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Lead Acetate Exposure Induces Growth Retardation, Immune Suppression, Oxidative/Antioxidant Imbalance, and Histological Alterations in *Oreochromis niloticus:* A Mitigation Trial via *Chlorella vulgaris*

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Abstract

LEAD POLLUTION is a common problem in aquatic environments and poses serious health hazards. Fish are extremely susceptible to Pb accumulation, which hinders their growth and general health. This study examined the effects of water-borne Pb acetate on the biological indices, tissue architecture, and growth of *Oreochromis niloticus*. The ability of dietary *Chlorella vulgaris* (CV) microalgae to protect against Pb exposure was also investigated. A total of 270 healthy *O. niloticus* (42.4±0.21 g) were divided into six groups (45 fish/group). The CTR (control) and Pb groups were fed a basal diet and exposed to 0, 5, or 10 mg/L Pb. The CV groups were fed a diet supplemented with 10% CV and exposed to 0, 5, or 10 mg/L Pb for 56 days. The results revealed that Pb exposure caused growth retardation in a dose-dependent manner, with significant decreases (P<0.05) in immunological and antioxidant indices. Pb exposure was associated with a significant increase (P<0.05) in the serum levels of lipid peroxidation and hepatorenal biomarkers. Exposure to Pb caused an increase in IL-12 gene expression and a significant, dose-dependent tissue-damaging effect. Compared with Pb-exposed non-fed fish, the growth, immunity, antioxidant status, hepatorenal functions, and expression of IL-12 of the Pb-exposed fish were considerably modulated when fed CV diets. The tissue architecture, chemical body composition, and Pb residue levels in the muscles of Pb-exposed fish were all enhanced by CV. Crucially, CV could be administered as a feed supplement to improve the health and productivity of Pb-exposed *O. niloticus*.

Keywords: *Oreochromis niloticus*, Lead toxicity, Immunity and Antioxidants, Histopathology, *Chlorella vulgaris*.

Introduction

Oreochromis niloticus is the most extensively farmed fish in the world and has significant economic value for the aquaculture and fisheries industries. *O. niloticus* alone accounts for more than 80% of the total tilapia production in aquaculture worldwide. Tilapia is a resilient fish that grows quickly in captivity and is seen as promising, particularly in low-income countries. It is crucial in providing the poorest residents of rural areas with dietary and financial support. It is a possible bioindicator of aquatic pollution because of its exceptional ability to live at a wide range of temperatures and unfavorable environmental circumstances [1]. As a result, tilapia is now used as a model fish to investigate their toxicity in specific aquatic environments. Pollution from anthropogenic activities, including domestic, industrial, and agricultural processes, commonly affects aquatic organisms. All living organisms are negatively impacted by heavy metal ions, which severely pollute the environment and cause both short-term and long-term biological, ecological, and financial issues [2]. The capacity of heavy metals to

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persist in the food chain is the greatest risk they pose. Lead (Pb) is one of the heavy metals that is most prevalent in aquatic environments. Fish are useful bioindicators of this type of contamination because they can both bioaccumulate and bio-magnify Pb [3]. Pb concentration, exposure duration, aquatic environment (dissolved oxygen, temperature, hardness, and pH), and fish physiological status (size and sex) influence the detrimental effects of Pb pollution differently [4]. Pb pollution disrupts fish health by preventing critical organs such as the kidneys, liver, brain, muscles, and gills from functioning normally. Among the various naturally occurring forms of Pb, Pb acetate is one of its most dangerous constituents. Pb significantly suppresses fish growth and alters the levels of glutathione [5], superoxide dismutase (SOD), and total protein. Furthermore, there has been a notable increase in the level of lipid peroxidation (MDA). Expensive chemical substances such as sodium selenite, ethylenediaminetetraacetic acid (EDTA), and sodium aluminosilicate (zeolite) are used to chelate and remove Pb pollution [6]; however, these agents are not economically viable for the aquaculture sector.

A variety of feed additives, including probiotics, algae, and vitamins with antioxidant qualities, have been added recently to help reduce the effects of Pb pollution. According to Ibrahim et al. [7], these substances regulate disease and pollution by inducing antioxidant and nonimmune-related defense mechanisms. The most prevalent category of unicellular freshwater microalgae is *Chlorella vulgaris* (CV). In aquaculture, it is frequently utilized as a probiotic nutritional supplement. Owing to its bioactive components, which include polysaccharides, proteins, minerals, vitamins, polyunsaturated fatty acids (omega-3 and omega-6), and photosynthetic pigments (chlorophylls and carotenoids), CV offers a wide range of nutritional, biological, and pharmacological qualities [8]. CV was added to the feed at rates of 2.5% [9], 5%, 7%, and 10% [10] for aquatic organisms. Dietary CV is employed in fish to preserve growth performance and biological parameters while also mitigating the negative effects of Pb exposure [11]. Abbas et al. [12] reported that CV can potentially have a therapeutic function by effectively mitigating the decreased immune responses and oxidative stress produced by arsenic exposure. According to Eissa et al. [13], the antioxidant property of CV can minimize oxidative stress and mitigate the toxicity generated by sodium nitrite.

To the best of our knowledge, this is the first study to compare and assess the probiotic benefits of CV against oxidative damage, immunosuppression, histological aberrations, hepatorenal toxicity, and growth performance changes following long-term Pb exposure in *O. niloticus.*

Material and Methods

Chemicals

The lead acetate $Pb(C_2H_3O_2)_2$ was obtained from the PIOCHEM Company, Egypt (product code: PCHL1028, CAS: 51404-69-4), with a purity of 99.8%.

A total of 1.002 g of lead acetate was dissolved in 1000 ml of deionized water to produce a Pb standard stock solution. Each ml of the stock solution contained 1 mg of Pb. Stock solutions were diluted to create the necessary test solutions for the investigation.

Chlorella vulgaris (CV) pure powder was provided by the Faculty of Agriculture, Kafr Elsheikh University.

Feed formulation

In accordance with Jobling [14], two experimental diets (Table 1) were prepared from commercial ingredients to satisfy the dietary requirements of *O. niloticus*. Various algal concentrations were included in the diets at 0 (CTR) and 100 g CV/kg diet. The dosage of CV was chosen as recommended by Galal et al. [10]. After thoroughly mixing the diet ingredients with CV for 30 min and adding 200 mL of water to each kg of feed to create pastes, the mixture was pelletized in an electrical mincer with a diameter of 2 mm. Afterwars, the meals were dried at 50°C until the moisture level was less than 10%. The experimental diets were then stored in a re frigerator at -4°C until use.

Feed samples from each experimental diet were collected at the beginning, middle and end of the experimental period. The chemical analysis of the prepared diets was conducted according to AOAC guidelines [15]. The chemical constituents of the feed ingredients are shown in Table 1. Two distinct isoenergetic and isonitrogenous meals were prepared, with crude protein contents ranging from 29.97 to 30.09% and gross energy from 3203.7 to 3233.2 kcal/kg, respectively.

Fish rearing conditions

A total of 270 healthy *O. niloticus* (42.4±0.21 g) were provided from a private farm and sent immediately to the Animal Health Research Institute's wet laboratory in the province of Kafr Elsheikh, Egypt. The fish were acclimated to the experimental setup for 14 days. To maintain appropriate water parameters, debris was removed, and ½ of the tank water was drained and replaced with clean, unchlorinated water daily. During the adaptation period, the fish were fed *ad libitum* on a control diet, and the feed was presented twice/day at 8.00 a.m. and 1.00 p.m. Water quality parameters were regularly monitored via a water quality checker and recorded as follows: water temperature, 24.5±2°C; pH, 7.7±0.2; dissolved oxygen, 6.5±1.1 mg/L; and salinity, 0.47±0.2 g/L.

Experimental design

For 56 days, the fish were kept in water contaminated with 10% or 20% of the median lethal concentration (LC_{50}) of lead acetate (Pb) in an attempt to mitigate Pb toxicity with dietary CV. The 96 h LC_{50} of Pb to *O. niloticus* was previously determined to be 51.96 mg/L [25]. In six groups, each in triplicate, the fish were haphazardly distributed into 18 glass aquaria (50 × 40 × 40 cm) (n=45 fish/group, 15/replicate). For the CTR and CV groups, the fish were fed *C. vulgaris* (CV) at rates of 0 and 100 g/kg fish feed, respectively, whereas for the 5 Pb and 10 Pb groups, the fish were exposed to Pb (5 and 10 mg/L Pb, respectively). 5 Pb + CV and 10 Pb + CV groups: Fish were exposed to Pb (5 and 10 mg/L Pb, respectively) and fed CV at a rate of 100 g/kg. The fish were fed to apparent visual satiation by hand twice a day. The fish were observed daily to monitor mortality rate (MR%) and clinical signs. The following equation is used to evaluate MR%:

$$\text{MR\%} = \frac{\text{No fish deathes in a certain period}}{\text{Total fish population in that period}} \text{ x } 100$$

To ensure that all of the feed was consumed, great care was taken. The fish were weighed at the beginning of the experiment (W1) and every two weeks for the following 56 days. The amount of feed consumed was adjusted for each period according to the average body weight. Figure 1 show the design of the experiment.

Growth performance and feed efficiency variables

Growth indices were computed at the end of the feeding trial according to the subsequent equations [16]:

$$\text{Total weight gain (WG, g)} = \text{Final weight (FBW)} - \text{Intial body weight (IBW)}$$

$$\text{Specific growth rate (SGR, \%)} = \frac{\text{In FBW} - \text{In IBW}}{\text{Days of the trial}} \times 100$$

$$\text{Gain \%} = \frac{(\text{FBW} - \text{IBW})}{\text{IBW}} \times 100$$

$$\text{Feed Conversion Ratio (FCR)} = \frac{\text{Feed intake (g)}}{\text{WG (g)}}$$

$$\text{Protein Effeciency Ratio} = \frac{\text{WG (g)}}{\text{Protein Intake (g)}}$$

Blood sampling and analyses

Following the 56-day feeding study, all the fish were subjected to a 24-hour starvation period before being sampled. Blood (n=9/group) was drawn from the caudal vein via an anticoagulant (EDTA) for the estimation of hematological parameters and phagocytosis (phagocytic index and activity), which was performed within 24 h. For serum analysis, another batch of blood was drawn without anticoagulant. After the serum was centrifuged for 5 min at 4000 rpm, it was collected in Eppendorf tubes and stored at -20°C until further analyses, which were performed within two weeks.

The fish were anesthetized by submersion in freezing water and a saturated benzocaine solution and then killed via spinal cord transection. Liver, gill, and intestine samples (n=9/group) were preserved in 10% formalin until histological analysis. The fish was sliced on ice to extract the liver tissues (n=9/group), which were then promptly frozen in liquid nitrogen and maintained at -80°C until RNA was extracted.

Hematological indices

All of the hematological variables were estimated according to Faggio et al. [17]. A modified Neubauer hemocytometer was used to count the red blood cell count (RBCs, $\times 10^6/mm^3$) and white blood cell count (WBCs, $\times 10^3/mm^3$). The cyanmethemoglobin technique was used to measure the hemoglobin concentration (Hb, g/dl), and a microhematocrit centrifuge was used to calculate the packed cell volume (PCV, %). Thin blood films were prepared on sterile slides to calculate the differential leucocyte count. A modified Wright's stain was applied to the slides once they had time to dry.

Immune-related and oxidative stress biomarkers

The techniques described by Abu-Elala et al. [18] and Kawahara et al. [19] were used to assess serum lysozyme (LYZ) activity and phagocytosis (activity % and index no), respectively.

The levels of GPx (glutathione peroxidase), SOD (superoxide dismutase), and CAT (catalase) activities were used to measure the serum antioxidant capacity. Diagnostic kits obtained from Biodiagnostic CO, Egypt, were used. The colorimetric methods used for estimation of GPx, SOD, and CAT activity were performed at 340, 560, and 510 nm, respectively, following the manufacture instructions. Malonaldehyde (MDA) levels were determined following the manufacturer's instructions via diagnostic reagent kits (Cusabio Biotech Co., Ltd.; China).

Gene expression

IL-12 gene expression was measured in the livers of the experimental *O. niloticus*. The primer sequences from the NCBI database are included in Table 2. All primers and kits were provided by Sigma–Aldrich (Sigma–Aldrich Chemie GmbH, Germany). Using an AbiPrism 7300 heat cycler (Applied Biosystems, USA), a quantitative polymerase chain reaction (qPCR) was performed. Using the $2^{-\Delta\Delta CT}$ approach, the quantitative fold changes in the investigated genes were computed concerning β-actin mRNA, a nonregulatory reference gene.

Serum biochemical parameters

Following Tietz [20], the serum biochemical indicators for kidney function (creatinine and urea) and liver function (ALT and AST) were estimated. The estimation of the levels of albumin and total protein was performed according to Doumas et al [21]. Albumen protein was subtracted from total protein to calculate globulin. Table 3 displays the kits used to estimate the serum biochemical parameters.

Proximate chemical analysis

To calculate the whole-body chemical composition, 9 fish/treatment were taken at random, weighed, and stored at -20 °C until analysis. The measurements were performed via the approved method of AOAC [22]. To achieve a constant weight, the moisture content was measured after oven drying at 105 °C, and the ash content was measured after burning for 6 h at 550 °C in a muffle furnace. A Micro-Kjeldahl apparatus (VELP Scientifca UDK149, Italy) was used to assay crude protein. A 16-h petroleum ether extraction process was used in the Soxhlet apparatus to assess the total lipid content.

Pb residue analysis

The Pb concentration in the fish muscles was analyzed via the technique described by Aljabryn [23]. For each group, three fish per replicate (n=9/group) were used to weigh ten grams of moist muscle tissue. The samples were then placed in crucibles and heated at 450°C overnight in a furnace. The tissue was dehydrated to room temperature and then heated to 80°C on a hot plate. Two milliliters of strong nitric acid (65%) and 20 mL of diluted HCL (10%) were added to a polytetrafluoroethylene (PTFE) beaker. Filtration of the resulting mixture with 0.45 µm pore size filter paper produced a 50 mL sample solution. Next, an atomic absorption method was applied to evaluate the Pb content.

Histological and intestinal histometric examination

The tissue samples (liver, gill, and intestine) were preserved in 10% neutral formalin, followed by dehydration with increasing alcohol concentrations, xylene clearing, and paraffin impregnation. Then, following Fischer et al. [24], 5 µm paraffin sections were made and stained with hematoxylin and eosin (H&E). Unaware of the groups' treatments, a histopathologist used a light microscope (Leica DM 5000) to examine three sections of each sample. Under a microscope fitted with a digital camera, the samples were inspected, images were snapped, and morphometric measurements were recorded. Using ImageJ analysis software (National Institutes of Health, MD, USA), all measurements (villus length and width) were recorded in micrometers.

Statistical analysis

The statistical analysis was conducted to evaluate the effects of Pb and CV on several health indices of the experimental *O. niloticus*. With SPSS software version 22, one-way ANOVA and Duncan's multiple range test were used to determine the means and standard errors of the obtained data. P values < 0.05 were used to determine statistical significance. The homogeneity and normality of variance were evaluated before the data analysis. SPSS version 22 software was used to draw the figures.

Method validation and biosafety protocol

Each treatment was carried out in triplicate, and three samples from different fish from each triplicate were used to evaluate the biological variance and repeatability (n=9 samples/treatment).

All of the fish that were still alive at the end of the trial were burned in the laboratory's stationary incinerator. Pb contamination must be managed since it can be extremely harmful to living organisms. To avoid contamination during the study, distilled water and acetone were used to clean all of the Petri plates and equipment.

Results

Mortality rate and growth performance

The main results of the trial are illustrated in Figure 1. The mortality rates of the fish exposed to 5 and 10 mg/L Pb acetate (Fig. 2) were significantly greater than those of the other treatment groups, and CV increased survival in the Pb-exposed groups. the CTR and CV-treated groups showed no mortalities throughout the trial (Fig. 2).

The data presented in Table 4 indicate that *O. niloticus* exposed to 5 or 10 mg Pb/L significantly (P<0.05) decreased FBW, TG, G%, FCR, PER and SGR compared with those in the CTR group. Moreover, compared with CTR, dietary addition of CV significantly (P<0.05) improved FBW, TG, G%, FCR, PER, and SGR by approximately 5.8%, 18.2%, 17.8%, 54.9%, 21.7%, and 14.3%, respectively. However, CV inclusion in the diets of the fish that were exposed to 5 or 10 mg Pb/L did not significantly improve growth performance parameters (FBW, TG, G%, PER and SGR) (P>0.05) in comparison with those of the fish in the groups exposed to the same Pb dose and fed the basal diet.

Hematological parameters

Table 5 shows the differences in hematological parameters (RBCs, Hb, PCV, and WBCs) and differential leukocyte counts after long-term exposure of *O. niloticus* to Pb and feeding on diets supplemented with CV. A dose-dependent reduction in RBC levels was observed in the Pb-exposed groups in addition to a significantly reduced Hb level and PCV% in the 10 Pb group, indicating that the fish were suffering from anemic conditions.

Compared with the CTR group, the Pb-intoxicated group presented considerably greater heterophil counts

(P<0.05) and significantly lower WBC and lymphocyte counts. When Pb-intoxicated fish were fed CV diets, their WBC and lymphocyte counts increased compared to the Pb groups (P<0.05). In addition, the heterophil count did not significantly differ between the Pb + CV groups and the CTR groups (P<0.05). Eosinophil, basophil, and monocyte counts were not significantly different between the groups (P>0.05).

Immune responses and antioxidant activity

The findings concerning the impact of chronic Pb exposure on *O. niloticus* immune status (Table 6) demonstrated immunotoxicity. Compared with the other groups, the Pb-exposed group presented considerably lower serum lysozyme activity (P<0.05). Treatment with CV increased lysozyme level (P<0.05) in Pb + CV toward control values (Table 6), in a dose-dependent manner. Moreover, Pb toxicity considerably reduced the phagocytic activity compared with that of Pb + CV (P<0.05). Compared with all the other groups, the fish-fed diets containing CV presented the highest levels of phagocytic and lysosomal activity (P<0.05). The phagocytic index did not significantly differ among the groups (P>0.05).

The serum oxidative indices of *O. niloticus* exposed to different concentrations of Pb acetate and fed diets containing CV are presented in Table 6. Compared with CTR, exposure to 5 or 10 mg Pb/L markedly (P<0.05) decreased serum GPx, SOD, and CAT activities but substantially (P<0.05) increased serum MDA activity. Moreover, dietary CV inclusion insignificantly increased serum SOD, CAT and GPx activities and insignificantly decreased serum MDA activity compared to CTR. When compared to Pb groups (P<0.05), feeding Pb-intoxicated fish with CV-supplemented diets resulted in a notable increase in liver SOD, CAT, and GPx levels together with a significant drop in blood MDA levels.

Gene expression

As shown in Figure 3, the gene expression of the cytokine IL-12 in the liver was evaluated to assess the effect of Pb on *O. niloticus* immunity. Exposure to Pb (5 Pb and 10 Pb groups) resulted in an increase in the level of proinflammatory IL-12 in a dose-dependent manner. There were no significant changes in either the CTR group or the CV group. Feeding Pb-exposed fish with CV-based diets induced significant (P<0.05) downregulation of the gene expression of IL-12.

Biochemical parameters

Table 7 shows that, compared with CTR, Pb exposure significantly (P<0.05) reduced the total serum protein, albumin and globulin levels and substantially elevated the serum creatinine, urea, AST and ALT levels. On the other hand, compared with CTR, CV addition to the *O. niloticus* diet significantly (P<0.05) increased the total protein concentration in the serum but had no effect on the serum ALB, globulin, creatinine, urea, AST or ALT levels. Moreover, CV inclusion in the diets of the fish exposed to 5 or 10 mg Pb acetate/L significantly (P<0.05) improved the abovementioned biochemical parameters toward CTR values compared with those of the fish exposed to the same Pb doses and fed the basal diet without CV.

Chemical body composition and Pb residues in muscle tissue

Table 8 shows the chemical body composition of *O. niloticus* exposed to chronic Pb toxicity and fed diets supplemented with CV. Body moisture and crude protein contents were significantly lower in the Pb-exposed groups than in the CTR group. Moreover, the ether extract, Ash, and carbohydrate contents increased significantly in the Pb-exposed fish compared with those in all the tested groups. The inclusion of CV in the diets of Pb-exposed fish significantly restored all the values of chemical body composition toward the CTR values, while it did not differ significantly between the CV group and CTR group.

As shown in Table 8, the lead residues in the muscle of the fish in the CV group presented the lowest level of Pb residue (0.01 ± 0.00) compared with those in the Pb groups (5 Pb and 10 Pb groups), which presented the highest quantities of Pb residue (1.78±0.07 and 3.80±0.15, respectively). Compared with those in the Pb group, the Pb + CV groups presented significant decreases (0.66±0.08 in the 5Pb + CV group and 2.06±0.05 in the 10Pb + CV group) in the Pb residue (P<0.05).

Clinical and internal examinations

For the CTR and CV groups, no changes in behavior were observed. However, the Pb-exposed groups presented notable behavioral changes related to feeding (loss of appetite), locomotion (sluggish movement and irregular swimming), skin (dark skin) and gill (pale gills) coloration. After 56 days of the trial, the majority of the behavioral impairments were very severe in the 5Pb and 10Pb groups. The most obvious clinical and P/M signs detected among the fish groups were briefly tabulated (Table 9). Clinical examination of the Pb-exposed fish revealed hemorrhagic spots on the skin, fin and tail erosions, scale detachment, ocular opacity and hemorrhage, and skin darkness (Figure 4a, b, c).

Internal examination of the Pb-exposed groups (Fig. 4d, e, f) revealed pale gills, pale hemorrhagic livers, splenomegaly, empty intestines, distended gall bladders, congested kidneys, and bile retention in the liver. The severity of the signs, which was dose-dependent, was lower in the 5Pb group than in the 10Pb group and was more severe. The inclusion of CV in the diets of the Pb-exposed groups markedly improved the picture of internal organs (Table 9).

Histopathological examination

The histomorphology of H&E-stained sections from the gills, hepatopancreases, and intestines of the experimental groups is described in detail in Figures 5-7. Normal histological features in the gills, consisting mainly of primary lamellae branching out into tiny secondary lamellae, were observed in the CTR and CV groups (Figure 5a, 5d). Conversely, the Pb-exposed groups exhibited significant necrosis of secondary lamellae, hyperplasia of the covering epithelium of primary lamellae with necrotic materials between primary lamellae (Figure 3b, 3c), and the severity of lesions differed in a dose-dependent manner. In the Pb + CV-treated groups, there was an apparent decrease in the extent and distribution of the lesions detected in the Pb-exposed groups, as the 5 Pb + CV group presented a normal histoarchitecture (Figure 5e) similar to that of the CTR group, even though certain alterations persisted in the 10 Pb + CV group (Figure 5f).

Like the gills, the hepatopancreases of the CTR, CV, and 5Pb + CV groups presented a normal histoarchitecture in which hepatocyte cords and pancreatic acini surrounded the central veins (Fig. 6a, 6d, and 6e). The Pb-exposed groups displayed pancreatic acini necrosis (varying from focal to massive according to the Pb dose) and massive hepatic necrosis with diffuse deposition of brown melanin pigments (in the 10Pb group) (Figure 6b-6c). In the Pb + CV-treated groups, a clear improvement in liver histology was observed, with a decrease in the distribution and severity of previously noted lesions (Figure 6e, 6f).

Once again, the intestines of the CTR and CV, 5 Pb + CV, and 10 Pb + CV groups presented typical histological structures with characteristic intestinal villi (Figure 7a, d, e), whereas those of the Pb-exposed groups presented diffuse catarrhal inflammation (Figure 7b) and massive villous necrosis (Figure 7c). The intestine of the Pb + CV-treated groups presented a marked reduction in the distribution and severity of previously detected lesions in the Pb-exposed groups, whereas the examined sections presented normal villous structures. The results of the intestinal morphological measurements are briefly tabulated in Table 10. Compared with CTR, long-term Pb toxicity caused a gradual reduction in villus length and the number of goblet cells. The lowest value was obtained for 10 Pb samples ($P<0.05$). In terms of villus width, the greatest difference was recorded for 10 Pb, indicating the severest form of disruption among the groups. The inclusion of CV in the diets of the Pb-exposed groups significantly improved the intestinal morphometry.

Discussion

O. niloticus is the most frequently farmed fish in Egypt. Heavy metal pollution, particularly Pb, builds up in fish organs, causing physiological and metabolic disruptions in fish and resulting in altered behavior and abnormal movements [25]. Most of these hazardous compounds end up in freshwaters, where they cause major symptoms in fish. Toxicants can alter an organism's growth, development, behavior, and reproduction or even cause it to die, even though aquatic ecosystems have several physiological and biological mechanisms to prevent or lessen their harmful effects.

In the present study, the maximum mortality in the highest treatment group, 10 mg Pb/L, was in agreement with that reported by Mahi et al. [25]. Nigar et al. [26] reported an increase in the mortality rates of *C. punctatus* and *H. fossilis* following exposure to Pb $(NO_3)_2$. Additionally, the dietary inclusion of CV decreased the proportion of fish that died, which is consistent with the findings of Andrews et al. [27], who reported that the dietary addition of CV to *rohu Labeo rohita* increased the survival rate, antioxidant capacity, and phagocytosis.

The lower growth performance in the present study due to Pb exposure was consistent with earlier studies [25; 28]. These studies revealed that fish exposed to Pb or other toxicants had reduced growth. Reduced feed intake and inadequate biological conditions associated with toxicity stress compensate for the poor growth performance of the Pb-exposed groups compared with that of the CTR group [29]. Measuring behavioral abnormalities in fish can help determine the toxicity of various hazardous contents [25]. The behavioral changes reported in this study were consistent with the studies conducted by Mahi et al. [25]. Our findings concur with those of Melebary and Elnaggar [30], who reported that the exposure of mice to Pb acetate resulted in decreased performance, a high mortality rate, and organ malfunction. Based on the results reported here, *O. niloticus* treated with CV and exposed to Pb presented significantly lower mortality rates than Pb-exposed groups fed on basal diets did, suggesting that CV had an ameliorative effect. Similarly, reduced mortality in *O. niloticus* fed supplemented diets has been described by Abu-Zahra et al. [31], who reported that the addition of *Spirulina platensis* to the *O. niloticus* diet decreased cumulative mortality.

Chlorella enhanced the growth performance of various fish and shellfish in several earlier studies [32; 33]. When *O. niloticus* were fed diets containing Chlorella, their body weight noticeably increased [32]. Galal et al. [10] reported similar results in *O. niloticus*, showing that dietary supplementation with Chlorella increased the resistance of these fish to penoxsulam exposure and improved their growth performance. The results of the present study demonstrated that the addition of CV to diets greatly increased the protein efficiency ratio of *O. niloticus*. This finding is in line with findings from previous study on *O. niloticus* by Peng et al. [33]. Fish growth is supported by the efficiency of protein, which is measured by the protein efficiency ratio [33]. The findings of the present study

regarding the increased protein efficiency ratio in fish fed diets containing CV suggest that the addition of CV to the diet enhances *O. niloticus* protein utilization. This is primarily because *Chlorella* contains abundant amino acids, vitamins, minerals, feeding attractants, antioxidants, fibers, and nucleic acids, which have been found to promote fish growth [33].

Hematological indices such as the PCV, Hb concentration, and RBC count can reveal an organism's secondary responses to pollutants. Hematological tests of *O. niloticus* subjected to 5 and 10 mg/L Pb revealed significant decreases in different blood parameters. Furthermore, the findings revealed that the inclusion of CV in fish diets could counteract these alterations. Hematological indices, including PCV, Hb, WBCs, and RBCs, have been employed as markers of osmotic shifts and metal contamination in aquatic environments [30]. The changes in hematological parameters were as follows: stress-induced release of RBCs from the spleen, hypoxia induced by Pb exposure, and direct or feedback response of structural impairment to RBC membranes, leading to hemolysis and impaired hemoglobin synthesis. The oxygen level of an organism is indicated by its hemoglobin concentration, which the body uses to sustain in the face of stress. According to the results of the present study, the Hb concentration significantly decreased in comparison with the CTR values. A decrease in hemoglobin levels in Pb-exposed fish typically results in a reduction in the ability of the blood to carry oxygen, which may also indicate anemia or support the detrimental impacts of Pb on *O. niloticus*. Moreover, as compensatory reactions to the onset of hypoxia, there may have been a decrease in RBC formation and an increase in RBC destruction, which led to decreases in RBC, PCV, Hb, and WBC counts.

WBC count changes are typical responses to toxicant exposure, according to Kori-Siakpere et al. [34], who also explained the decline in WBCs observed in our investigation. In the present study, feeding fish CV-supplemented diets improved *O. niloticus* hematological marker levels. This may be related to the high iron content of algae, which has an impact on erythropoiesis. According to our findings, fish treated with 10% CV presented insignificantly higher levels of Hb, PCV%, RBCs, and WBCs. The RBC count is an immune cell characteristic that is commonly used to assess potential undesirable side effects, such anemia. This finding is inconsistent with that of Galal et al. [10], who reported that adding CV to the diet of *O. niloticus* for 15–30 days markedly increased the RBC count, Hb concentration, PCV%, and total leucocyte count.

The phagocytotic activity of fish, a nonspecific immunological response, is a crucial defense mechanism against infectious agents such bacteria, viruses, and parasites. Hepatic dysfunction combined with low WBC counts, total serum protein, and globulin results in the production of less immunoglobulin. When fish are subjected to stressors and hazardous substances, their immune systems become impaired, and WBC counts are significantly reduced. When *O. niloticus* was supplemented with CV in their diets, their immunological state improved. Similarly, when *O. niloticus* [31] was fed diets containing other algae, *S. platensis*, marked increases in immune-related parameters and phagocytic activity were detected.

The findings of the PA and lysosome assays revealed immunosuppression in *O. niloticus* exposed to Pb acetate, and they also improved when CV was added to the diet of the fish. These results demonstrated the poor health of the fish following Pb exposure and were in line with those reported for liver enzyme levels and hematological markers. In this work, elevated serum lysosomal activity in fish fed diets containing CV revealed that dietary CV increased the immunological potential of *O. niloticus*. Additionally, when *Chlorella* was added to the diets of *O. niloticus*, this lysosome-increasing effect was previously shown by Pang et al. [33]. The lysosome is a crucial defense molecule that plays a key role in mediating defense against microbial invasion [33]. The increase in lysosomes caused by dietary *Chlorella* raised the possibility that algae contain bioactive compounds that regulate the immune response in fish. Notably, immunostimulants derived from hot water extraction of *Chlorella* cells, such as the potent antioxidants vitamin C, β-1,3-glucan, and astaxanthin [35], increase the immunological resistance of fish. Moreover, the immune response of fish may be regulated by bioactive components such as Chlorella growth factor (CGF) [33]. On the other hand, the gene expression of the proinflammatory cytokine IL-12 was observed. A comparable study by Galal et al. [10] demonstrated the immunomodulatory impacts of CV on fish exposed to penoxsulam.

The current findings showed that prolonged exposure to Pb acetate considerably reduced the activity of the antioxidants GPx, SOD, and CAT and increased the levels of MDA, but CV supplementation dramatically reduced the effects of Pb on the oxidative/antioxidant balance. Reactive oxygen species (ROS) are produced when exposed fish tissues bioaccumulate heavy metals such as Pb [36]. These ROS can have detrimental impacts. ROS are known to target unsaturated fatty acids in cell membranes and induce lipid peroxidation, which is the cause of many disorders associated with oxidative stress. The results confirmed that dietary incorporation of CV decreased lipid peroxidation and increased antioxidant enzyme activity. Furthermore, CV protects the kidney from cellular damage and HgCl2-induced oxidative stress, as shown by Blas-Valdivia et al. [37]. They predicted that because CV carotenes scavenge free radicals, they would prevent lipid peroxidation caused by HgCl2.

According to Ibrahim et al. [38], improvements in MDA and antioxidant activities, histological and histochemical alterations, and improved immunological function are signs that *S. plantensis* and CV have protective qualities against Pb acetate.

The initial line of defense against oxidative stress is thought to involve antioxidant enzymes. Consequently, alterations in blood levels of GPx, CAT, and SOD serve as reliable markers of this type of stress. According to Ding et al. [39], long-term Pb exposure of 13.13 mg/L may cause oxidative stress and increase hepatic antioxidant activity; in contrast, exposure to 26.26 mg/L may cause this activity to decrease, suggesting that antioxidant function has been inhibited.

These data demonstrated that Pb increased oxidative stress by increasing the serum MDA content in *O. niloticus*. These results are consistent with earlier studies showing that Pb exposure causes oxidative stress and excess ROS generation. CV may have a protective effect against heavy metal toxicity because of its antioxidant qualities, which stimulate GPx, CAT, and SOD activity and decrease the MDA content. The ability of CV to scavenge ROS may be attributed to its combination of phenolic acids, minerals, and vitamins, which are recognized for their antioxidant qualities.

According to Galal et al. [10], dietary supplementation with *Chlorella* can increase serum GPx activity and protect *O. niloticus* against penoxsulam toxicity. This effect may be attributed to the antioxidant properties of the flavonoids and polyphenols found in CV [33]. Furthermore, our results corroborated other studies' findings that *O. niloticus* was protected from arsenic-induced oxidative stress by dietary *Chlorella* [40]. Notably, *Chlorella* possesses antioxidant qualities and a relatively strong ability to suppress lipid peroxidation [33].

According to this study, Pb considerably and dose-dependently elevates *O. niloticus* liver enzyme levels, as measured by ALT and AST levels. These results were comparable to those of Khalefa et al. [5]. On the other hand, Melebary and Elnaggar [30] reported substantial decreases in ALT, AST, and ALP levels in Pb-exposed mice. This was explained by liver damage that resulted in a loss of enzyme production. These contradictory findings across studies may be attributed to varying Pb concentrations and exposure periods because longer Pb exposure periods exacerbate Pb toxicity. The findings presented here demonstrated that prolonged exposure to Pb led to an increase in liver enzyme levels. The hepatorenal functions (ALT, AST, creatinine, and urea levels) in this study were augmented by chronic Pb exposure; numerous hepatic lesions corroborated these deteriorations in terms of biochemical indicators. Pb exposure may be the cause of these effects because it can accumulate in the liver. Hepatic membrane permeability and functional integrity may be lost due to the strong pro-oxidative effects of Pb, which can lead to aberrant liver function and hepatic enzyme outflow into the bloodstream. Dietary inclusion of CV resulted in improvements in liver and kidney function. Therefore, adding CV to the diet did not have any negative effects on normal physiological parameters. similarly, Fadl et al. [32] reported that the serum ALT, AST, creatinine, and uric acid levels did not change when 15% CV was added to *O. niloticus* diets.

The current results demonstrated that whereas CV dietary supplementation significantly increased the serum total protein, globulin, and albumin levels, Pb exposure caused a decrease in these parameters. The findings of Fadl et al. [32] are comparable to ours in that they discovered that supplementing diets with CV for 9 weeks may considerably increase the levels of total protein, albumin, and globulins in the serum of treated *O. niloticus*. The improvement in liver function induced by CV may be the cause of this increase in blood protein levels.

Similarly, Sayed et al. [41] reported that feeding African sharp-tooth catfish subjected to microplastic toxicity (500 mg/kg fish feed) on diets supplemented with 5% CV for 15 days improved the serum total protein, globulin, and albumin levels. Furthermore, Galal et al. [10] reported that CV might preserve fish health by preserving *O. niloticus* blood parameters at normal levels when they are exposed to penoxsulam.

In this study, the addition of CV to the diet did not significantly affect the body composition of *O. niloticus*. Similarly, feeding *Chlorella* to *O. niloticus* [33] did not significantly alter their body composition. Pb exposure significantly reduced moisture and crude protein contents and increased ether extract and carbohydrate contents. According to Mahmood et al. [42], dietary exposure of *O. niloticus* to another pollutant (microplastics) significantly reduces the body composition of the fish. They reported that protein content was significantly lower while fat content was significantly greater in MP-exposed fish than in CTR fish. An essential part of nutrient metabolism involves vital organs such as the intestine and liver. According to Mahmood et al. [42], damage to these organs impairs their ability to function properly, which decreases the nutritional value of fish. These findings, which include decreased immunity, oxidative stress, decreased growth, and altered body composition of fish, suggest that Pb may negatively impact market demand and production, potentially causing enormous financial losses. However, dietary inclusion of CV significantly restored the body composition of the *O. niloticus* in the Pb-exposed groups.

Clinical and internal examination of Pb-exposed fish revealed hemorrhagic spots on the skin, fin and tail erosions, scale detachment, ocular opacity and hemorrhage, skin darkness, pale gills, pale

hemorrhagic liver, splenomegaly, an empty intestine, a distended gall bladder, a congested kidney, and bile retention in the liver. These findings may be attributed to the high accumulation of Pb in hepatic, renal, and gill tissues. As discussed previously, Pb accumulation in fish tissues promotes oxidative stress due to increased ROS generation. Oxidative stress caused by Pb exposure promotes neuronal damage and neurotransmitter dysfunction, as neurotoxicity in fish, which may be the cause of the several aberrant clinical symptoms observed in this study, including slow movement and erratic swimming. The behavioral alterations increased with increasing Pb concentration, which is in line with Abdelzaher et al. [43].

Several researchers have previously conducted histological analyses of important fish organs to determine the degree of heavy metal toxicity [25]. In environmental toxicity, fish gill and liver histology are important biomarkers [44]. Because of their wide surface area and persistent water absorption from their surroundings, gills are extremely vulnerable to all kinds of toxins. Its distinct feature makes it a highly reliable bioindicator of aquatic contamination [44]. According to Hossain et al. [28], the main pathological signs in *O. niloticus* gills subjected to chlorpyrifos are mucous cell diffusion, epithelial lifting, filament damage, gill lamellae shortening, and necrosis in epithelial tissue. When *C. carpio* was exposed to Pb $(NO_3)_2$, there were noticeable changes in both the primary and secondary gill lamellae [44]. Similar pathological lesions have been noted in the case of Pb intoxication in *O. niloticus* by Mahi et al. [25]. The present findings concur with the previously mentioned results. Gill toxicity-induced O2 deprivation is frequently cited as the main factor contributing to the deterioration of gill filaments. The oxygen exchange process is hampered when the gills are exposed to toxic substances, which results in an inadequate supply of oxygen to the gill tissue. In gill filaments, this oxygen shortage may cause cellular deterioration and injury [45].

Hepatic necrosis and vacuolation have been observed in fish exposed to varying degrees of Pb [28]. Very similar pathologies were observed in the present study. Hepatic necrosis refers to damage to the liver, an organ involved in excretion, detoxification, and the synthesis of binding proteins such as metallothionein. Since the liver of fish accumulates environmental pollutants at a higher rate than other organs do, it is extremely susceptible to toxic substances [45]. Fish contain metallothionein, a sequestering agent that aids in binding and storing trace elements. However, the ability of the liver to operate can be compromised by the mechanism by which these trace elements accumulate in critical amounts. This may cause liver cells to deteriorate and lose their syncytial organization [45]. There is a decrease in the surface area of liver cells in conjunction with the aforementioned degenerative process, which causes an increase in intrabiliary fiber-connective tissue.

The present study provides evidence of pathological changes in Pb-exposed fish; in particular, these changes include hepatocyte degradation and vacuolation. According to Elnaggar et al. [45], this hepatocyte vacuolation can cause protein synthesis to be inhibited, energy to be depleted, and the aggregation of microtubules. These results point to a possible discrepancy between parenchymal cell synthesis of materials and their release into the bloodstream [45]. Atrophy results from a loss of contact between hepatocytes and pancreocytes, which indicates injury to the hepatopancreas.

According to Mahi et al. [25], the intestine is a crucial organ for digestion and has a significant role in the nutritional absorption of fish. The intestinal tissue of *O. niloticus* exposed to Pb presented evidence of tissue rupture, larger vacuoles, an enlarged lumen, and increased villus width [25]. Exposure to higher Pb concentrations in the present study was associated with notable intestinal alterations, such as villous necrosis, catarrhal inflammation, villi height shortening, and a decrease in the goblet cell count. Thus, the histological data from this study indicated that Pb exposure significantly altered the histomorphology of *O. niloticus* intestinal, hepatic, and gill tissues.

Moreover, there was a strong correlation between the concentration of Pb acetate and the severity of the lesions it induced. These findings add to the growing body of evidence suggesting that fish mortality during the exposure period may have been influenced by histological alterations in the intestine, gills, and hepatopancreas.

The adverse effects of Pb were reduced and mitigated in the Pb + CV groups by the administration of CV. In particular, in the 5 mg/L Pb + CV group, very few pathological alterations were observed, and the histological appearance returned to normal. According to Latif et al. [46], CV lessens the negative effects of paracetamol intoxication on the levels of oxidative stress and hematological, biochemical, and histological parameters. Furthermore, they reported that CV protected Wistar rats against the damaging effects of paracetamol through its antioxidant, hepato-, nephro-, and cardioprotective properties.

CV significantly reduced the lead residue in the Pb + CV groups compared with that in the Pbs group, demonstrating the bioremediation impact of CV on Pb muscle residue in *O. niloticus*. The ability of CV to absorb heavy metals such as Hg and Pb may be due to the presence of vital constituents such as minerals, complex polysaccharides, flavonoids, carotene, and vitamins, along with phenolic substances such as gallic acid [38].

Conclusion

According to this study, Pb exposure caused *O. niloticus* to exhibit behavioral abnormalities, growth retardation, hepatorenal toxicity, hematological dysfunction, oxidative stress, and downregulation of immune-related and antioxidant indicators.

Moreover, a relatively high mortality rate was observed in the high-dose treatment group, indicating that Pb induced lethality in the studied species. Significant histological alterations in the structure of vital organs have been noted. Additionally, *O. niloticus* fed a diet supplemented with CV presented increased growth performance, whole-body composition, and antioxidant and immune capacity, all of which help to protect against Pb toxicity. As a result, the negative consequences of lead acetate decreased, which improved the tissue pathology caused by Pb-induced alterations. However, more investigations are needed to examine the immunostimulatory effects of CV on the immune system of fish, taking into account various species and pathogen virulence levels. Therefore, to reduce Pb toxicity, we recommend incorporating CV into fish diets.

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Declaration of Conflict of Interest

The authors declare that they have no competing financial interests or personal relationships that could influence the work reported in this paper.

Ethical of approval

The methodology outlined above was implemented in compliance with the European Union directive 2010/63UE and applicable rules and regulations of the Animal Health Research Institute's Ethics Committee at the Agriculture Research Centre in Egypt. The reporting of this study complies with the ARRIVE criteria (https://arriveguidelines.org). None of the authors' investigations involving human subjects are included in this paper. There were no protected or endangered species in the field studies.

Authorship contribution statement

Nagwa I.S. Abu-Zahra: Methodology, Formal analysis, Writing-original draft, Writing-Review & editing, Resources, Supervision, Investigation, Visualization, Abeer M. ElShenawy: Ideas, Writing-original draft, Formulation of overarching research goals and aims, Writing-Review. Gehan I.E Ali: Resources, Investigation, Visualization, Validation, Writing-Review, Shereen A. Yassin: Ideas, Formulation of overarching research goals and aims, Project administration, Writing-Review, Ayman Attia: Resources, Investigation, Visualization, Validation, Writing-Review.

TABLE 1. Proximate analysis of the basal diet.

Physical Composition			Chemical Composition		
Ingredients %	**Basal diet**	**CV diet**	**Items**	**Basal diet**	**CV diet**
Yellow corn	37.5	36.75			
Soybean meal 44%	31.25	32.0			
Fish Meal 60%	10.0	0.0	Moisture%	11.53	11.09
Chlorella vulgaris[*]	0.0	10.0	Crude protein%	30.09	29.97
Corn gluten (60%)	10.0	10.0	Ether extract%	4.56	4.95
wheat flour	7.0	7.0	Ash%	5.76	6.09
Soybean oil	2.0	2.0	Crude fiber%	5.96	5.75
DCP	0.3	0.3	NFE%	42.1	42.15
DL-methionine	0.2	0.2	Calcium%	1.21	1.11
Lysine	0.1	0.1	Total phosphorus%	0.89	0.75
Choline chloride	0.1	0.1	DE[1] Kcal/kg	3203.7	3233.2
Salt	0.25	0.25	P/E ratio[2]	106.5	107.9
Vitamins mixture**	0.15	0.15			
Mineral mixture***	0.15	0.15			
Carboxy methyl cellulose	1.0	1.0			

*Chemical analysis of *Chlorella vulgaris*: 5.6% moisture, 57.3% CP, 6.8% fat, 4.5% ash, 0.85% crude fiber and 24.95 NFE.
**Vitamins blend- Each one kg contains: vitamin D3 2200000 IU, vitamin A 12000000 IU, vitamin K3 2 g, α tocopherol 10 g, riboflavin 5 g, thiamine 1 g, cobalamin 0.01 g, pyridoxine 1.5 g, ascorbate 250 g, biotin 0.050 g, niacin 30 g, pantothenic acid 10 g, folic acid 1 g, and carrier to 1000 g.
***Mineral blend - each one kg contains: Mn 60 g, Zn 50 g, Cu 4 g, Fe 5 g, Se 0.1 g, Co 0.1 g, I 1 g, and CaCO3 carrier to 1000 g.
[1]Digestible energy: carbohydrate (3.48 kcal/g), ether extract (8.5 kcal/g), protein (4.49 kcal/g).
[2]P/E proportion = mg of protein/Kcal of DE (Jobling 2011).

TABLE 2. Primers used in the study of IL-12.

Primers	(5'-3')	NCBI accession No.
IL-12	F- GGGTGCGAGTCAGCTATGAG R- GGTTGTGGATTGGTTGCGTC	XM_003437924.4
β-actin	F-CCACACAGTGCCCATCTACGA R-CACGCTCTGTCAGGATCTTCA	EU887951.1

TABLE 3. Details on the kits used and the serum biochemical markers examined.

Parameters	Kits used	Method	Cat No.
Total protein	Spinreact, Spain	Biuret. Colorimetric	SP1001291
Albumin	Spinreact, Spain	Bromocresol green. Colorimetric	SP1001020
ALT	Spectrum Diagnostics, Egypt	Colorimetric	264 001
AST	Spectrum Diagnostics, Egypt	Colorimetric	260 001
Urea	Spinreact, Spain	Urease - GLDH. Kinetic	SP41041
Creatinine	Spinreact, Spain	Jaffé Colorimetric-Kinetic	SP1001111

TABLE 4. Growth indices of *O. niloticus* subjected to long-term Pb acetate exposure for 56 days and fed diets containing CV.

Item	CTR	5 Pb	10 Pb	CV	5 Pb + CV	10 Pb + CV	*P* value
IBW (g)	42.50±0.19	42.44±0.18	42.44±0.10	42.71±0.17	42.15±0.4	42.16±0.01	0.090
FBW (g)	60.70±1.30^{b}	53.68±0.71^{c}	52.29±0.12^{c}	64.23±.67^{a}	55.72±0.43^{c}	54.73±0.31^{c}	<0.001
TG (g)	18.20±1.08^{b}	11.24±0.67^{c}	9.85±0.20^{c}	21.52±0.84^{a}	13.57±0.46^{c}	12.57±0.31^{c}	<0.001
G%	42.80±2.34^{b}	26.49±1.58^{c}	23.20±0.52^{c}	50.40±2.17^{a}	32.19±1.13^{c}	29.82±2.36^{c}	0.000
TFI (g)	44.03±0.21^{a}	42.34±0.05^{b}	41.21±0.08^{c}	42.68±0.15^{b}	41.40±0.23^{c}	40.95±0.05^{c}	0.000
FCR	2.44±0.14^{d}	3.79±0.21^{ab}	4.19±0.09^{a}	1.10±0.07^{d}	3.06±0.12^{bc}	3.26±0.08^{b}	0.000
PER	1.38±0.08^{b}	0.89±0.05^{cd}	0.80±0.02^{d}	1.68±0.06^{a}	1.09±0.04^{c}	1.02±0.02^{c}	<0.001
SGR	0.28±0.01^{a}	0.18±0.00^{bc}	0.16±0.00^{c}	0.32±0.01^{a}	0.22±0.01^{b}	0.20±0.00^{b}	0.000

$^{a...d.}$ Means ± SEs (n=9/group), within trait and source of variation (S.O.V), followed by different superscripts, differ significantly at $P<0.05$ [47]. IBW, initial body weight; FBW, final body weight; TG, total gain; G%, gain percent; TFI, total feed intake; FCR, feed conversion ratio; PER, protein efficiency ratio; SGR, specific growth rate.

TABLE 5. Hematological parameters of *O. niloticus* subjected to long-term Pb acetate exposure for 56 days and fed diets containing CV

Item	CTR	5 Pb	10 Pb	CV	5 Pb + CV	10 Pb + CV	*P* value
RBCs (x10^{6}/mm^{3})	3.55±0.17^{a}	2.77±0.12^{b}	2.24±0.07^{c}	3.74±0.04^{a}	3.53±0.05^{a}	3.11±0.12^{ab}	0.00
Hb (g/dl)	9.94±0.47^{ab}	7.76±0.32^{cb}	6.27±0.19^{d}	10.48±0.12^{a}	9.88±0.15^{ab}	8.70±0.32^{b}	0.00
PCV (%)	30.80±1.45^{ab}	24.07±1.00^{cb}	19.45±0.60^{d}	32.47±0.38^{a}	30.63±0.47^{ab}	26.97±1.01^{b}	0.00
WBCs (x10^{3}/mm^{3})	28.82±1.43^{a}	22.43±1.25^{cb}	19.33±1.34^{c}	31.16±1.19^{a}	25.65±0.54^{ab}	21.43±0.62^{b}	0.00
Lymphocytes (%)	60.67±0.33^{b}	55.33±1.20^{c}	54.00±1.00^{cd}	65.67±0.67^{a}	60.33±1.30^{b}	59.00±0.58^{bc}	0.00
Neutrophil (%)	26±0.38^{b}	31.77±1.47^{a}	33.47±1.34^{a}	21.17±0.12^{c}	26.50±1.32^{b}	27.67±1.17^{b}	0.00
Eosinophil (%)	0.90±0.21	0.77±0.12	0.67±0.09	0.83±0.12	0.60±0.06	0.70±0.15	0.639
Basophil (%)	3.63±0.12	3.27±0.13	2.90±0.30	3.50±0.29	3.50±0.17	3.40±0.57	0.621
Monocytes (%)	8.80±0.20	8.87±0.47	8.97±0.49	8.83±0.44	9.07±0.27	9.23±0.32	0.961

$^{a...d.}$ Means ± SEs (n=9/group), within trait and source of variation (S.O.V), followed by different superscripts, differ significantly at $P<0.05$ [47]. RBC, red blood cell count; Hb, hemoglobin; PCV, packed cell volume; WBC, white blood cell count.

TABLE 6. Immune responses and antioxidant activity of *O. niloticus* subjected to long-term Pb acetate exposure for 56 days and fed diets containing CV.

Item	CTR	5 Pb	10 Pb	CV	5 Pb + CV	10 Pb + CV	*P* value
SOD(u/ml)	241.41±14.42^{ab}	194.40± 9.13^{bc}	164.96±3.40^{c}	271.81±7.06^{a}	228.84±6.10^{ab}	224.45±10.77^{b}	0.000
CAT(u/ml)	62.84±2.57^{b}	50.30±2.27^{c}	40.52±1.49^{d}	73.49±3.33^{a}	59.55±0.39^{bc}	53.00±1.96^{bc}	0.000
GPx (u/ml)	106.59±3.55^{ab}	82.49±3.18^{bc}	67.10±4.01^{c}	121.12±5.67^{a}	97.49±5.03^{b}	86.99±3.65^{b}	0.000
MDA (mmol/ml)	9.63±0.48^{b}	14.16±1.11^{a}	15.14±1.80^{a}	8.73±0.47^{bc}	11.37±0.68^{ab}	12.10±0.84^{ab}	0.008
PA%	88.33±4.10^{ab}	70.67±4.63^{b}	63.33±6.69^{c}	96.67±2.96^{a}	84.33±3.28^{ab}	78.33±2.91^{ab}	0.002
PI	1.10±0.03	1.03±0.01	1.07±0.03	1.10±0.01	1.07±0.03	1.10±0.01	0.259
LYZ(µg/ml)	0.60±0.02^{b}	0.31±0.02^{d}	0.29±0.01^{d}	0.88±0.04^{a}	0.46±0.04^{c}	0.37±0.02^{cd}	0.000

$^{a...d.}$ Means ± SEs (n=9/group), within trait and source of variation (S.O.V), followed by different superscripts, differ significantly at $P<0.05$ [47]. SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; MDA, malondialdehyde; PA, phagocytic activity; PI, phagocytic index; LYZ, lysosome activity.

TABLE 7. Biochemical indices of *O. niloticus* subjected to long-term Pb acetate exposure for 56 days and fed diets containing CV

Item	CTR	5 Pb	10 Pb	CV	5 Pb + CV	10 Pb + CV	*P* value
TP (g/dl)	5.21±0.03^{b}	3.78±0.11^{c}	3.36±0.13^{c}	6.02±0.29^{a}	4.71±0.08^{b}	4.59±0.19^{b}	<0.001
ALB (g/dl)	3.19±0.09^{a}	2.55±0.25^{ab}	2.08±0.04^{b}	3.98±0.29^{a}	2.95±0.02^{a}	3.36±0.19^{a}	0.000
GLO (g/dl)	2.03±0.06^{a}	1.23±0.19^{b}	1.28±0.10^{b}	2.04±0.29^{a}	1.76±0.07^{ab}	1.22±0.06^{b}	0.004
A/G ratio	1.58±0.09	2.20±0.47	1.65±0.12	2.07±0.41	1.68±0.06	2.76±0.14	0.074
Creatinine (mg/dl)	0.64±0.09^{d}	1.31±0.07^{b}	1.82±0.15^{a}	0.56±0.09^{d}	0.88±0.06^{cd}	1.13±0.05^{c}	0.000
Urea (mg/dl)	14.33±0.47^{d}	25.43±0.73^{b}	28.61±0.89^{a}	13.73±0.99^{d}	16.93±1.02^{cd}	20.37±1.03^{c}	0.000
AST (IU/L)	55.03±0.33^{cd}	71.59±2.15^{b}	83.40±4.18^{a}	53.27±1.68^{cd}	60.29±2.10^{c}	69.76±1.13^{bc}	<0.001
ALT (IU/L)	29.45±2.82^{d}	53.48±2.89^{b}	74.57±4.78^{a}	24.64±1.55^{d}	41.11±1.23^{c}	51.21±1.50^{bc}	0.000

$^{a...d.}$ Means ± SEs (n=9/group), within trait and source of variation (S.O.V), followed by different superscripts, differ significantly at $P<0.05$ [47]. TP, total protein; ALB, albumin; GLO, globulin; A/G ratio, albumin/globulin ratio; AST; aspartate aminotransferase, ALT; alanine aminotransferase.

TABLE 8. Chemical composition (on a dry basis) and Pb residue in *O. niloticus* subjected to long-term Pb acetate exposure for 56 days and fed diets containing CV.

Item	CTR	5 Pb	10 Pb	CV	5 Pb + CV	10 Pb + CV	*P* value
Moisture %	73.77±0.23^{a}	72.30±0.26^{cb}	71.23±0.20^{c}	74.70±0.23^{a}	73.47±0.32^{ab}	73.30±0.35^{b}	<0.001
DM %	26.23±0.23^{bc}	27.70±0.26^{ab}	28.77±0.20^{a}	25.30±0.23^{bc}	26.53±0.32^{b}	26.70±0.35^{b}	0.000
CP %	75.77±0.47^{a}	66.50±0.60^{c}	63.67±0.55^{d}	80.40±0.60^{a}	73.70±0.59^{b}	70.27±0.78^{bc}	0.000
EE %	7.90±0.35^{c}	11.93±0.20^{a}	13.00±0.15^{a}	8.17±0.24^{c}	10.87±0.18^{b}	11.10±0.12^{b}	<0.001
Ash %	12.43±0.44^{c}	17.47±0.09^{a}	18.57±0.41^{a}	9.97±0.37^{d}	13.03±0.24^{bc}	14.67±0.43^{b}	0.000
CHO %	3.90±0.25^{b}	4.10±0.62^{a}	4.77±0.22^{a}	1.47±0.32^{b}	2.40±0.89^{b}	3.97±1.03^{b}	0.030
Pb residues (µg/g)	0.013±0.01^{e}	1.78±0.07^{b}	3.80±0.15^{a}	0.01±0.00^{e}	0.66±0.08^{d}	2.06±0.05^{c}	0.000

$^{a...d.}$ Means ± SEs (n=9/group), within trait and source of variation (S.O.V), followed by different superscripts, differ significantly at $P<0.05$ [47]. DM, dry matter; CP, crude protein; EE, ether extract; CHO, carbohydrate.

TABLE 9. Clinical and internal examination of *O. niloticus* subjected to long-term lead acetate exposure for 56 days and treated with CV.

Item	CTR	5 Pb	10 Pb	CV	5 Pb + CV	10 Pb + CV
Fin/tail erosions	-	-	++	-	-	-
Scale detachment	-	-	++	-	-	-
Skin hemorrhage	-	+	+	-	-	+
Ocular opacity/hemorrhage	-	-	+	-	-	-
Dark skin	-	+	++	-	-	+
Hemorrhagic liver	-	+	+	-	-	-
Retention of bile in the liver	-	-	++	-	-	+
Distended gall bladder	-	+	+++	-	+	+
Splenomegaly	-	++	+++	-	-	+
Congested kidney	-	-	+	-	-	-
Empty intestine	-	+	++	-	+	+
Pale gills	-	+	++	-	-	+

-, Absent (no lesions); +, Mild; ++, Moderate; and +++, Severe. The data are shown as the mean ± SE.

TABLE 10. Intestinal morphometry of *O. niloticus* subjected to long-term Pb exposure for 56 days and fed diets containing CV.

Item	CTR	5 Pb	10 Pb	CV	5 Pb + CV	10 Pb + CV	*P* value
Villi length (µm)	488.83±9.22^{a}	371.67±6.65^{c}	247.64±14.22^{d}	513.05±6.53^{a}	435.64±8.23^{b}	343.45±8.23^{c}	0.000
Villi width (µm)	109.41±7.72^{b}	121.52±9.8^{ab}	176.21±10.22^{a}	97.75±4.97^{b}	137.46±11.25^{ab}	150.84±22.95^{ab}	0.009
Goblet cell number(no/mm^{2})	230.67±8.83^{b}	209.91±5.50^{b}	122.77±10.13^{c}	286.11±10.64^{a}	224.74±12.90^{b}	187.13±12.39^{b}	0.000

$^{a...d.}$ Means ± SEs (n=9/group), within trait and source of variation (S.O.V), followed by different superscripts, differ significantly at $P<0.05$ [47].

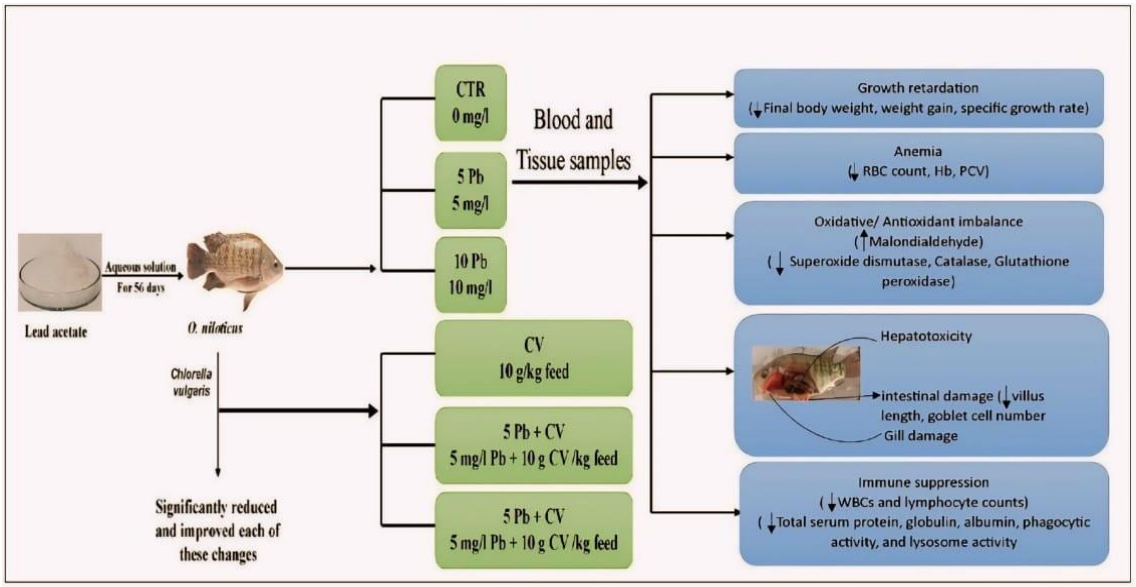

Fig. 1. Flow chart showing the main steps and the results of the trial.

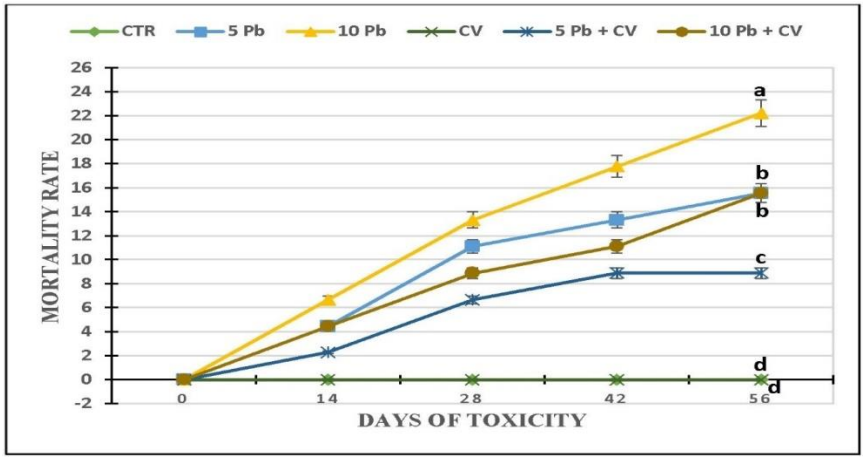

Fig. 2 Mortality rates of *O. niloticus* subjected to long-term Pb exposure for 56 days and fed diets containing CV.

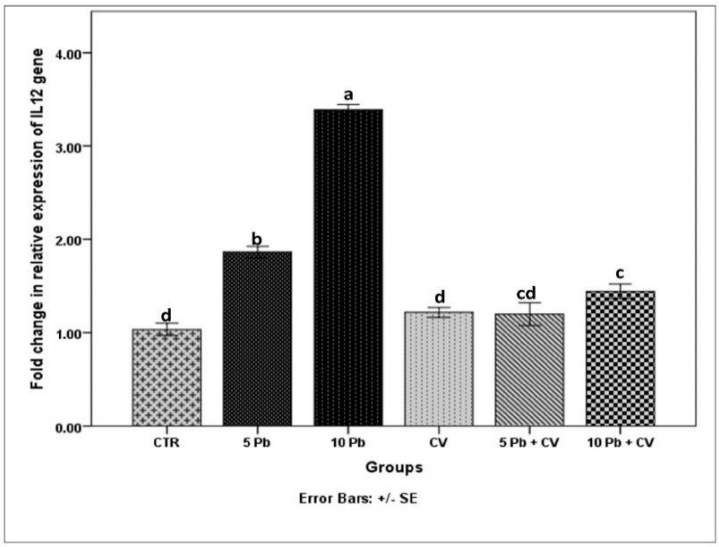

Fig. 3. Gene expression of IL-12 in the liver of *O. niloticus* subjected to long term Pb acetate exposure for 56 days and fed diets containing CV.

Different letters indicate significant differences at $P<0.05$. error bars indicate SE (n=9/group).

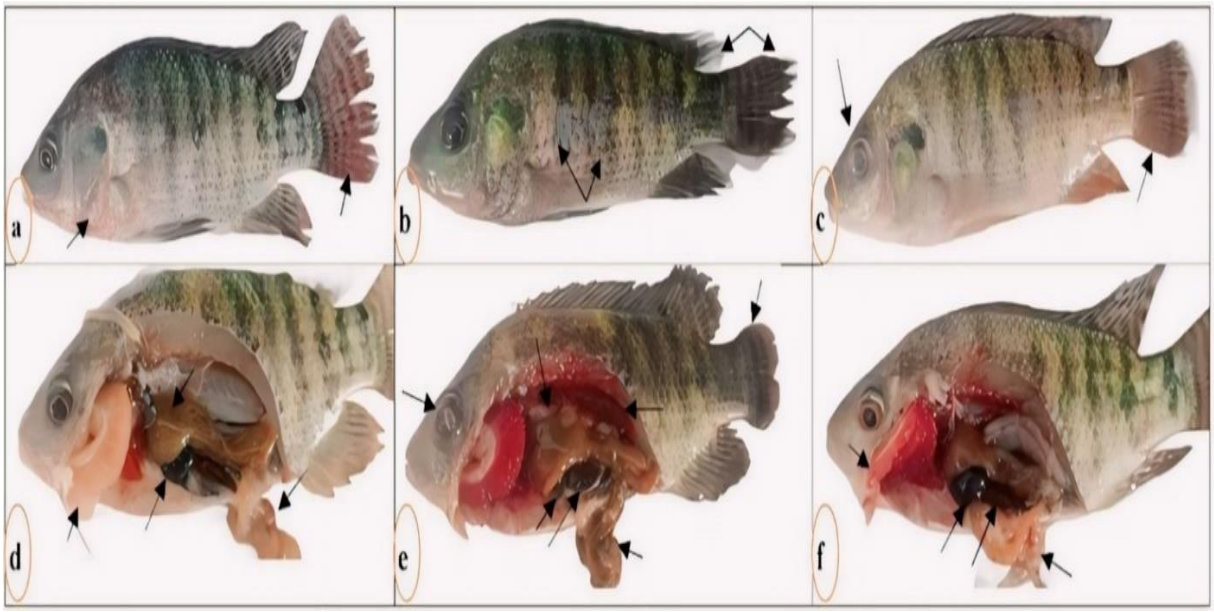

Fig. 4. Clinical and internal examination of *O. niloticus in the* experimental groups. a) 5 Pb group showing skin hemorrhage and a hemorrhagic tail; **b)** 10 Pb group showing fin erosion, dark skin, and scale detachment; **c)** 10 Pb group showing ocular opacity and tail erosion; **d)** 5 Pb group showing pale hemorrhagic liver, distension of the gall bladder, pale gills, and empty intestine; **e)** 10 Pb group showing ocular hemorrhage, tail erosion, bile retention in the liver, distended gall bladder, splenomegaly, congested kidney, and empty intestine; **f)** 10 Pb + CV group showing pale gills, distended gall bladder, and splenomegaly.

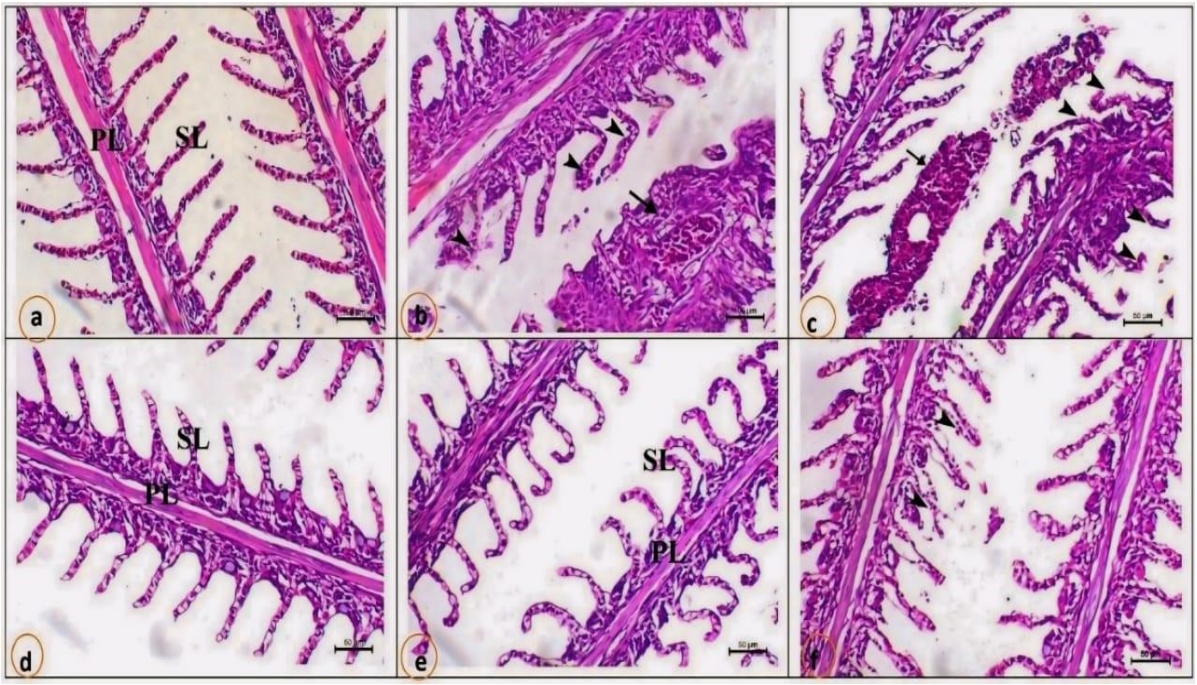

Fig. 5. Representative photomicrograph of H&E-stained gill tissue (H indicates hepatocytes, and HP indicates hepatopancreas). Scale bar = 50 µm. a) CTR group showing normal histoarchitecture consisting mainly of primary lamellae (PL) branching into tiny secondary lamellae (SL), **b)** 5 Pb group showing multifocal necrosis of secondary lamellae (arrowhead) and hyperplasia of the covering epithelium of primary lamellae (arrow), **c)** 10 Pb group showing diffuse massive necrosis of secondary lamellae (arrowhead) with necrotic materials in between primary lamellae (arrow), **d)** CV group showing normal histoarchitecture consisting mainly of primary lamellae (PL) branching out into tiny secondary lamellae (SL), **e)** 5 Pb+ CV group showing normal histoarchitecture consisting mainly of primary lamellae (PL) branching out into tiny secondary lamellae (SL), **f)** 10 Pb +CV group showing multifocal secondary lamellar necrosis of some filaments (arrowheads).

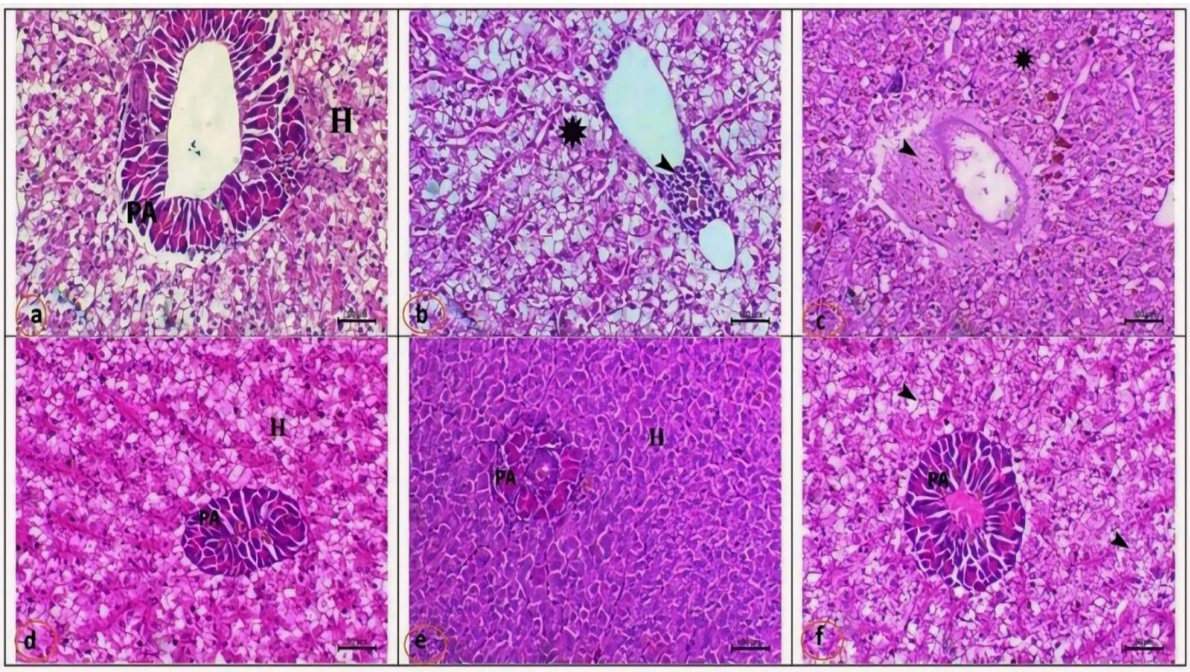

Fig. 6. Representative photomicrograph of H&E-stained hepatopancreas. Scale bar = 50 µm. a) CTR group showing normal histoarchitecture where hepatocyte cords (H) and pancreatic acini surround central veins (PA), **b)** 5 Pb group showing focal pancreatic acini necrosis (arrowhead) and massive hepatic necrosis (asterisk), **c)** 10 Pb group showing massive pancreatic acini necrosis (arrowhead) and massive hepatic necrosis with diffuse deposition of brown melanin pigments (asterisk), **d)** CV group showing normal histoarchitecture where hepatocyte cords (H) and pancreatic acini surrounding central veins (PA), **e)** 5 Pb + CV group showing normal histoarchitecture where hepatocyte cords (H) and pancreatic acini surrounding central veins (PAs), **f)** 10 Pb + CV group showing multifocal hepatic necrosis (arrowheads). Note that the pancreatic acini (PA) are normal.

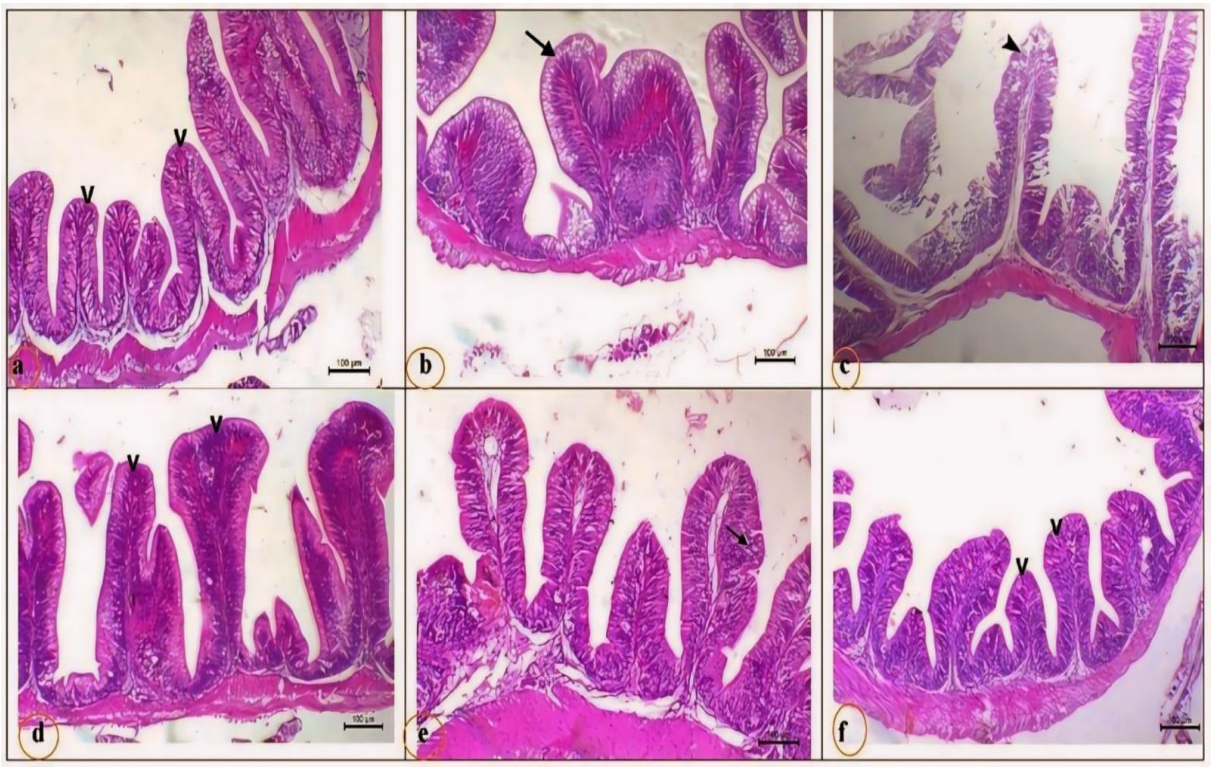

Fig. 7. Representative photomicrograph of H&E-stained intestine. Scale bar = 100 µm. a) CTR group showing normal histoarchitecture with characteristic intestinal villi (V), **b)** 5 Pb group showing diffuse cerebral inflammation, **c)** 10 Pb group showing massive villous necrosis, **d)** CV group showing normal histoarchitecture with characteristic intestinal villi (V), **e)** 5 Pb + CV group showing normal histoarchitecture with characteristic intestinal villi (arrow), **f)** 10 Pb + CV group showing normal villous structures (V).

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التعرض لأسيتات الرصاص يؤدي إلى تأخر النمو ، وتثبيط المناعة ، وعدم التوازن التأكسدي/مضادات الأكسدة ، والتغيرات النسيجية في أسماك البلطي النيلي مع حاولة التخفيف عبر الكلوريلا فولجارز

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الملخص

يعد التلوث بالرصاص احدى المشاكل الشائعة في البيئات المائية ويشكل مخاطر صحية خطيرة لكل من البشر والأسماك. الأسماك معرضة بشدة لتراكم الرصاص ، مما يعيق نموها وصحتها العامة. هذه الدراسة هدفت الى فحص تأثير أسيتات الرصاص على المؤشرات البيولوجية ، و التغيرات النسيجية ، ونمو أسماك البلطي النيلي. كما تم التحقيق من قدرة الطحالب الدقيقة (الكلوريلا فولجارز) الغذائية على الحماية من التعرض للرصاص. تم تقسيم ٢٧٠ من أسماك البلطي النيلي (٤٢,٤±٠,٢١ جم) إلى ست مجموعات (٤٥ سمكة / مجموعة). تم تغذية المجموعة الضابطة و مجموعتي الرصاص بنظام غذائي أساسي وتعريضها ل ٠ أو ٥ أو ١٠ مجم رصاص/ لتر. تم تغذية مجموعات الكلوريلا بنظام غذائي مكمل ب ١٠٪ كلوريلا وتعرضوا ل ٠ أو ٥ أو ١٠ مجم رصاص/ لتر لمدة ٥٦ يوما. كشفت النتائج أن التعرض للرصاص تسبب في تأخر النمو بطريقة تعتمد على الجرعة ، مع انخفاض كبير في المؤشرات المناعية ومضادات الأكسدة. كما تبين أن التعرض للرصاص مرتبط بزيادة كبيرة في مستويات بيروكسيد الدهون والوظائف الكلوية والكبدية. علاوة على ذلك ، تسبب التعرض للرصاص في زيادة التعبير عن الجينات التي تشفر السيتوكين الالتهابي IL-12 وتأثير ضار كبير يعتمد على الجرعة. بالمقارنة مع تلك المجموعات المعرضة للرصاص و غير مغذاة بالكلوريلا ، تم تعديل النمو والمناعة وحالة مضادات الأكسدة ووظائف الكبد و الكلي والتعبير عن IL-12 في الأسماك المعرضة للرصاص بشكل كبير عندما تم تغذيتها على الكلوريلا. تم تعزيز التغيرات النسيجية وتكوين الجسم الكيميائي ومستويات بقايا الرصاص في عضلات الأسماك المعرضة للرصاص من خلال التغذية علي الكلوريلا. بشكل حاسم ، لتحسين صحة وإنتاجية أسماك البلطي النيلي المعرضة للرصاص، يمكن إعطاء الكلوريلا كمكمل غذائي.

الكلمات الدالة: أسماك البلطي النيلي، تسمم الرصاص ، المناعة ، مضادات الأكسدة ، التغيرات النسيجية ، الكلوريلا فولجارز.