercial farming and enabling the global trade of tilapia products. China, Indonesia, and Egypt were the leading producers, with production volumes of 1.6 million tons, 1.3 million tons, and 1.1 million tons, respectively [1]. However, the intensification of tilapia farming has also resulted in the emergence of disease outbreaks, particularly bacterial infections, which pose a threat to sustainable production. With their high nutrient concentrations, water temperatures, and fish densities, Tilapia farms create favourable conditions for bacterial growth, including the development of virulent bacterial strains that can potentially cause zoonotic infections.

Streptococcosis is one of the significant infectious bacterial diseases affecting freshwater and marine aquaculture worldwide, with a detrimental impact on its sustainability [2]. Moreover, streptococcal infection has a zoonotic potential that significantly impacts human food safety [1]. Chief among streptococcal species is *Streptococcus parauberis*, gram-positive cocci, which has an increased prevalence worldwide. Clinically, S. parauberis infects the body surface, fins, and muscles, causing haemorrhages and leading to various pathological changes in host tissues, including pericarditis, meningitis, pale gills, darkening of the skin, and abnormal spleen pictures as reported earlier in olive flounder (*Paralichthys olivaceus*) [3]

Although vaccines are highly effective in preventing the spread of infectious diseases, bacterial vaccines are not fully effective due to the variety of species and strains involved in the infections [4]. Furthermore, vaccination failure is also common in the aquatic industry due to the diversity of fish species, fish size, vaccine formulation, and the route of administration [5]. Adding more layer of complexity, serotypic variations in streptococcal species are also involved in vaccination failure [6]. Fish are commonly treated with various antibiotics to combat streptococcal infections, including ampicillin, florfenicol, erythromycin, doxycycline, and oxytetracycline. Accordingly, frequent antibiotic treatments are necessary due to repeated bacterial infections. These facts result in a significant accumulation of antibiotics in fish carcasses and the release of drugs into aquatic environments, increasing the likelihood of bacterial resistance, which poses a global threat to public health [7; 8]. All this information encourages scientists to search for environmentally friendly antimicrobial agents to combat antibiotic-resistant pathogenic microorganisms, including *S. parauberis*.

Nanotechnology has provided great potential to improve aquaculture through innovative nanoparticles, referred to as nano-aquaculture [9]. Using nanoparticles in aquaculture can help improve fish production, limit disease spread, and enhance water and wastewater treatment. Mechanistically, nanoparticles can increase the absorption of nutrients by the gut tissue of fish, leading to a decrease in unabsorbed feed that would otherwise be excreted. Moreover, minerals in the form of nanoparticles present in aquafeeds can be easily absorbed by the cells compared to their larger counterparts, contributing to better growth performance, improved fish health, and immune response [10; 11; 12]. We and others have previously reported that inorganic compounds in nano size have demonstrated remarkable antimicrobial properties in vitro, with zinc oxide nanoparticles (ZnO-NPs) being particularly effective against a range of bacteria, including Streptococcus parauberis [13], Staphylococcus *aureus, Escherichia coli* [14], *Campylobacter jejuni, Salmonella typhimurium* [15] and *Acinetobacter baumannii* [16]. However, their efficacy in live fish infected with bacteria remained unclear. In this study, we aim to evaluate the potential effects of different dietary doses of ZnO-NPs in Nile tilapia (safety experiment) and to evaluate their therapeutic effectiveness against pathogenic *S. parauberis* infection in Nile tilapia (challenge experiment).

Material and Methods

Ethical considerations:

The Institutional Animal Care and Use Committee (IACUC) approved the present study. The approval number is (CU/ II/ F /15 /21).

Nanoparticles

Chemically synthesized ZnO-NPs of average size 30±5 nm was purchased from the Nanotech centre, 6th October, Giza, Egypt. Our group previously tested these synthesized nanoparticles in vitro for their antimicrobial activity against *Streptococcus parauberis* [13]; a certificate of analysis of the utilized nanoparticles was included in the supplementary materials.

Experimental diet

Standard commercial pelletized fish ration was mixed with different concentrations of ZnO-NPs using gelatin as a binding material and appetizer. The chemical composition of the standard commercial fish ration was included in the supplementary data. Four experimental diets were prepared, labelled as D1, D2, D3, and D4. D1 was the control diet without any ZnO-NPs, while D2, D3, and D4 were formulated to contain 125, 250, and 500 mg ZnO-NPs powder /kg dry feed, respectively.

Safety experiment

This experiment aimed to assess the impact of different doses of ZnO-NPs on growth performance**,** haematological, blood biochemical parameters, and histopathological changes in various organs of Nile tilapia (Oreochromis niloticus). A total of 120 fish with an average weight of 88±20 g were randomly divided into four groups with three replicates each. The first group was fed a basal diet without ZnO-NPs supplementation and served as a control group. The second, third, and fourth groups were fed a diet supplemented with 125, 250, and 500 mg ZnO-NPs powder/kg basal diet for 7 consecutive days. Blood and tissue samples were collected from the fish of each group at the end of the feeding trial.

Growth performance parameters

To assess the impact of ZnO-NPs on growth performance, the weight of fish was recorded at the beginning and end of the experimental period. Parameters such as final body weight (FBW), weight gain (WG), weight gain percentage, specific growth rate (SGR), and feed intake (FI) were calculated using the method outlined by Kishawy et al. [17]

Haematological analysis

Blood samples were collected on EDTA anticoagulant from the caudal vein of fish (5 fish/group). They were used for analysis of haematological parameters, haemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte count (RBCs), total leucocyte count (TLC), and differential leucocyte count (DLC) according to Feldman et al. [18].

Blood biochemical analysis

The biochemical parameters were analyzed in the following ways: serum total proteins (Spectrum, Egypt, Cat no. # 310 001) were tested according to Gornall et al. [19], while albumin (Spectrum, Egypt, Cat no. # 210 001) was estimated according to Dumas et al. [20]. To determine the level of globulins, serum albumin was deducted from the value of serum total proteins. The Albumin/Globulin ratio was obtained by dividing the serum albumin values by those of serum globulins. Creatinine (Spectrum, Egypt, Cat no. # 234 001) value was determined according to Bowers and Wong. [21]. Glucose (Spectrum, Egypt, Cat no. # 250 001) was determined after Trinder. [22]. Furthermore, alkaline phosphatase (ALP) (Spectrum, Egypt, Cat no. # 216 001) was determined after Moss. [23].

Ammonia concentration (Spectrum, Egypt, Cat no. # 220 000) was measured according to Tietz. [24]. When combined with Glutamate Dehydrogenase (GLDH), ammonia reacts with α-ketoglutarate and NADPH to produce Glutamate and NADP+. The ammonia level in the plasma can be determined by measuring the decrease in absorbance (NADPH to NADP+) at 340 nm. It is essential to quickly separate the plasma from blood cells to obtain accurate results. It is recommended to perform the analysis within 30 minutes of sample collection.

Histopathological examination

Three fish from each group were euthanised, and tissue samples were collected from the liver, kidney, spleen, gills, and brain at the end of the experimental period for histopathological examination [25]. In brief, fresh tissue specimens were fixed in 10% neutral buffered formalin. The formalin-fixed tissues were then processed and embedded in paraffine wax. Five-micron tissue sections were then stained with H&E. using the method described by Bancroft et al., [25].

Tissue residue determination

Muscle specimens from three fish in each group were obtained to determine the Zn residues according to Christian, and Feldman [26]. Muscle specimens were weighed and digested with 7 ml of H_2SO_4 and 2 ml of H_2O_2. The sample was then assayed using an atomic absorption spectrometer (Thermo Scientific iCE 3300, German) to determine the metal content. This method involves the acid digestion of the sample in digestion tubes using a temperature-controlled hot plate.

Challenge experiment

Microbial isolate, media, and growth conditions

Streptococcus parauberis (Genbank accession number: MW534372) was used in the present study. The bacterial cultures were maintained on

trypticase soy agar (TSA; Oxide, UK) or Müller Hinton agar (MHA; Sigma-Aldrich, USA). The clinical isolate was previously recovered from the brain of Nile tilapia positively diagnosed with a bacterial infection, collected during disease outbreaks in a fish farm in Ismailia governorate, Egypt [13].

Determination of S. parauberis LD50 %:

Before the challenge, the lethal dose-50 (LD50) of *S. parauberis* in Nile tilapia was determined experimentally by intraperitoneal (I/P) injection to verify the optimum bacterial concentration to perform the bacterial challenge. Sixty (n=60) apparently healthy Nile tilapia (*O. niloticus*) fish with an average weight of 88±20 g were used in the pathogenicity test to determine the median lethal dose of *S. parauberis* according to [27]. In brief, the 60 fish were randomly and equally divided into six groups (i.e., 10 fish/group). Fish were maintained in six glass aquariums; the first group was I/P injected with 0.2 ml of sterile (PBS) and served as a control group. Meanwhile, the second group was I/P injected with 0.2 ml of *S. parauberis* at a dose of 6*108 CFU/ml in sterile (PBS). Each following group was inoculated with 10-fold serial dilution. Fish were observed for mortalities for 10 days.

Experimental design:

One hundred twenty (120) apparently healthy Nile Tilapia (*O. niloticus*) of average weight 88±20 g were randomly divided into 4 equal groups (duplicate/group). Fish in group (1) were I/P injected with 0.2 ml of PBS and were served as the negative control group. Group (2) was I/P infected with 0.2 ml of 6×106 CFU/ml (LD 50%) of the pathogenic *S. parauberis* bacterial suspension, fed on a basal diet and served as the positive control group. Group (3) was I/P infected with 0.2 ml of 6×106 CFU/ml (LD 50%) of the pathogenic *S. parauberis* bacterial suspension, treated with ZnO-NPs supplemented diet at a dose of 125 mg powder/kg as soon as the appearance of clinical signs (48 h post-challenge) for 7 successive days. Group (4) was I/P infected with 0.2 ml of 6×106 CFU/ml (LD 50%) of the pathogenic *S. parauberis* bacterial suspension, treated with ampicillin supplemented diet at a dose of 50 mg/ kg [27] as soon as the appearance of clinical signs (48 h post-challenge) for 7 successive days, which followed by another 7 days of observation. The entire period of the challenge experiment was 16 days.

Blood and tissue samples were collected from different experimental groups on the 2nd, 3rd , 10th and 16th day post-infection.

Fish were carefully observed throughout the experimental period for any abnormalities in fish behaviour and clinical alterations for 16 days post-challenge. Moreover, the mortality/survival rate % in each group was recorded. Brian tissue of the freshly dead fish was collected for microbiological examination by bacterial re-isolation followed by biochemical identification.

Haematological analysis

Blood samples were collected on EDTA anticoagulant from the caudal vein of fish (5 fish/ group). They were used for analysis of haematological parameters, haemoglobin concentration (Hb), packed cell volume (PCV), erythrocyte count (RBCs), total leucocyte count (TLC), and differential leucocyte count (DLC) according to [18].

Biochemical analysis

The biochemical parameters were following ways: serum total proteins (Spectrum, Egypt, Cat no. # 310 001) were tested according to Gornall et al. [19], while albumin (Spectrum, Egypt, Cat no. # 210 001) was estimated according to Dumas et al. [20]. To determine the level of globulins, serum albumin was deducted from the value of serum total proteins. The Albumin/Globulin ratio was obtained by dividing the serum albumin values by those of serum globulins. Creatinine (Spectrum, Egypt, Cat no. # 234 001) value was determined according to Bowers and Wong. [21]. Glucose (Spectrum, Egypt, Cat no. # 250 001) was determined after Trinder. [22]. Furthermore, alkaline phosphatase (ALP) (Spectrum, Egypt, Cat no. # 216 001) and CK activities (Spectrum, Egypt, Cat no. # 238 001) were determined after Moss. 1982 and Burtis 1999 [23] and [28], respectively. Ammonia concentration (Spectrum, Egypt, Cat no. # 220 000) was measured according to Tietz. [24].

Immunological parameters

Serum Lysozyme Activity (SLA), Interleukin – 6 (IL – 6) and Serum Amyloid A (SAA).

Serum lysozyme activity (SLA) (Sigma Aldrich, USA) test was performed according to [29]. Briefly, twenty-five (25) µl serum was added to 175 µl (0.75 mg/ml) Micrococcus Lysodeikitcus in the reaction buffer in a flat-bottomed 96-well plate. The reduction in absorbance at 450 nm was measured from 0 to 15 min at 25oC in an ELISA reader. One unit of lysozyme activity was defined as a reduction in absorbance per minute. The units

of lysozyme activity were calculated using the hen egg white lysozyme standard curve.

Serum IL-6 (SunRed, Shanghai, Cat no.# 201-00-0007-48t) and SAA (SunRed, Shanghai, Cat no.# 201-00-2144-48t) were determined using the double sandwich ELISA technique. Briefly, The serum samples were added to monoclonal antibody enzyme pre-coated with fish IL-6 or SAA monoclonal antibody. The ELISA plate was incubated, and IL-6 or SAA antibodies labelled with biotin and combined with streptavidin-HRP were added to form an immune complex. The plates were incubated again and washed to remove the un-combined enzyme. Chromogen solutions A and B were added, so the colour of the liquid changed into blue, and finally, at the effect of acid, the colour became yellow. The chroma of colour and concentration of fish IL 6 or SAA of the sample were positively correlated.

Flowcytometric Characterization of CD79b

Spleen specimens were prepared for flowcytometric characterisation of CD79b according to [30]. After weighing spleen specimens, they were rinsed with PBS, placed in a dish, and cut into small particles (1~2 mm3) with scissors. Then, the tissue was ground with a syringe plunger to obtain single-cell suspension. The cells were suspended in an appropriate amount of PBS. At room temperature, the cells were incubated with anti-CD79b antibodies for 30 min. The cells were centrifuged again at 3000 rpm for 5 min. The pellet was resuspended in live/dead marker (Thermo Fisher, USA) according to the manufacturer's protocol for 30 min. The cells were washed and fixed with 4% paraformaldehyde for 30 min. Then, the cells were washed and resuspended in 1x permeabilisation buffer for 15 min. Cells were washed and resuspended in 0.25% BSA for 30 min. The cells were washed and resuspended in 1x PBS before being analysed by flow cytometry (Beckman coulter cytoflex). Live, and singlet cells were gated based on forward and side scatters. Data was analysed using Cytexpert software.

Histopathological examination

Three fish from each group were euthanised, and tissue samples were collected from the liver, kidney, spleen, gills, and brain on the 2^{nd} , 3^{rd} , 10^{th} and 16^{th} -day post-infection for histopathological examination [25]. In brief, fresh tissue specimens were fixed in 10% neutral buffered formalin. The formalin-fixed tissues were then processed and embedded in paraffin wax. Five-micron tissue sections were then stained with H&E. using the method described by Bancroft et al., [25].

Statistical analysis

All data were examined for normality and homogeneity of variance through one-way ANOVA followed by the LSD test at a significance level of 0.05 [31]

Results

Safety experiment results

Growth performance analysis

The effect of different concentrations of ZnO-NPs on the growth performance of Nile tilapia is illustrated in Table (1). Compared to the control group, there were no significant changes in the growth performance parameters of fish groups fed on a diet supplemented with ZnO-NPs.

Hematological results

Statistical analysis of erythrogram revealed a significant increase in PCV%, Hb concentration and RBCs count in fish supplemented with the 125 mg ZnO-NPs/Kg diet compared with the control group. Meanwhile, compared to the control group, non-significant changes were recorded in fish fed on a diet supplemented with the 250 mg ZnO-NPs/Kg diet. Nevertheless, the group of fish supplemented with a 500 mg ZnO-NPs/Kg diet showed significant microcytosis (decreased MCV) with a significant increase in RBCs count compared with the negative control group (Table 2). No significant changes were observed in the leukogram results of different experimental groups.

Biochemical results

Statistical analysis of the tested biochemical parameters (total proteins, albumin, globulins, creatinine, glucose, ALP and ammonia) revealed non-significant changes in fish fed on a diet supplemented with different doses of ZnO-NPs compared to the control group except for significant hypoglycemia in fish supplemented with 500 mg ZnO-NPs/Kg in comparable with the negative control group (Table 3).

Tissue residue result

Statistical analysis of Zn residue revealed a significant increase in muscle Zn concentration in fish groups fed on ZnO-NPs supplemented diet compared to the negative control group (Table 4). However, the highest concentration of Zn (20.49±1.75 ppm) was in the fourth group fed on 500 mg ZnO-NPs/Kg dry feed.

Histopathological findings

Histopathological alterations demonstrated in the control negative group.

Generally, no marked histopathological alterations were demonstrated in the control negative group. The gills revealed normal gill lamellae, with mild dilation of the blood capillaries (Fig. 1a). The liver showed normal hepatocytes, with normal rounded nuclei and normal hepatopancreas (Fig. 2a). Slightly brown intracytoplasmic granules were demonstrated within the hepatocytes of some examined sections, in addition to focal aggregation of melanomacrophages in the hepatopancreas. The spleen of this group has a normal white pulp with normal lymphoid cells and melanomacrophages (Fig. 3a).

Similarly, no alterations were demonstrated in the kidney and brain of this group, which revealed normal renal tubules and normal aggregation of melanomacrophages (Fig. 4a). Normal brain tissue was also demonstrated (Fig. 5a).

Histopathological alterations demonstrated in Oreocromis niloticus fed 125 mg ZnO.

Normal lamellar epithelium, with mild congestion of the central venous sinuses, was demonstrated in the gills of this group (Fig. 1b). The liver showed normally vacuolated hepatocytes with round basophilic nuclei. The hepatopanacreas in some examined sections were focally infiltrated with a few mononuclear cells and melanomacrophages (Fig. 2b). Normal splenic ellipsoid and melanomacrophage centres were demonstrated in the spleen of this group (Fig. 3b). Normal renal tubular epithelial cells lined with round basophilic nuclei were demonstrated in the kidneys (Fig. 4b). The brain revealed normal optic tectum and mesencephalon (Fig. 5b).

Histopathological alterations demonstrated in Oreocromis niloticus fed 250 mg ZnO.

No significant alterations were demonstrated in the gills of this group (Fig. 1c). Normal hepatocytes with few melanomacrophages infiltrating the hepatopancreas were demonstrated in the liver of this group (Fig. 2c). Spleen showed normal red and white pulp and normal melanomacrophages (Fig. 3c). The kidney appeared normal in most examined sections. Only mild vacuolar degeneration of some renal tubular epithelium was demonstrated in some examined sections (Fig. 4c). Normal brain tissue was also demonstrated (Fig. 5c).

Histopathological alterations demonstrated in Oreocromis niloticus fed 500 mg ZnO.

Generally, mild alterations were demonstrated in this group, particularly in the gills and kidneys. The gills revealed focal telangiectasis (Fig. 1d). Normal liver and spleen with no pronounced histopathological alterations were demonstrated in this group (Fig. 2d & 3d). The kidney showed normal histological structure in most examined sections. Only single-cell necrosis was demonstrated in a few examine sections (Fig. 4d). Normal mesencephalon and other brain tissues were demonstrated (Fig. 5d).

Challenge experiment results

LD 50% of S. parauberis

The pathogenicity test result revealed that the LD50 of *S. parauberis* in *Oreochromis niloticus* (Nile tilapia) fish weighing 88±20g was 0.2ml of 6×106 CFU/ml by intraperitoneal inoculation. No mortalities were detected in the negative control group and in the fish group intraperitoneally injected with 0.2 ml of *S. parauberis* at a 6*104 CFU/ml dose in sterile PBS. Meanwhile, the fish group intraperitoneally injected with 0.2 ml of *S. parauberis* at a 6*108 CFU/ml dose in sterile PBS revealed 100% mortality.

Clinical signs and Mortalities:

The first death in the challenged groups was recorded on the 2nd day post-challenge. The mortality stopped on the 12th day post-challenge. The negative control group had no mortalities, clinical signs, or pathological changes throughout the experimental period. On the contrary, high mortality rates were recorded in all challenged groups, ranging between 53.33% in Group 2 (the positive control group) and 46.66% in Groups 3 and 4 (challenged-treated groups; ZnO NP and Amp, respectively). All challenged fish exhibited clinical signs of streptococcosis (haemorrhages in skin, fins and around the brain, detachment of scales, abnormal swimming behaviour, and loss of appetite). Moreover, Gram-positive diplococci oxidase-positive and catalase-negative were reisolated from the brains of freshly dead fish. Bacterial chains were observed in the blood films of the challenged groups (Figure 6).

Haematology findings:

Statistical analysis of erythrogram data revealed a significant decrease in Hb concentration, PCV, and RBCs count of *Streptococcus parauberis* infected groups (groups 2, 3, and 4) compared to the negative control group on the 2nd and 3rd day post-challenge. Meanwhile, no significant

changes in MCV and MCHC were observed. On the 10th day post-challenge, there was a significant improvement in Hb concentration, PCV, and RBCs count of the group (3) treated with ZnO-NPs supplemented diet comparable with the positive control group. Fish treated with an ampicillin-supplemented diet showed significant improvement in Hb concentration, PCV and insignificant improvement in RBCs count compared to the positive control group. At the end of the experimental period, all the infected groups showed significant microcytosis compared to the negative control group. In the treated groups; group 3 and group 4 showed significant improvement in PCV and RBCs count Compared to the positive control group. (Table 5).

Leukogram data revealed significant leukocytosis with heterophilia and monocytosis on the 2nd and 3rd day post-challenge in all infected groups compared to the negative one. Lymphocyte count showed insignificant changes on the 2nd day, though significant lymphopenia was observed on the 3rd day in the infected groups comparable with the negative control group. Significant leukopenia with neutropenia, lymphopenia, and monocytopenia was observed in groups (2) and (4) on the 10th and 16th day post-challenge compared to the negative control group. Meanwhile, group (3) showed significant leukopenia with neutropenia and lymphopenia. Compared to the positive control group, groups (3) and (4) showed significant improvement in TLC and absolute lymphocyte count on the 10th and 16th days post-challenge (Table 6).

Biochemistry results:

Statistical analysis of the protein profile revealed a significant decrease in total proteins, albumin and estimated globulins concentration with a non-significant change in the A/G ratio of the infected groups (groups 2, 3 and 4) on the 2nd and 3rd day post-challenge compared with the negative control group. The positive control group showed significant hypoproteinemia and hypoalbuminemia with a decreased A/G ratio on the 10th day post-challenge, comparable with the negative control group. Meanwhile, the treated groups (groups 3 and 4) showed significantly improved total proteins, albumin concentration and A/G ratio on the 10th post-challenge compared to the positive control group.

Statistical analysis of creatinine concentration revealed a significant increase on the 3rd and 10th day post-challenge in the infected groups (groups 2, 3 and 4) compared to the negative control group. Treated groups either fed on ZnO-NPs or an Ampicillin-supplemented diet showed insignificant improvement in creatinine concentration on the 16th day post-challenge.

Concerning the blood glucose level, significant hyperglycemia was reported in all infected groups on the 2nd, 3rd, 10th and 16th day post-challenge compared to the negative control group. Regarding enzyme activities, significant elevations in serum ALP and CK activity were observed on the 2nd, 3rd, 10th, and 16th day post-challenge in all infected groups compared to the control group. Treated groups showed a significant improvement in CK activity compared to the positive control group on the 10th and 16th day post-challenge (Table 7).

A significant increase in ammonia concentration was observed during the entire experiment in the infected fish compared to the negative control group. A remarkable improvement in its concentration was observed in the treated (group 3 and group 4) fish on the 10th and 16th day post-infection compared to the positive control group.

Immunological results:

Statistical analysis of serum immunological parameters revealed a significant increase in SLA concentration of the infected groups (groups 2, 3 and 4) on the 2nd and 3rd day post-challenge compared to the negative control group. The group of fish fed on a ZnO-NPs supplemented diet showed a significant increase in SLA compared with the positive control group. However, fish fed on an ampicillin-supplemented diet showed a marked tendency to restore SLA on the 10th day post-challenge. Non-significant increases were observed in IL -6 and SAA values on the 2nd day post-challenge in infected groups (Table 8).

Flow cytometric analysis of CD79b expression revealed a significant increase in all infected groups on the 2nd day post-challenge compared to the negative control group. Nevertheless, a significant decrease was observed in the treated groups on the 10th day post-challenge compared to the positive control group Fig. (7).

Histopathological findings:

The examined tissues (i.e., liver, kidneys, spleen, brain and gills) of the negative control group were apparently normal. In contrast, the control positive group showed various pathological lesions that exaggerated throughout the experimental period. The liver of the control

negative group revealed normal hepatocytes and a normal hepatopancreas (Fig. 8a). In contrast, severe deleterious histopathological alterations were demonstrated in the liver of the control positive group. The liver showed extensive vacuolar degeneration of hepatocytes, which appeared markedly swollen with the presence of bacterial cocci, and massive necrosis of the pancreas, which is heavily colonised with the bacterial cocci (Figs. 8b and 8c). Expansive loss of pancreatic acinar cells with the aggregation of bacterial colonies was demonstrated in most examined sections (Fig. 8d). On the other side, the liver of the ZnO NP-treated group showed considerable improvement, in which the hepatocytes revealed mild vacuolar degeneration and the pancreas revealed focal necrosis (Fig. 8e). Normal hepatocytes and pancreas were demonstrated in the liver of the ampicillin-treated group (Fig. 8f). Only a minute focal area of hepatocellular necrosis was demonstrated in this group.

The kidneys of the control negative group revealed normal glomeruli and renal tubules (Fig. 9a). In contrast, the kidney of the control positive group revealed necrosis of renal tubules, which revealed intensely eosinophilic cytoplasm and pyknotic nuclei (Fig. 9b). Significant improvement was demonstrated in the ZnO NP-treated group, which revealed vacuolar degeneration of the epithelial lining renal tubules associated with the presence of the bacterial cocci in the renal tubules and melanomacrophages (Figs. 9c, 9d, and 9e). Little improvement was demonstrated in the kidneys of the ampicillin-treated group, which revealed coaggulative necrosis of some renal tubules (Fig. 9f).

The spleen of the control negative group revealed normal red and white bulbs (Fig. 10a). While the spleen of the control positive group revealed marked reticular cell proliferation with aggregation of the bacterial cocci in the reticular cells and melanomacrophages (Fig. 10b & 10c). Pronounced amelioration was demonstrated in the spleen of the ZnO NP- and ampicillin-treated group, in which a few bacterial cocci were demonstrated in the reticular cells and melanomacrophages (Fig. 10d & 10e).

The gills of the control negative group revealed normal gill lamellae (Fig. 11a). While the gills of the control positive group revealed hyperplastic proliferation of the epithelial lining the secondary lamellae, which is associated with the presence of the bacterial colonies (Fig. 11b). The proliferating epithelial cells revealed large vesicular nuclei with prominent nucleoli (Fig. 11c). Mucous cell hyperplasia was also demonstrated in the gills of this group (Fig. 11d). Normal gill lamellae were demonstrated in the ZnO NP-treated group (Fig. 11e). Mild proliferation of the secondary lamellar epithelium was demonstrated in the ampicillin-treated group (Fig. 11f).

The brain of the control negative group revealed normal mesencephalon and metencephalon (Figs. 12a and 13a). In contrast, the mesencephalon of the control positive group revealed extensive dissociation and vacuolation of the optic tectum associated with massive aggregation of bacterial colonies (Figs. 12b and 12c). The periventricular tissue revealed marked congestion of the blood vessels associated with oedema and intense infiltration of lymphocytes and eosinophilic granular cells (Fig. 12d). The metencephalon (cerebellum) of this group revealed congestion of blood vessels associated with oedema, massive necrosis of Purkinje cells, and aggregation of numerous bacterial colonies in the ganglionic and granular cell layers (Figs. 13b, 13c, 13d, and 13e).

A normal optic tectum with scant eosinophilic granular cells in the periventricular tissue was demonstrated in the mesencephalon of the ZnO NP-treated group (Fig. 12e). The cerebellum revealed degeneration of Purkinje cells in the absence of vascular congestion and vacuolation of the molecular cell layer (Fig. 13f). The brain of the ampicillin-treated group revealed focal aggregation of the bacterial colonies in the optic tectum and sparse degeneration of the Purkinje cells of the cerebellum (Figs. 12f and 13g)**.**

Discussion

Zinc (Zn) is an essential micronutrient for fish, as it plays a vital role in biological activities. As a microelement, Zn participates in several enzymatic processes of fish metabolism, such as growth, immune response, and enzyme function. It restrains the generation of reactive oxygen species (ROS) such as superoxide anion radical, hydroxyl radical, and hydrogen peroxide. Zn cannot be stored in the body, necessitating regular dietary intake [32]. The effects of Zn depend on the dose, source, duration of feeding, sizes of the animals, and experimental conditions [33]. Several studies have indicated that some fish species, such as catfish, common carp and rainbow trout, can tolerate high dietary ZnO-NPs

intake levels up to 1.2, 1.7 and 1.9 g/Kg diet, respectively [11] without showing any adverse effect on their growth performance and survival. In this study, the safety experiment aimed to evaluate the potential effects of high dietary doses of ZnO-NPs on Nile tilapia.

Concerning the effect of high doses of ZnO-NPs on the growth performance parameters of Nile tilapia, there were no significant changes in the growth performance parameters of fish groups fed on a diet supplemented with ZnO-NPs compared to the control group. These findings might be attributed to the short duration of supplementation.

Regarding the clinicopathological changes associated with dietary supplementation of high doses of ZnO-NPs, there was a significant increase in PCV%, Hb concentration, and RBCs count in fish supplemented with 125 mg ZnO-NPs/Kg diet comparable with the negative control group. These findings are consistent with Yaqub et al. [12], who recorded a significant increase in PCV%, Hb concentration, and RBCs count in Nile tilapia fed on ZnO-NPs supplemented diet at a dose of 40 mg/ kg diet for 8 weeks. Authors reported that deficiency of Zn in the control group might lead to enhanced lipid peroxidation in the mitochondrial and microsomal membranes, resulting in the osmotic fragility of erythrocyte membranes [13]. Ghazi et al. [10] recorded a significant increase in Hb concentration and RBCs count of Nile tilapia fed on a ZnO-NPs supplemented 10 mg/kg diet for 60 days.

Regarding the results of the tested biochemical parameters (total proteins, albumin, globulins, creatinine, glucose, ALP and ammonia), non-significant changes were recorded in fish fed on a diet supplemented with different doses of ZnO-NPs compared to the control group except for significant hypoglycemia in fish supplemented with 500 mg ZnO-NPs/Kg in comparable with the control group. These findings could be attributed to the role of Zn in glucose metabolism. It enhances glucose metabolism and storage. Further, Zinc-α2-glycoproteins mediated the insulinomimetic action, which enhanced the cellular GLUT4 levels in muscles and fatty tissue and facilitated glucose absorption [34]. Safety experiment findings are consistent with reports of Yaqub et al. [12] demonstrating the nontoxic impacts of ZnO-NPs (40 mg ZnO-NPs/kg diet for 8 weeks) on haematological and blood biochemistry levels in Nile Tilapia.

In this study, muscle tissues of O. niloticus, which received dietary ZnO-NPs, retained higher Zn levels than fish in the control group. In all treatments, Zn concentration in the muscle showed higher values than the controls. However, it was still within the permissible limit (40 ppm) set by the Food and Agriculture Organization (FAO). These findings agree with a previous study by [35], who reported that Zn accumulation occurred in the intestine, followed by the liver, kidney, gills, brain and muscle tissue.

Regarding the challenge results, the erythrogram revealed normocytic normochromic anemia in the experimentally infected groups (groups 2, 3, and 4) compared to the negative control group on the 2nd and 3rd days post-infection. This finding may be attributed to the acute blood loss (severe haemorrhages in the skin, base of fins) induced by S. parauberis infection. At the end of the experimental period, all the infected groups showed significant microcytosis compared to the negative control group. This finding could be attributed to the regenerative anemia in response to the marked hemorrhage caused by S. parauberis. [36] reviewed that marked hemorrhage or hemolysis often results in microcytic anemia because regenerating immature erythrocytes make up the majority of cells in peripheral circulation, and they are smaller in size than mature erythrocytes.

Comparing all infected groups to the negative control group on the 2nd and 3rd days post-challenge, leukogram analysis showed remarkable leukocytosis with heterophilia and monocytosis. This finding might be explained by the bacteremia that has shown up obviously on the blood film. Leukocytosis with neutrophilia, monocytosis, and lymphopenia on the 3rd day post-challenge are typical pictures of stress correlating with the recorded hyperglycemia. Our results are consistent with Abd-Allah [37], who recorded significant neutrophila and lymphopenia in Nile tilapia infected with Streptococcus sanguinis 4 days post-infection.

On the 10th and 16th day post-challenge, significant leukopenia with neutropenia and lymphopenia were recorded in the infected groups, which might be attributed to the migration of white blood cells to the different tissue as a predominant cell in the defence reactions of the organism, and this is confirmed by intense infiltration of lymphocytes and eosinophilic granular cells in the mesencephalon of the infected groups. This

result is consistent with a previous study by [37], who observed leukopenia with neutropenia in Nile tilapia infected with Streptococcus sanguinis on the 11th day post-infection. Monocytopenia was also reported in groups 2 and 4 on the 10th and 16th days post-challenge. This finding is consistent with [38], who observed increased macrophages and aggregated lymphocytes around the ellipsoids in the spleen of S. parauberis infected fish. Significant improvement in TLC and absolute lymphocyte count in the treated groups could be attributed to the ability of ZnO-NPs or ampicillin to reduce the bacterial load in different examined tissues.

Regarding protein profile, the infected groups showed significant hypoproteinemia, hypoalbuminemia and hypoglobulinemia with normal A/G ratio on the 2nd and 3rd day post-challenge. This finding could be attributed to the extensive blood loss (protein loosing dermatopathy) caused by the S. parauberis infection in addition to extensive necrosis of hepatocytes, which appeared intensely eosinophilic with pyknotic nuclei and was associated with focal haemorrhage and aggregation of bacterial colonies. Treated groups (groups 3 and 4) showed significant improvement in total proteins and albumin concentration and A/G ratio on the 10th and 16th day post-challenge compared with the positive control group. These findings are correlated with the pathological findings of the liver of the treated groups. The liver showed considerable improvement, and the hepatocytes revealed mild vacuolar degeneration. Meanwhile, the liver of the positive control group showed congestion of the central vein and hepatic sinusoids with severe diffuse vacuolar degeneration of hepatocytes and aggregation of bacterial colonies. The results are consistent with those of [39], who reported a significant decrease in total proteins, albumin, and globulins concentrations in Nile tilapia infected with pathogenic S. iniae 15 days post-challenge.

Regarding the creatinine concentration, significant elevation was recorded in groups 2, 3, and 4 on the 3rd and 10th day post-challenge compared with the negative control group. The increased creatinine concentration is correlated to renal function impairment (necrosis of renal tubules (pyknotic nuclei and highly eosinophilic cytoplasm). This finding agrees with Ali et al., [40], who recorded elevated creatinine concentration associated with reduced kidney function in cultured tilapia infected with streptococcosis. Treated groups showed insignificant improvement in creatinine concentration on the 16th day post-challenge, comparable with the positive control group. These findings correlate with the pathological finding observed in the kidneys of the treated groups. Regenerative renal tubules were demonstrated in the kidneys of the ampicillin-treated group mixed with a few bacterial cocci in the surrounding renal tubules.

Concerning the blood glucose level, significant hyperglycemia was reported in the infected groups on the 2nd , 3rd , 10th and 16th day post-challenge compared to the negative control group. This finding could be attributed to the massive necrosis of the pancreas, which is heavily colonised with the bacterial cocci, and stressful conditions caused by bacterial infection, as glucose is one of the most critical stress indicators. Abd-Allah [37] recorded stress-induced hyperglycemia in Nile tilapia experimentally infected with Streptococcus sanguis or Enterococcus durans 4- and 11-days post-challenge.

Alkaline phosphatase (ALP) is an excellent biological indicator for assessing cellular stress. Its activity is a helpful bioindicator to assess the physiological health of cellular membranes, cell growth, apoptosis and cell migration, cellular metabolic status, hepatocyte function, and detoxification activity in hepatocytes [41]. Therefore, increased ALP activity in the infected groups may be attributed to the effect of Streptococcus parauberis on hepatic and renal tissues. This finding agrees with [39], who reported a significant increase in ALP activity of Nile tilapia infected with S. iniae as the harmful bacteria secret toxins that damage the hepatocytes in the liver. Molina et al. [42] recorded a significant increase in renal and hepatic ALP activity due to cyanobacterial cell-induced toxicity in tilapia fish.

Regarding creatine kinase (CK), it serves as a non-invasive, functional indicator for brain and skeletal muscle damage, which could indicate disease or environmental stress. Three CK isoforms have been described in fish; muscle CK (M-CK), brain CK (B-CK) and mitochondrial sarcomeric, and the release of any of them could be detected in the serum [43]. Significant elevation in the activity CK was observed in all infected groups compared to the negative control group. This finding is correlated with the pathological finding of the brains of S. parauberis-infected groups. The brain showed extensive dissociation and vacuolation of the optic tectum

in addition to oedema, degeneration and massive necrosis of Purkinje cells. Treated groups showed a significant decrease in CK activity compared to the positive control group (Table 7). This finding could be attributed to the ameliorative effect of ZnO or Ampicillin against streptococcus effect on brain tissue. Although the exact mechanism by which ZnO-NPs mitigate the harmful effects of bacteria is still unknown, Fadl et al. [13] reported that ZnO-NPs could adhere to the bacterial cell membrane, disrupting its permeability and causing intracellular content to leak out and bacterial cell rupture. Other investigations claim that ZnO-NPs could fight off bacterial infection because of their capacity to scavenge free radicals like ROS, which cause oxidative stress during infection [17].

A significant increase in ammonia concentration was observed in all infected groups over the experimental period. This finding might be attributed to the hyperplastic proliferation of the epithelial lining of the gill secondary lamellae, which is associated with bacterial colonies. Excretion of ammonia into the water by fish is carried out mainly through their gills by simple diffusion as NH3 because they have a large surface area, perfusion by 100% of cardiac output, significant ventilation rates, small diffusion distances, and contact with a voluminous mucosal medium [44]. Therefore, gill damage might result in increased concentrations or interference with NH3 excretion by fish, resulting in toxicity. Ammonia is believed to represent a major factor responsible for astrocyte swelling, although the mechanisms by which ammonia causes such swelling are not entirely understood. It has been hypothesised that in hyperammonemic conditions, glutamine generated in astrocytes from ammonia and glutamate in a reaction catalysed by glutamine synthetase could exert osmotic effects and contribute to brain swelling [44]

One of the key components of the innate immune response to an infection or injury is serum lysozymes. Lysozymes main function is their contribution to antibacterial defense, causing the lysis of pathogens. The increased activities of lysozymes following infection, or the injection of foreign materials or pathogens, have been reported by many researchers. Hence, the increased SLA in the infected groups is a strong evidence of infection. This finding agrees with Woo and Park [3], who reported that lysozyme activities following injection of S. parauberis in olive flounder were higher than that of the control group at 1 and 3 days after injection.

Interleukin 6 cytokine plays an essential role in the immune response, such as inducing the B cells' differentiation into plasma cells and promoting antibody production by antibody-secreting cells. A non-significant increase in the values of IL 6 was noticed in the infected groups on the 2nd day post-challenge. Wei et al. [45] reported that IL-6 might get involved in host defense against bacterial infection in Nile tilapia, and this is confirmed by the up-regulated expression of IL-6 Following S. agalactiae challenge in vivo (head kidney and spleen) and in vitro (head kidney and spleen leukocytes). CD79 is a transmembrane protein that forms a complex with BCR and generates a signal following recognition of antigen by the BCR, which plays a critical role in B cell differentiation, proliferation, and effector functions. Wu et al. [46] reported that CD79 was likely to get involved in host defence against bacterial infection in Nile tilapia and play a role in BCR signalling. They also reported that upon S. agalactiae infection, the transcriptional expressions of OnCD79a and OnCD79b were significantly up regulated in both the spleen and anterior kidney [46]. Concerning the flow cytometric analysis of CD79b expression, there was a significant increase in all the infected groups on the 2nd day post-challenge compared to the negative control group. This finding might be attributed to the host defence against bacterial infection. Nevertheless, a significant decrease was observed in the treated groups on the 10th day post-challenge compared to the positive control group. Taken together, these data might reflect the ability of ZnO-NPs to ameliorate S.parauberis infection in Nile tilabia via decreasing the bacterial load in different tissues.

Conflicts of interest

"There are no conflicts to declare".

Funding statement

The study was supported by Cairo university research funds for postgraduate students.

TABLE 1. Effect of different high dietary doses of ZnO ON growth performance parameters of Nile tilapia

Groups	initial average weight (g/ fish)	final average weight (g/ fish)	weight gain (g/ fish)	Weight gain percent (%)	SGR	FI (g/ fish)
Negative control	82 [a]	90.5 [a]	8.5 [a]	10.36585 [a]	0.611925 [a]	11.48 [a]
125 mg ZnO NPs/ Kg diet	90.5 [a]	101.4 [a]	10.9 [a]	12.0442 [a]	0.705563 [a]	12.67 [a]
250 mg ZnO NPs/ Kg diet	94 [a]	104 [a]	10 [a]	10.6383 [a]	0.627221 [a]	13.16 [a]
500 mg ZnO NPs/ Kg diet	85 [a]	93 [a]	8 [a]	9.411765 [a]	0.558057 [a]	11.9 [a]

Values represent means, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05.

TABLE 2. Effect of different high dietary doses of ZnO on haematological parameters of Nile tilapia (means ± SD).

GroupS	PCV (%)	Hb (g/dL)	RBCs count (10^6 Cell/µL)	MCV (fL)	MCHC (g/dL)	TLC (10^3 Cell/ µL)	Heterophils (10^3 Cell/ µL)	Lymphocytes (10^3 Cell/µL)	Esinophils (10^3 Cell/ µL)	Monocytes (10^3 Cell/µL)
Negative control	25.43 ± 2.99 [bc]	6.04 ± 2.47 [b]	1.44 ± 0.25 [b]	181.54 ± 39.82 [a]	23.69 ± 8.51 [b]	30.86 ± 7.17 [a]	8.13 ± 4.56 [a]	21.85 ± 3.73 [a]	0.00 ± 0.00 [a]	0.81 ± 0.55 [a]
125 mg ZnO NPs/ Kg diet	29.71 ± 2.98 [a]	9.05 ± 1.88 [a]	1.93 ± 0.42 [a]	157.70 ± 23.18 [ab]	30.28 ± 3.80 [a]	32.29 ± 19.03 [a]	7.50 ± 5.88 [a]	23.73 ± 13.44 [a]	0.34 ± 0.58 [a]	0.78 ± 0.37 [a]
250 mg ZnO NPs/ Kg diet	23.00 ± 1.41 [c]	5.50 ± 1.28 [b]	1.61 ± 0.23 [ab]	144.86 ± 18.61 [b]	23.73 ± 4.41 [b]	26.57 ± 4.12 [a]	7.68 ± 4.51 [a]	17.74 ± 6.72 [a]	0.00 ± 0.00 [a]	1.15 ± 0.71 [a]
500 mg ZnO NPs/ Kg diet	26.14 ± 2.54 [b]	6.15 ± 0.83 [b]	2.02 ± 0.55 [a]	135.65 ± 29.05 [b]	23.71 ± 3.99 [b]	27.00 ± 9.47 [a]	11.91 ± 7.79 [a]	14.12 ± 9.66 [a]	0.18 ± 0.34 [a]	0.79 ± 0.31 [a]

Values represent means ± SD, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05

TABLE 3. Effect of different high dietary doses of ZnO on blood biochemical parameters of Nile tilapia (means ± SD).

GroupS	Total protein (g/dl)	Albumin (g/dl)	Globulins (g/dl)	A/G ratio	creatinine (mg/dl)	glucose(mg/dl)	ALP (U/L)	Ammonia (µmol/L)
Negative control	3.25 ± 0.63 [a]	1.37 ± 0.27 [a]	1.88 ± 0.76 [a]	1.01 ± 0.97 [a]	0.37 ± 0.09 [a]	86.01 ± 17.97 [a]	43.29 ± 11.44 [a]	457.60 ± 49.22 [a]
125 mg ZnO NPs/ Kg diet	3.46 ± 0.52 [a]	1.33 ± 0.22 [a]	2.13 ± 0.44 [a]	0.65 ± 0.15 [a]	0.30 ± 0.07 [a]	80.28 ± 12.90 [ab]	45.71 ± 11.74 [a]	512.20 ± 141.70 [a]
250 mg ZnO NPs/ Kg diet	2.92 ± 0.40 [a]	1.30 ± 0.18 [a]	1.62 ± 0.50 [a]	0.88 ± 0.30 [a]	0.32 ± 0.05 [a]	74.21 ± 18.04 [ab]	37.29 ± 13.86 [a]	453.20 ± 67.55 [a]
500 mg ZnO NPs/ Kg diet	2.93 ± 0.56 [a]	1.41 ± 0.21 [a]	1.52 ± 0.54 [a]	1.06 ± 0.51 [a]	0.35 ± 0.04 [a]	69.20 ± 10.24 [b]	42.29 ± 8.85 [a]	561.40 ± 72.52 [a]

Values represent means ± SD, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05

TABLE4. Effect of different high dietary doses of ZnO on tissue residue of Nile tilapia (means ± SD).

Groups	Zn Concentration (ppm)
Negative control	7.99 ± 0.54 [c]
125 mg ZnO NPs/ Kg diet	14.33 ± 1.08 [b]
250 mg ZnO NPs/ Kg diet	18.65 ± 0.79 [a]
500 mg ZnO NPs/ Kg diet	20.49 ± 1.75 [a]

Values represent means ± SD, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05

TABLE5. Effect of *S. parauberis* on erythrogram of different experimental groups of Nile tilapia (means ± SD).

Experimental period	Groups	Hb g/dL	PCV %	RBCs (10^6 cell/µl)	MCV (fL)	MCHC (g/dL)
2nd-day post-challenge	Group 1	6.28 ± 0.37 [a]	23.20 ± 1.10 [a]	1.35 ± 0.10 [a]	172.81 ± 7.21 [a]	27.08 ± 1.41 [a]
	Group 2	3.84 ± 1.15 [c]	17.40 ± 2.61 [b]	0.93 ± 0.19 [c]	200.19 ± 86.41 [a]	22.98 ± 8.52 [a]
	Group 3	4.92 ± 0.56 [b]	19.60 ± 2.97 [b]	1.11 ± 0.03 [b]	176.43 ± 28.35 [a]	25.82 ± 6.13 [a]
	Group 4	4.40 ± 0.19 [bc]	19.00 ± 1.41 [b]	1.10 ± 0.09 [b]	173.54 ± 17.53 [a]	23.20 ± 0.83 [a]
3rd-day post-challenge	Group 1	6.12 ± 0.42 [a]	26.20 ± 1.92 [a]	1.70 ± 0.22 [a]	155.60 ± 18.57 [a]	23.45 ± 2.01 [ab]
	Group 2	2.01 ± 0.84 [c]	7.20 ± 1.30 [d]	0.49 ± 0.11 [c]	146.80 ± 9.08 [a]	29.61 ± 14.61 [a]
	Group 3	1.92 ± 0.73 [c]	13.20 ± 1.92 [c]	0.93 ± 0.11 [b]	142.94 ± 16.46 [a]	14.18 ± 4.13 [b]
	Group 4	2.87 ± 0.14 [b]	16.20 ± 0.84 [b]	1.10 ± 0.20 [b]	151.68 ± 30.92 [a]	17.77 ± 1.38 [b]
10th-day post-challenge	Group 1	5.35 ± 0.21 [a]	28.80 ± 1.92 [a]	1.54 ± 0.11 [a]	188.05 ± 18.13 [a]	18.61 ± 0.65 [a]
	Group 2	2.98 ± 0.61 [c]	20.60 ± 0.55 [c]	1.17 ± 0.17 [c]	179.62 ± 24.97 [ab]	14.53 ± 3.23 [b]
	Group 3	4.58 ± 0.21 [b]	22.64 ± 2.04 [b]	1.43 ± 0.17 [ab]	160.52 ± 26.61 [ab]	20.32 ± 1.25 [a]
	Group 4	4.15 ± 0.45 [b]	20.60 ± 0.89 [c]	1.32 ± 0.12 [bc]	156.80 ± 12.82 [b]	20.16 ± 2.43 [a]
16th-day post-challenge	Group 1	4.96 ± 0.35 [a]	25.20 ± 0.45 [a]	1.30 ± 0.06 [b]	194.07 ± 11.16 [a]	19.67 ± 1.15 [a]
	Group 2	3.45 ± 0.38 [b]	17.60 ± 0.55 [c]	0.96 ± 0.12 [c]	185.41 ± 24.39 [b]	19.64 ± 2.35 [a]
	Group 3	4.08 ± 0.97 [b]	22.60 ± 1.52 [b]	1.48 ± 0.13 [a]	153.76 ± 18.20 [b]	17.94 ± 3.25 [a]
	Group 4	3.93 ± 0.53 [b]	22.40 ± 1.52 [b]	1.41 ± 0.16 [ab]	160.92 ± 18.90 [b]	17.66 ± 3.04 [a]

Values represent means ± SD, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05

TABLE 6. Effect of *S. parauberis* on leukogram of different experimental groups of Nile tilapia (means ± SD).

Experimental period	Groups	TLC (10^3 cell/µL)	Heterophil (10^3 cell/µL)	Lymphocytes (10^3 cell/µL)	Monocytes (10^3 cell/µL)	Esinohil (10^3 cell/µL)
2nd-day post-challenge	Group 1	67.86 ± 2.48 c	17.19 ± 2.29 d	49.32 ± 1.76 ab	1.35 ± 0.04 b	0.00 ± 0.00
	Group 2	83.14 ± 5.34 b	29.43 ± 4.77 c	48.67 ± 3.76 ab	5.04 ± 0.79 a	0.00 ± 0.00
	Group 3	93.14 ± 4.63 a	34.25 ± 3.03 b	54.52 ± 5.52 a	4.37 ± 1.04 a	0.00 ± 0.00
	Group 4	97.14 ± 8.07 a	46.52 ± 2.58 a	46.21 ± 8.30 b	4.42 ± 0.65 a	0.00 ± 0.00
3rd-day post-challenge	Group 1	34.00 ± 1.22 b	6.60 ± 1.27 c	26.71 ± 0.56 a	0.69 ± 0.01 c	0.00 ± 0.00
	Group 2	41.20 ± 2.86 a	22.86 ± 4.29 ab	15.68 ± 1.99 c	2.66 ± 0.82 a	0.00 ± 0.00
	Group 3	42.40 ± 5.77 a	27.07 ± 5.31 a	13.52 ± 2.11 d	1.81 ± 0.42 b	0.00 ± 0.00
	Group 4	40.40 ± 2.07 a	19.04 ± 2.12 b	19.08 ± 0.62 b	2.28 ± 0.76 ab	0.00 ± 0.00
10th-day post-challenge	Group 1	55.00 ± 1.87 a	11.55 ± 2.36 a	41.64 ± 2.53 a	1.80 ± 0.41 a	0.00 ± 0.00
	Group 2	22.00 ± 4.30 d	4.86 ± 1.33 b	16.57 ± 4.93 d	0.56 ± 0.21 d	0.00 ± 0.00
	Group 3	37.20 ± 3.35 b	4.61 ± 1.45 b	30.79 ± 3.48 b	1.80 ± 0.40 a	0.00 ± 0.00
	Group 4	30.20 ± 4.38 c	4.32 ± 0.18 b	24.56 ± 4.26 c	1.32 ± 0.08 b	0.00 ± 0.00
16th-day post-challenge	Group 1	41.60 ± 1.14 a	19.00 ± 1.73 a	19.93 ± 1.80 ab	2.66 ± 0.35 a	0.00 ± 0.00
	Group 2	26.40 ± 2.51 d	10.44 ± 3.08 b	14.58 ± 2.04 c	1.38 ± 0.33 b	0.00 ± 0.00
	Group 3	35.40 ± 2.07 b	12.88 ± 3.71 b	21.40 ± 2.82 a	1.12 ± 0.49 b	0.00 ± 0.00
	Group 4	29.00 ± 1.22 c	12.16 ± 3.73 b	15.80 ± 4.87 b	1.04 ± 0.10 b	0.00 ± 0.00

Values represent means ± SD, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05

TABLE 7. Effect of streptococossis on blood biochemical parameters of different experimental groups of Nile tilapia (means ± SD).

Experimental period	Groups	TP (g/dl)	Albumin (g/ dL)	Globulins (g/ dL)	A/G ratio	Creatinine (mg/dL)	Glucose (mg/dL)	ALP (U/ L)	CK (U/ L)	Ammonia (µmol/L)
2nd-day post-challenge	Group 1	3.43 ± 0.21 a	1.69 ± 0.01 a	1.75 ± 0.21 a	0.98 ± 0.11 ab	0.62 ± 0.11 a	29.60 ± 3.05 b	39.00 ± 4.53 c	194.80 ± 27.88 c	630.20 ± 115.86 d
	Group 2	2.60 ± 0.11 bc	1.35 ± 0.09 b	1.25 ± 0.15 c	1.10 ± 0.19 a	0.57 ± 0.03 a	49.20 ± 6.87 a	57.60 ± 4.28 b	1246.00 ± 150.18 b	1231.20 ± 85.10 c
	Group 3	2.51 ± 0.22 c	1.30 ± 0.11 bc	1.21 ± 0.24 c	1.12 ± 0.27 a	0.62 ± 0.10 a	49.00 ± 6.04 a	71.80 ± 16.22 a	1191.60 ± 145.02 b	1376.00 ± 66.93 b
	Group 4	2.75 ± 0.01 b	1.26 ± 0.05 c	1.49 ± 0.05 b	0.85 ± 0.06 b	0.56 ± 0.02 a	44.20 ± 6.02 a	59.20 ± 9.18 ab	4242.40 ± 356.93 a	1759.00 ± 106.54 a
3rd day post-challenge	Group 1	3.23 ± 0.06 a	1.54 ± 0.09 a	1.69 ± 0.08 a	0.91 ± 0.09 a	0.44 ± 0.06 c	62.20 ± 3.49c	35.60 ± 10.74 b	110.00 ± 43.90 d	345.60 ± 86.06 c
	Group 2	2.37 ± 0.06 b	1.25 ± 0.05 b	1.12 ± 0.08 b	1.13 ± 0.13 a	0.93 ± 0.13 a	118.00± 16.72 a	56.25 ± 14.86 a	1281.20 ± 143.19 a	2334.20 ± 759.36 a
	Group 3	2.43 ± 0.24 b	1.26 ± 0.07 b	1.16 ± 0.28 b	1.16 ± 0.41 a	0.81 ± 0.18 ab	98.20± 18.79 b	69.20 ± 19.15 a	395.40 ± 36.65 c	1060.60 ± 101.17 b
	Group 4	2.43 ± 0.01 b	1.28 ± 0.03 b	1.15 ± 0.04 b	1.12 ± 0.07 a	0.70 ± 0.11 b	86.00 ± 3.94 b	55.20 ± 6.34 a	1105.00 ± 94.75 b	1127.20 ± 161.96 b
10th day post-challenge	Group 1	3.10 ± 0.10 a	1.92 ± 0.03 a	1.18 ± 0.13 a	1.64 ± 0.19 a	0.49 ± 0.16 b	108.40 ± 4.88 b	37.40 ± 5.13 c	82.20 ± 53.29 d	271.20 ± 66.57 c
	Group 2	2.61 ± 0.08 b	1.42 ± 0.04 b	1.19 ± 0.04 a	1.19 ± 0.01 b	a0.07 ± 0.80	134.20 ± 5.85 a	68.20 ± 9.68 a	690.40 ± 133.37 a	1058.80 ± 170.19 a
	Group 3	2.80 ± 0.22 ab	1.75 ± 0.10 a	1.05 ± 0.20 a	1.72 ± 0.33 a	0.65 ± 0.07 a	129.40 ± 3.21 a	53.80 ± 4.55 b	343.60 ± 103.12 c	510.40 ± 113.48 b
	Group 4	2.97 ± 0.39 a	1.77 ± 0.28 a	1.20 ± 0.14 a	1.47 ± 0.17 a	0.75 ± 0.11 a	128.80 ± 9.18 a	53.60 ± 5.94 b	497.40 ± 100.08 b	562.20 ± 71.47 b
16th day post-challenge	Group 1	3.89 ± 0.37 a	2.14 ± 0.04 a	1.74 ± 0.35 a	1.27 ± 0.25 a	0.54 ± 0.18 b	105.40 ± 9.91 b	51.20 ± 5.02 b	60.80 ± 23.86 d	183.80 ± 95.31 c
	Group 2	2.75 ± 0.52 b	1.66 ± 0.21 b	1.09 ± 0.62 b	2.22 ± 1.71 a	0.88 ± 0.24 a	136.20 ± 4.66 a	55.20 ± 5.67 ab	443.80 ± 59.29 a	674.20 ± 76.78 a
	Group 3	3.99 ± 0.41 a	2.15 ± 0.26 a	1.85 ± 0.22 a	1.17 ± 0.13 a	0.73 ± 0.05 ab	136.40 ± 3.65 a	62.24 ± 15.30 ab	171.00 ± 30.60 c	480.60 ± 92.25 b
	Group 4	3.49 ± 0.26 a	2.14 ± 0.10 a	1.35 ± 0.27 ab	1.65 ± 0.39 a	0.86 ± 0.37 ab	134.00 ± 6.16 a	64.60 ± 4.93 a	239.00 ± 48.15 b	527.60 ± 95.42 b

Values represent means ± SD, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05

TABLE 8. Effect of *S.parauberis* on serum immunological parameters of different experimental groups of Nile tilapia (means ± SD).

Experimental period	Groups	SLA (mg/ml)	IL-6 (ng/L)	SAA (ng/L)
2nd day post challenge	Group 1	0.29 ± 0.06 c	18.14 ± 1.79 a	2.92 ± 0.08 a
	Group 2	5.34 ± 2.09 a	18.76 ± 0.72 a	3.16 ± 0.22 a
	Group 3	5.77 ± 0.30 a	18.79 ± 1.94 a	3.19 ± 0.08 a
	Group 4	2.49 ± 0.28 b	18.42 ± 1.33 a	2.95 ± 0.13 a
3rd day post challenge	Group 1	0.63 ± 0.25 c	18.91 ± 2.64 a	3.37 ± 0.53 a
	Group 2	8.38 ± 1.58 a	19.89 ± 0.44 a	3.99 ± 0.41 a
	Group 3	3.82 ± 1.86 b	18.05 ± 2.41 a	3.46 ± 0.27 a
	Group 4	5.47 ± 0.61 b	16.39 ± 0.98 a	3.69 ± 1.09 a
10th day post challenge	Group 1	0.22 ± 0.12 c	15.47 ± 2.97 a	3.57 ± 0.07 a
	Group 2	2.56 ± 0.12 b	15.67 ± 3.36 a	5.27 ± 2.10 a
	Group 3	3.93 ± 0.70 a	18.10 ± 1.55 a	3.82 ± 0.33 a
	Group 4	0.89 ± 0.12 c	18.95 ± 3.76 a	3.38 ± 0.26 a

Values represent means ± SD, means with different superscripts (a and b) within the same column are significantly different at P value < 0.05.

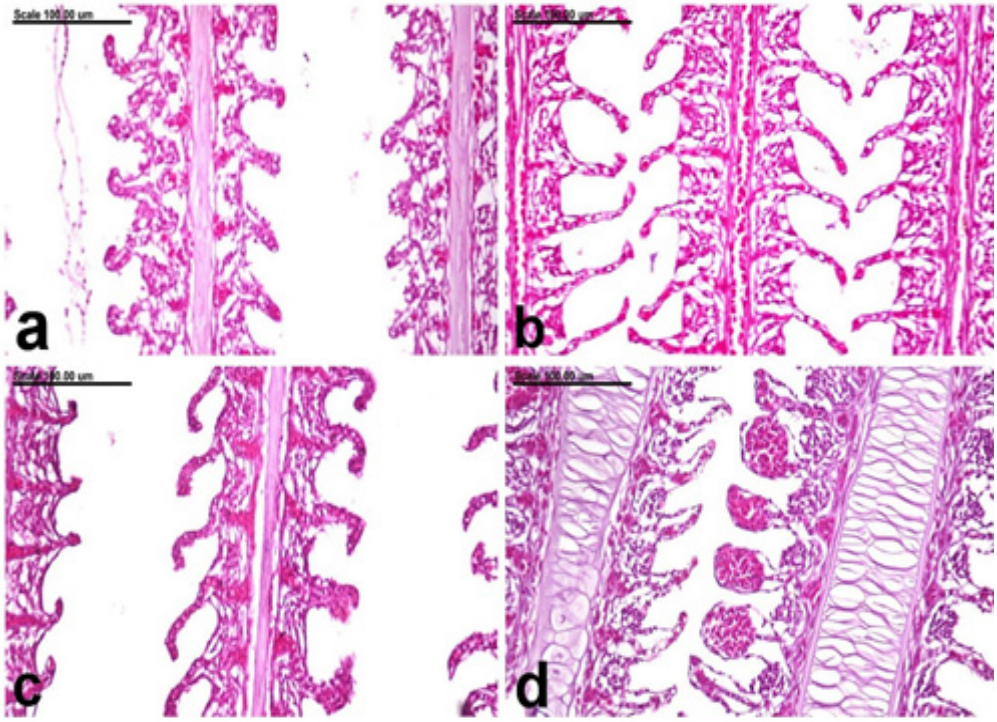

Fig. 1. The gills of O. niloticus, of the (a) control negative group showing normal gill lamellae, with mild dilation of the blood capillaries, (b) 125 mg ZnO group showing normal lamellar epithelium, with mild congestion of the central venous sinuses, (c) 250 mg ZnO group showing normal gill lamellae, and (d) 500 mg ZnO group showing focal telangiectasis. (Stain: H&E; scale bar=100µm)

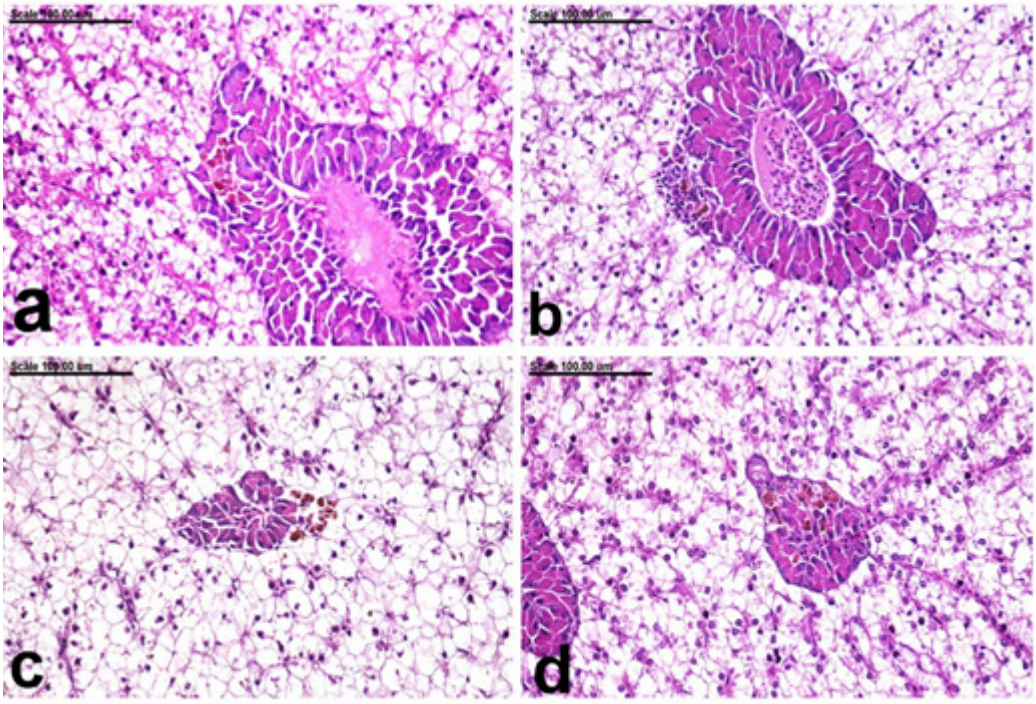

Fig. 2. The liver of O. niloticus, of the (a) control negative group showing normal hepatocytes, with normal rounded nuclei and normal hepatopancreas, (b) 125 mg ZnO group showing normally vacuolated hepatocytes and focal infiltration of the hepatopancreas with mononuclear cells, (c) 250 mg ZnO group showing normal hepatocytes with few melanomacrophages infiltrating the hepatopancreas, and (d) 500 mg ZnO group showing normal hepatic structure. (Stain: H&E; scale bar=100µm)

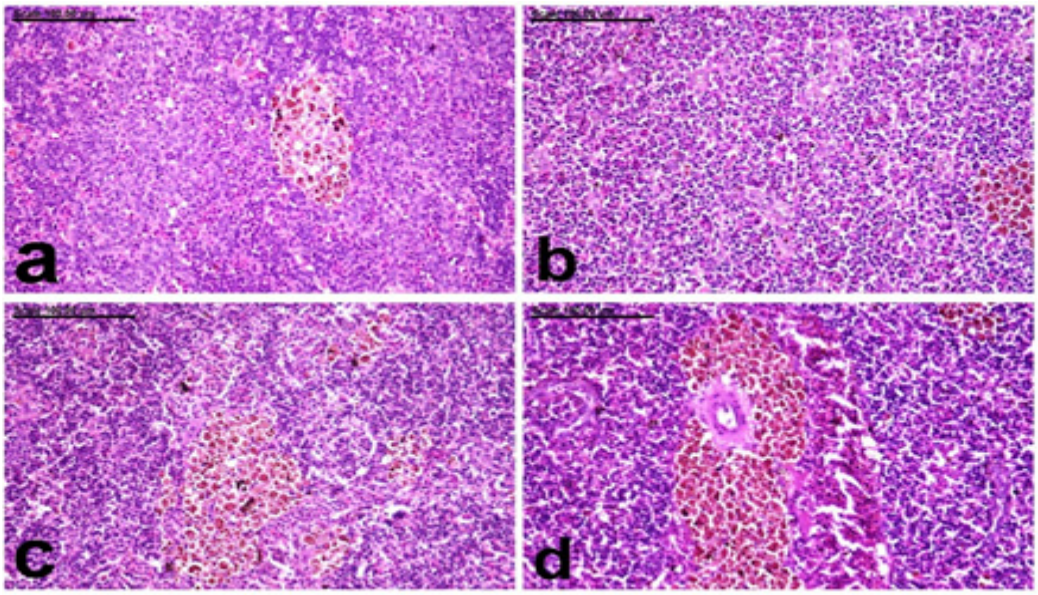

Fig. 3. The spleen of O. niloticus, of the (a) control negative group showing normal white pulp with normal lymphoid cells and melanomacrophages, (b) 125 mg ZnO group showing normal splenic ellipsoid and melanomacrophage centres, (c) 250 mg ZnO group showing normal red and white pulp and normal melanomacrophages, and (d) 500 mg ZnO group showing normal splenic parenchyma. (Stain: H&E; scale bar=100µm)

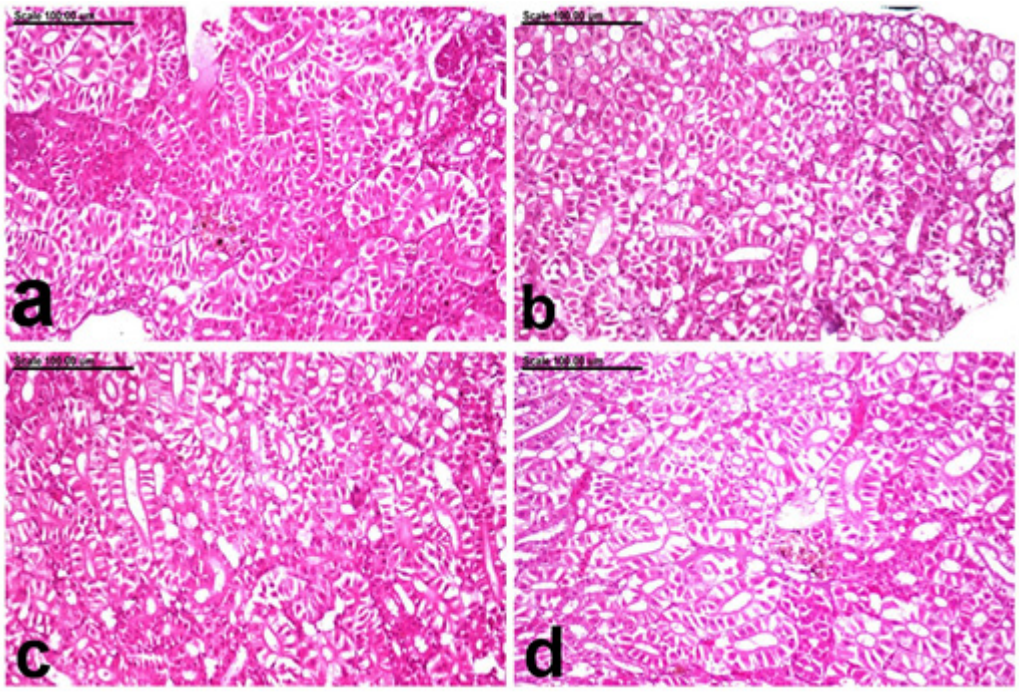

Fig. 4. The kidney of O. niloticus, of the (a) control negative group showing normal renal tubules and normal aggregation of melanomacrophages, (b) 125 mg ZnO group showing normal renal tubular epithelial cells lined with round basophilic nuclei, (c) 250 mg ZnO group showing mild vacuolar degeneration of some renal tubular epithelium, and (d) 500 mg ZnO group showing single cell necrosis. (Stain: H&E; scale bar=100µm)

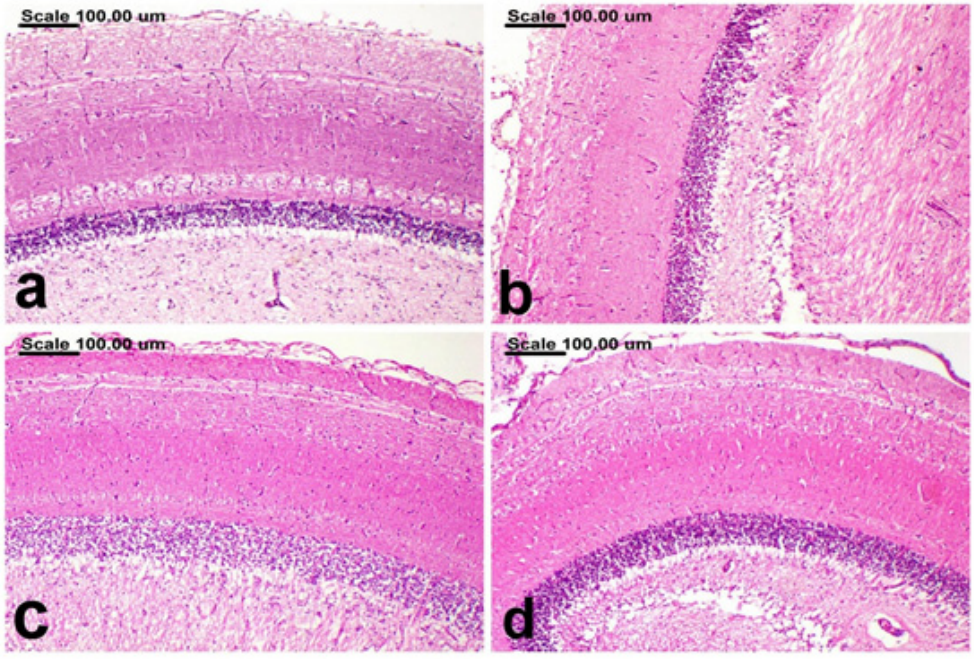

Fig. 5. The brain of O. niloticus, of the (a) control negative group showing normal brain tissue (optic tectum and mesencephalon), (b) 125 mg ZnO group showing normal optic tectum and mesencephalon, (c) 250 mg ZnO group showing normal brain tissue, and (d) 500 mg ZnO group showing normal brain tissue. (Stain: H&E; scale bar=100µm)

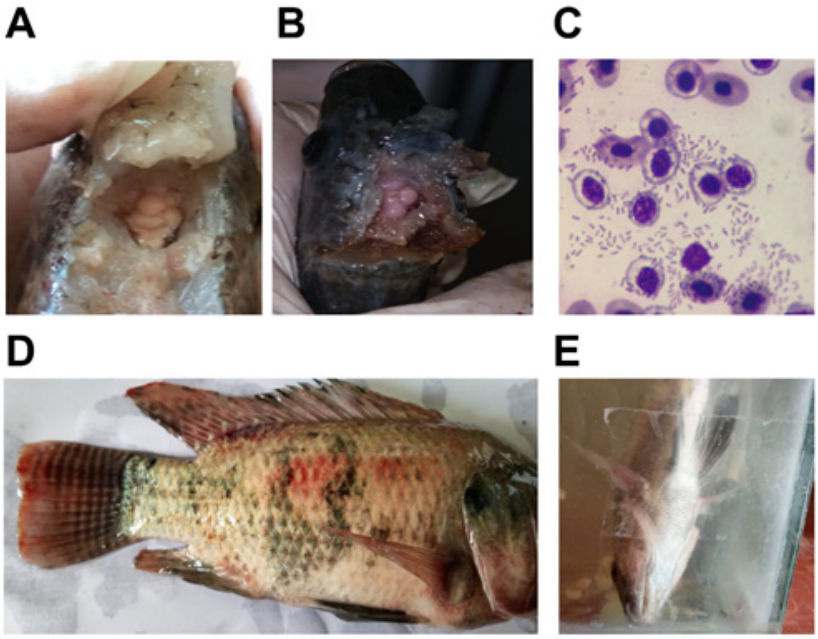

Fig. 6. Clinical signs of streptococossis. (A) brain of the negative control group showing a normal gross appearance, meanwhile challenged fish exhibited (B) congested brain, (C) bacterial cocci in the blood smear, (D) haemorrhages in skin fins, and (E) abnormal swimming behaviour.

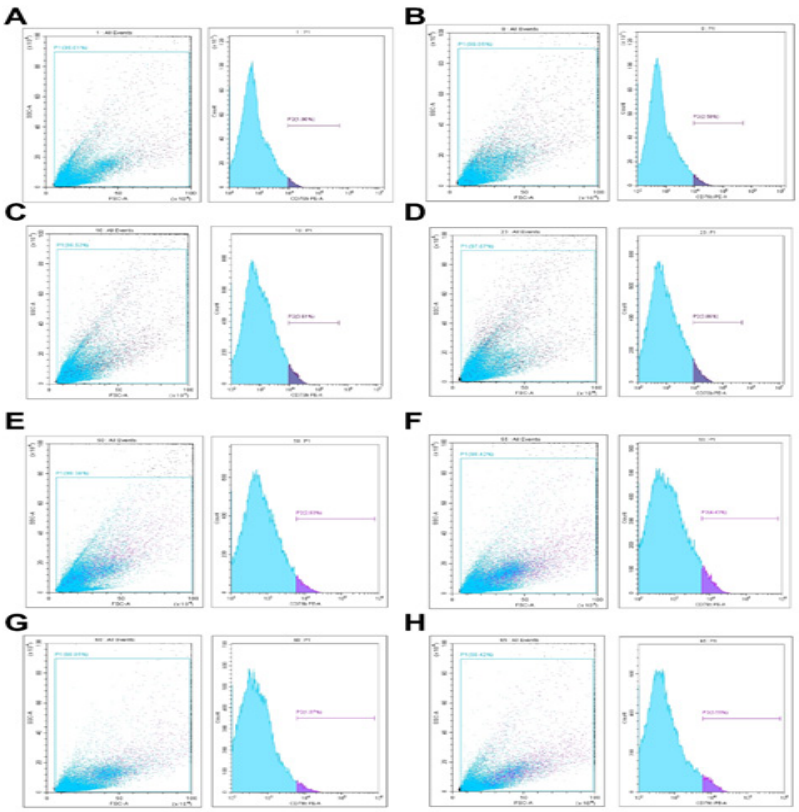

Fig. 7. Flow cytometric analysis of CD79b expression of spleen shows a significant increase in the expression of CD79 b of all challenged groups on the 2nd day post-challenge (B, C, D) compared to the negative control group (A). However, on the 10th day post-challenge infected fish of group 2 and 3 showed significant increase in the expression (F and H) compared to the negative control group (E). ZnO-NPs treated group showed significant decrease (G) in the expression of CD 79b compared to the positive control group (F).

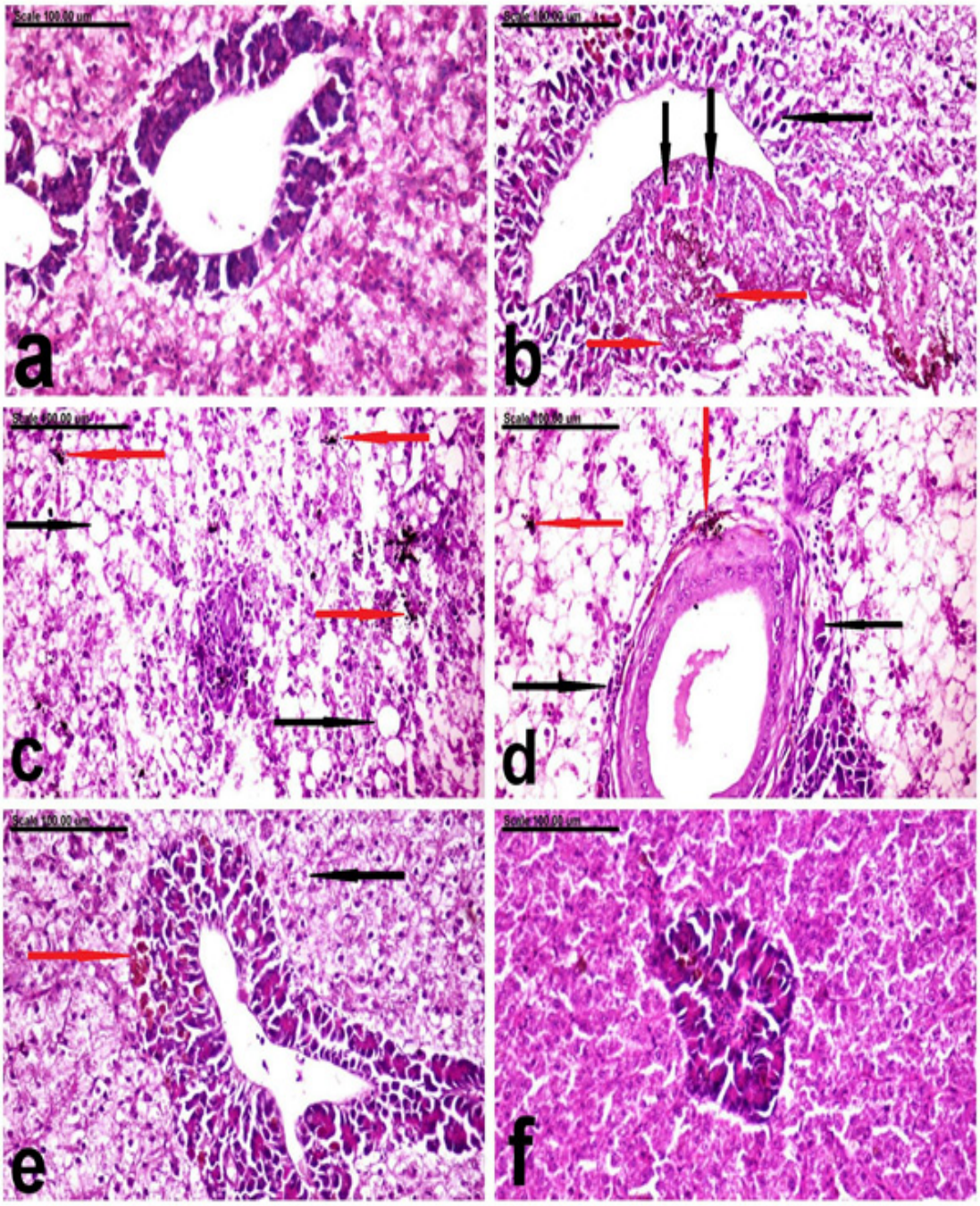

Fig. 8. photomicrograph of the liver of, (a) control negative group showing normal histological structures with normal hepatocytes and a normal hepatopancreas, (b, c, d) control positive group showing extensive vacuolar degeneration of hepatocytes, which appeared markedly swollen (black arrows) with the presence of bacterial cocci (red arrows) (c), and massive necrosis of the pancreas (black arrows), which is heavily colonised with the bacterial cocci (red arrows) (b) and expansive necrosis with loss of pancreatic acinar cells (black arrows) as well as the aggregation of bacterial colonies (red arrows) (d), (e) ZnO NP-treated group showing mild vacuolar degeneration of hepatocytes (black arrow) and focal necrosis of the pancreas (red arrow) , and (f) the ampicillin-treated group showing normal hepatocytes and pancreas.

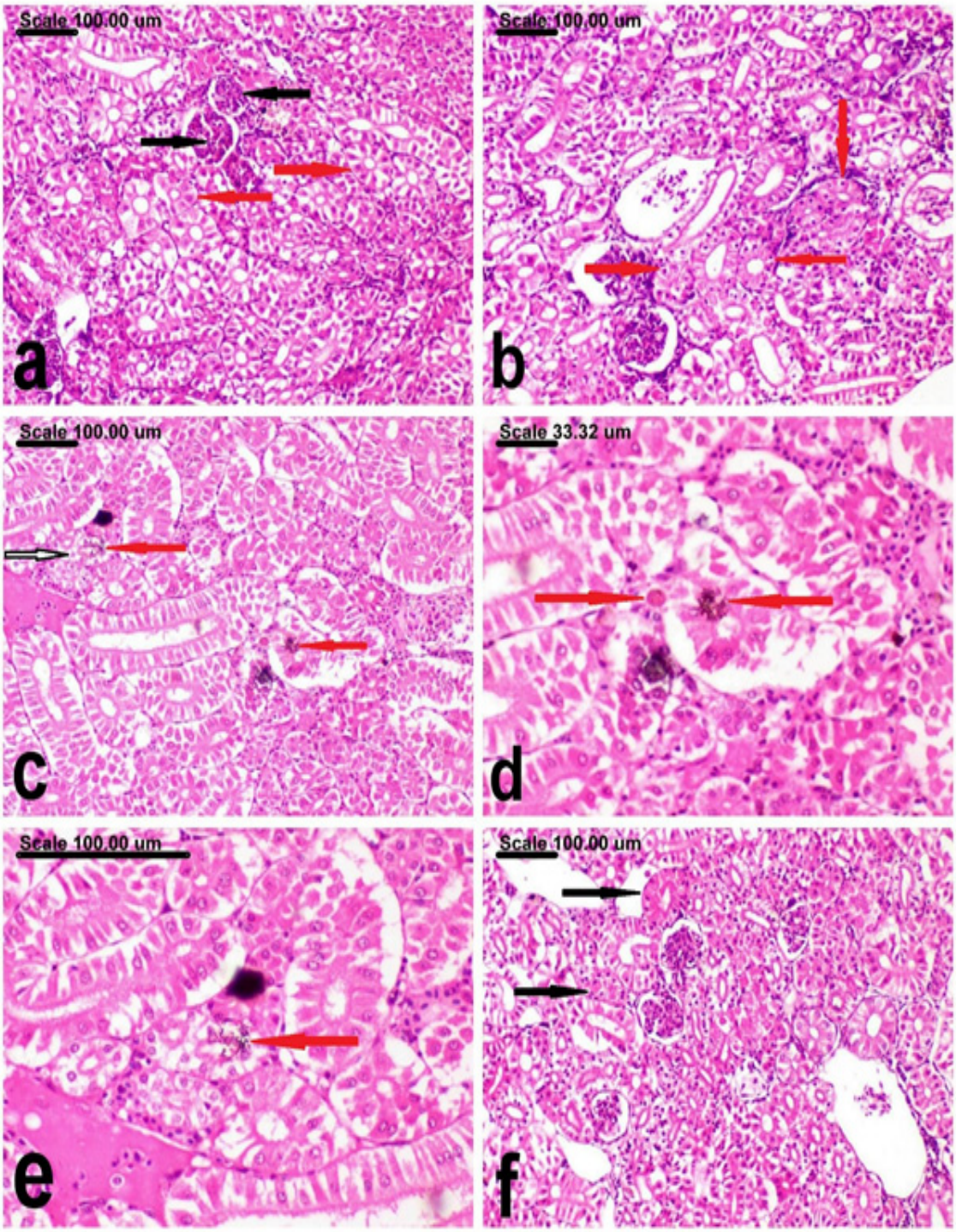

Fig. 9. photomicrograph of the kidney of, (a) control negative group showing normal glomeruli (black arrows) and renal tubules (red arrows), (b) control positive group showing necrosis of renal tubules, which revealed intensely eosinophilic cytoplasm and pyknotic nuclei(red arrows), (c, d, e) ZnO NP-treated group showing vacuolar degeneration of the epithelial lining renal tubules (black arrow) (c) associated with the presence of the bacterial cocci in the renal tubules (red arrows) and melanomacrophages (red arrows) (d & e), and (f) the ampicillin-treated group showing coagulative necrosis of some renal tubules (black arrows).

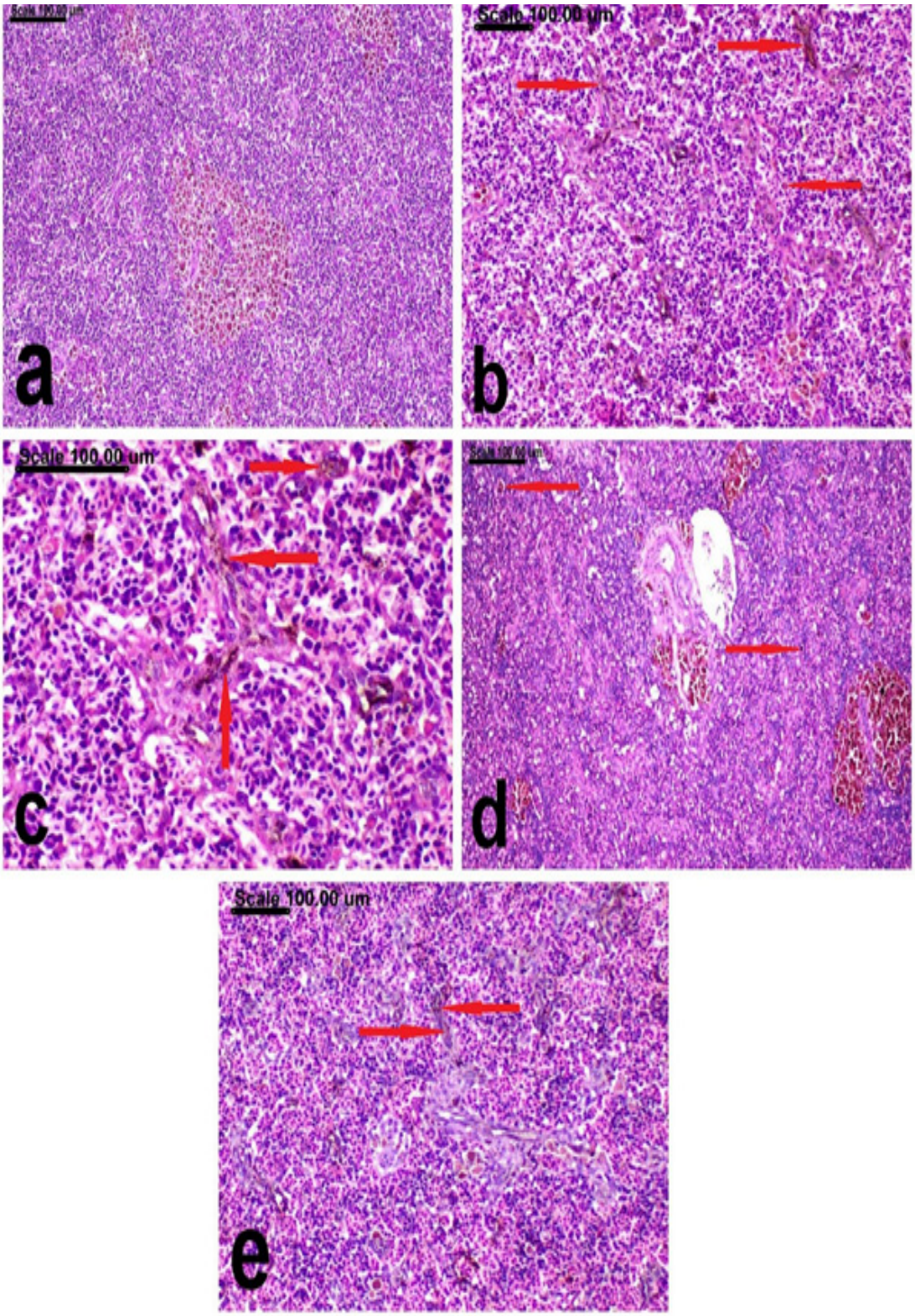

Fig. 10. Photomicrograph of the spleen of, (a) control negative group showing normal red and white bulbs and normal melanomacrophage centre, (b, c) control positive group showing marked reticular cell proliferation with aggregation of the bacterial cocci in the reticular cells and melanomacrophages (red arrows) (b & c), (d) ZnO NP-treated group showing few bacterial cocci in the reticular cells and melanomacrophages (red arrows), and (e) the ampicillin-treated group showing bacterial cocci in the reticular cells (red arrows).

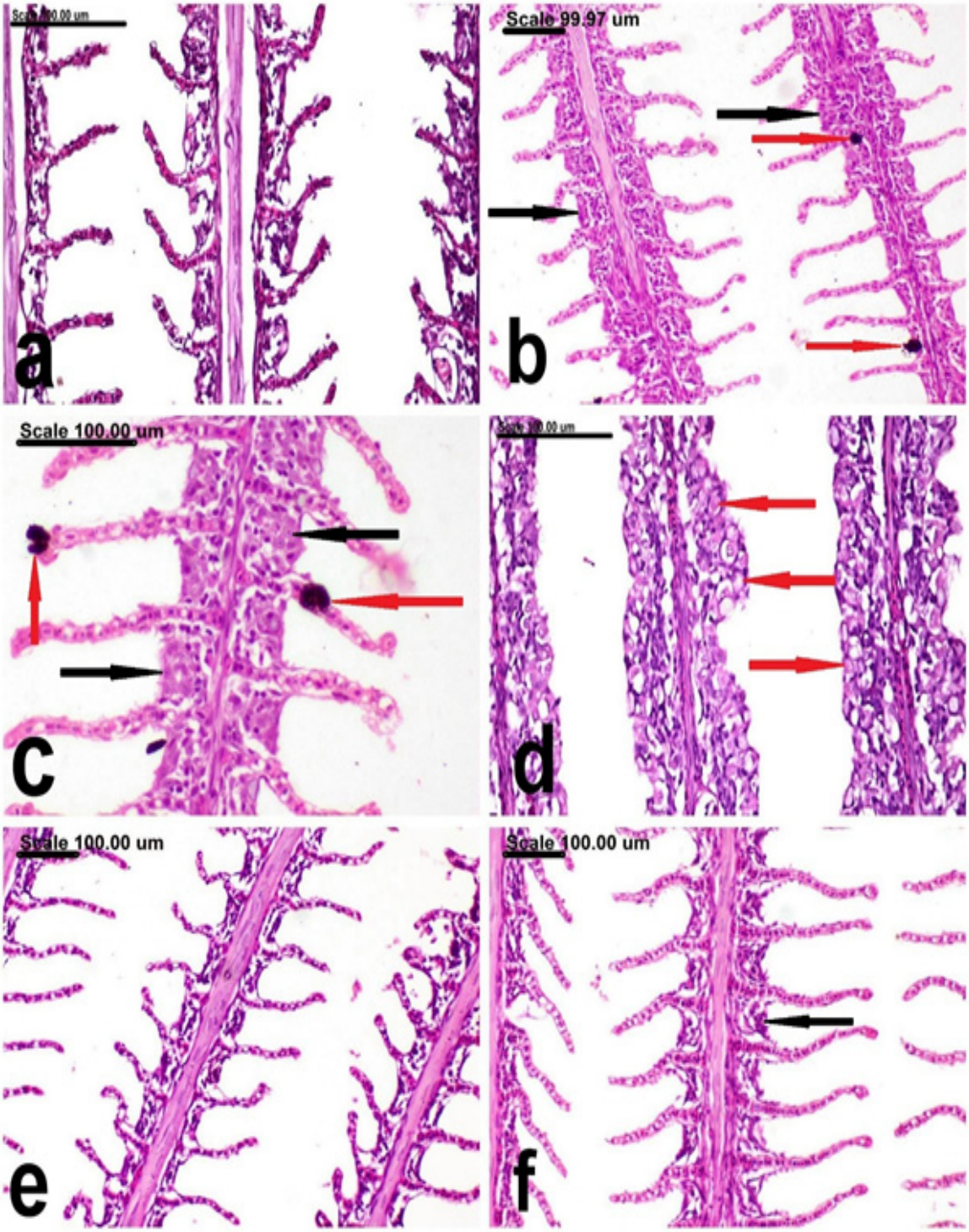

Fig. 11. Photomicrograph of the gills of, (a) control negative group showing normal gill lamellae, (b,c, d) control positive group showing hyperplastic proliferation of the epithelial lining the secondary lamellae (black arrows), which is associated with the presence of the bacterial colonies (red arrows) (b), the proliferating epithelial cells revealed large vesicular nuclei with prominent nucleoli (black arrows) (c) and mucous cell hyperplasia (red arrows)(d), (e) ZnO NP-treated group showing normal gill lamellae, and (f) the ampicillin-treated group showing mild proliferation of the secondary lamellar epithelium (black arrow).

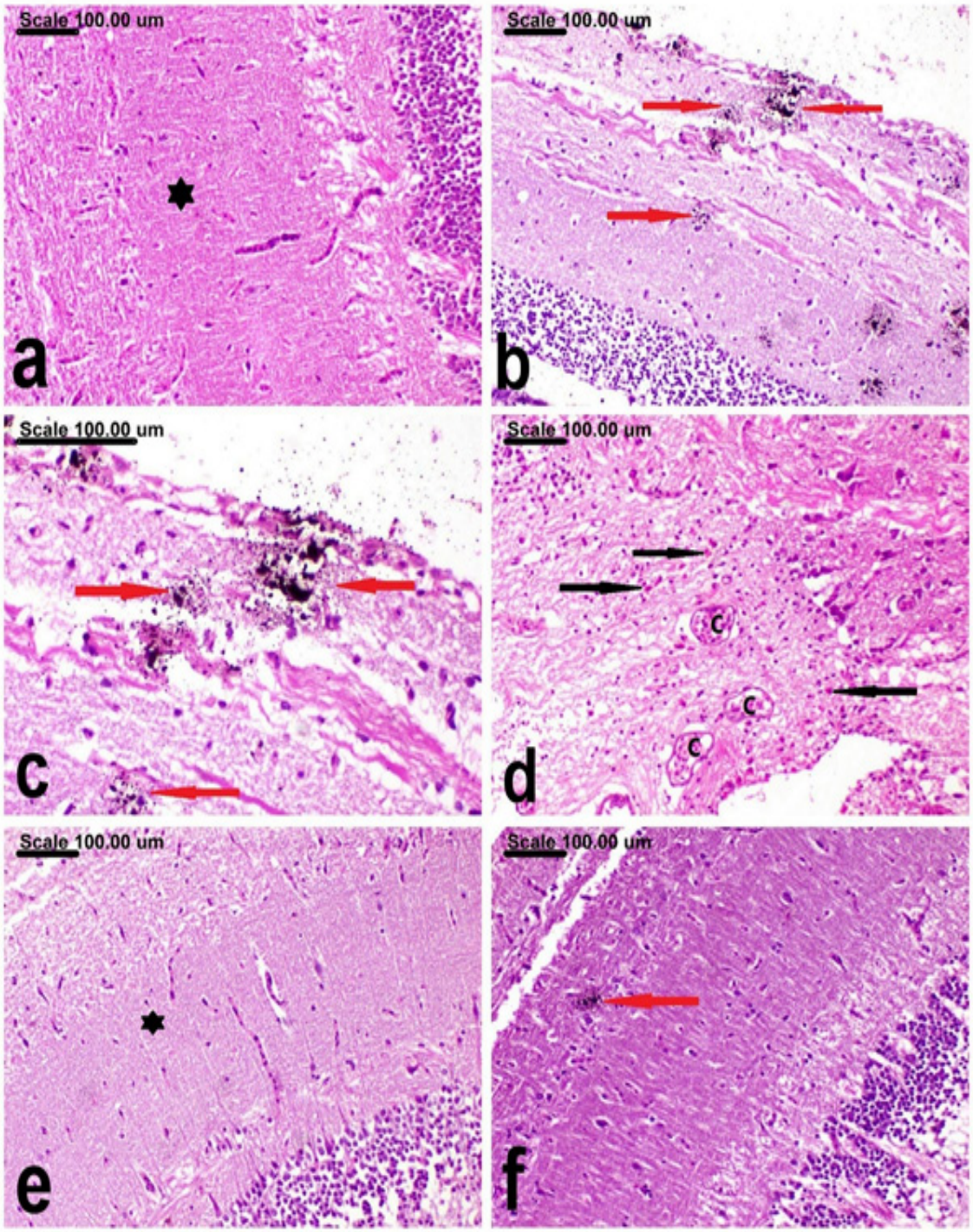

Fig. 12. photomicrograph of the brain mesencephalon of, (a) the control negative group showing a normal mesencephalon with normal optic tectum (astrix), (a), (b, c, d) the control positive group showing extensive dissociation and vacuolation of the optic tectum associated with massive aggregation of bacterial colonies (red arrows) (b & c) and marked congestion of the blood vessels (C) in the periventricular tissue revealed associated with edoema and intense infiltration of lymphocytes and eosinophilic granular cells (black arrows) (d), (e) ZnO NP-treated group showing a normal optic tectum (astrix), and (f) the ampicillin-treated group showing focal aggregation of the bacterial colonies in the optic tectum (red arrow).

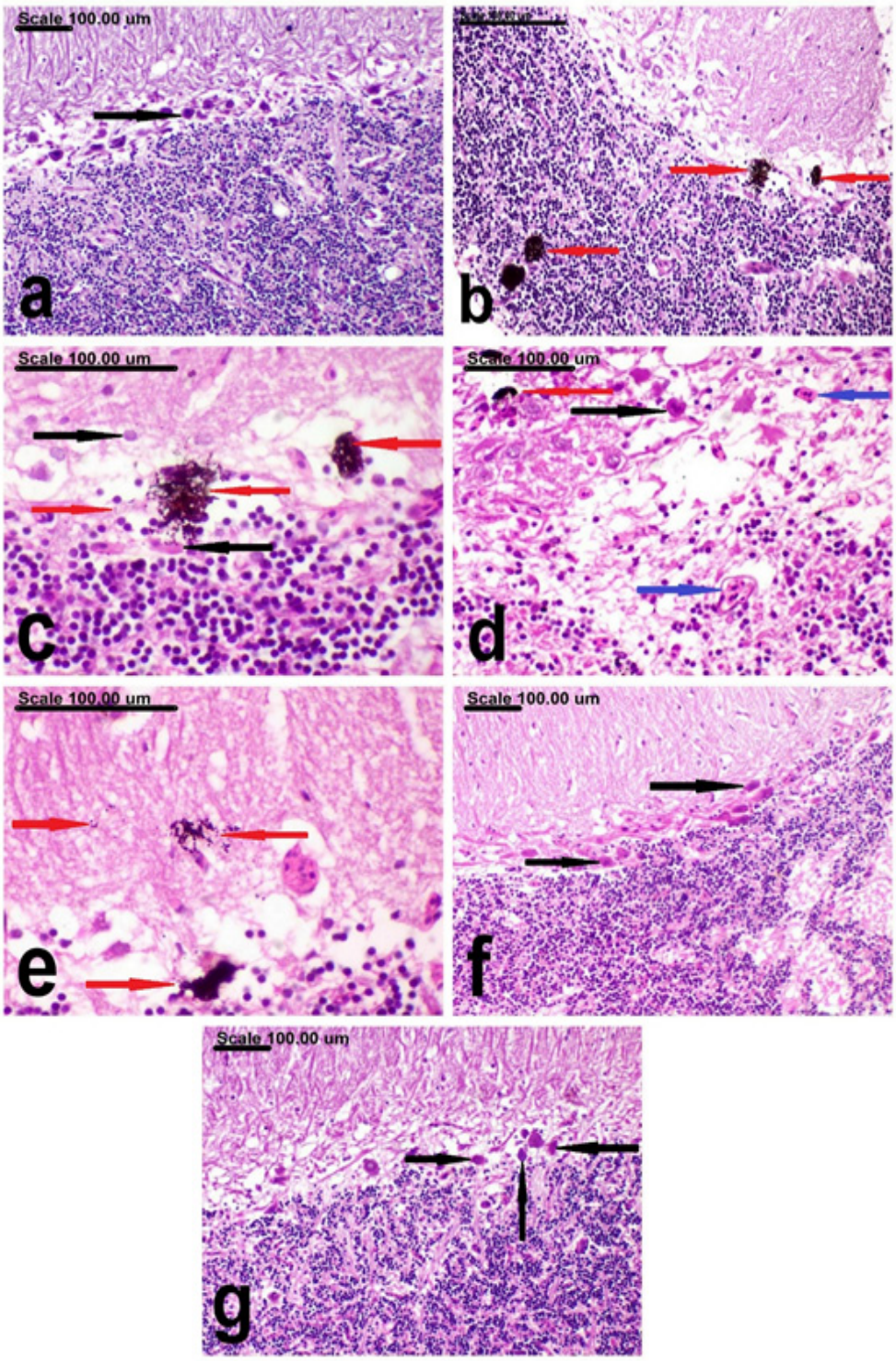

Fig. 13. photomicrograph of the cerebellum (metencephalon) of, (a) the control negative group showing normal cerebellum, with normal Purkinje (ganglionic) cells (black arrow), (b, c, d, e) the control positive group showing congestion of blood vessels (blue arrows) associated with edoema, massive necrosis of Purkinje cells (black arrows), and aggregation of numerous bacterial colonies in the ganglionic and granular cell layers (red arrows) (b, c, d, e), (f) ZnO NP-treated group showing degeneration of Purkinje cells (black arrows) in the absence of vascular congestion and vacuolation of the molecular cell layer, and (g) the ampicillin-treated group showing sparse degeneration of the Purkinje cells (black arrows).

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التأثير التحسيني لجسيمات أكسيد الزنك متناهية الصغر لعدوى البكتيريا العقدية باروبريس في أسماك البلطي النيلي

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قسم الباثولوجيا - كلية الطب البيطري – جامعة القاهرة – الجيزة –١٢٢١١ - مصر

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أظهرت جسيمات أكسيد الزنك متناهية الصغر تأثيرا مضادا لأنواع متنوعة من البكتريا في العديد من الدراسات المختبرية ومع ذلك لم يتم التحقق من صحة آثارها المضادة للميكروبات في أسماك البلطي النيلي ولذلك هدفت هذه الدراسة إلى تقييم التأثيرات المحتملة للجرعات الغذائية العالية من جسيمات أكسيد الزنك متناهية الصغر على أسماك البلطي النيلي بالإضافة إلى دراسة فاعليتها العلاجية ضد عدوى المكورات العقدية في البلطي النيلي المصاب تجريبيا بمكورات الباروبريس. ولاختبار سلامة الجسيمات متناهية الصغر داخل الجسم الحي، تم تقسيم ١٢٠ سمكة من أسماك البلطي النيلي إلى أربع مجموعات تتناول جرعات غذائية مختلفة (٠، ١٢٥، ٢٥٠ و٥٠٠ ملغم/كغم من العلف الجاف) من الجسيمات متناهية الصغر بمتوسط حجم (٣٠±٥ نانومتر) لمدة ٧ أيام متتالية. أظهرت نتائج تجارب السلامة أن تغذية البلطي النيلي على علائق مكملة بـجسيمات زنك متناهية الصغر (125 مجم/كجم من العلف الجاف) أدى إلى زيادة ملحوظة في تركيز الهيموجلوبين ونسبة الهيماتوكريت وعدد كرات الدم الحمراء. كما أظهرت النتائج زيادة تركيز بقايا الزنك في عضلات الأسماك التى تغذت على علف مكمل بجسيمات أكسيد الزنك وكان أعلى تركيز لبقايا الزنك في العضلات (٢٠,٤٩ ± ١,٧٥ جزء في المليون) في المجموعة التي تم تغذيتها على ٥٠٠ ملجم من أكسيد الزنك متناهى الصغر/كجم من العلف الجاف. وبعد دراسات السلامة، تم تقسيم 120 سمكة إلى أربع مجموعات. كانت المجموعة الأولى (G1) بمثابة مجموعة ضبط السلبية، في حين تمت عدوى المجموعات الثلاثة الأخرى داخل التجويف البروتوني باستخدام *S. parauberis*. وقد كانت المجموعة الثانية (G2) بمثابة المجموعة الضابطة الإيجابية وتم تغذيتها على نظام غذائي أساسي بدون أدوية. كما عولجت المجموعتين الثالثة والرابعة باستخدام جسيمات أكسيد الزنك متناهية الصغر (125 مجم/كجم من النظام الغذائي الأساسي) والأمبيسيلين (50 مجم/كجم من النظام الغذائي الأساسي)، على التوالي، لمدة 7 أيام متتالية بمجرد ظهور العلامات السريرية لداء المكورات العقدية. تمت ملاحظة الأسماك بعناية طوال الفترة التجريبية بحثًا عن أي تشوهات أو تغيرات في سلوك الأسماك والتغيرات السريرية وملاحظة الوفيات لمدة ١٦ يومًا بعد التحدي. تم جمع عينات الدم والأنسجة من مجموعات تجريبية مختلفة لتقييم التغيرات الدموية والكيميائية الحيوية والمناعية والنسيجية طوال فترة التجربة. وكانت معدلات البقاء على قيد الحياة ١٠٠٪ (G1)، ٤٦,٦٧٪ (G2)، ٥٣,٣٤٪ (G3)، و٥٣,٣٤٪ (G4). تم تسجيل العديد من التغيرات السلبية في عوامل الدم والكيمياء الحيوية والمناعية والتغيرات التشريحية المرضية في المجموعات المرضية. أدى علاج البلطي النيلي بجرعات غذائية من ZnO NPs أو الأمبيسلين إلى تحسين تأثير عدوى *S. parauberis* في البلطي النيلي. ولكن على الرغم من ذلك إلا أنها تحتاج إلى مزيد من التحسين لتحقيق معدلات بقاء أعلى للسيطرة على عدوى *S. parauberis*

الكلمات الدالة: مقاومة المضادات الحيوية؛ بكتريا باراوبيريس العقدية ؛ جسيمات أكسيد الزنك متناهية الصغر.