Egypt. J. Vet. Sci. **Vol. 56**, No. 5, pp. 961-978 (2025)

Egyptian Journal of Veterinary Sciences

https://ejvs.journals.ekb.eg/

Dietary N-acetylcysteine Improved Nile tilapia (*Oreochromis niloticus)* Performance and Health Status Against Heavy Metals-induced Oxidative Stress

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Abstract

N-acetylcysteine (NAC) is a derived form of the naturally occurring amino acid L-cysteine. The present work investigated the effect of NAC on Cd and Pb exposed fish. Nile tilapia received NAC supplementations 200 mg/kg fish diet and cultured for 40 days in presence of metals. Fish exposed metals recorded decrease of growth performance and feed utilization, but NAC reversed the impairment of fish growth performance, but NAC reversed the impairment of fish growth performance. Metals also arise WBCs count and decreased HTC and RBCs count, unlike, NAC restored white blood cells count and HTC. Heavy metals also induced deterioration of biochemical parameters (Aspartate aminotransferase "AST", alanine aminotransferase "ALT", alkaline triphosphatase "ALP", urea, triglycerides, total protein "TP", bilirubin, and high-density lipoprotein "HDL"), but NAC succeeded impede this effect through decreasing ALT, AST, ALP, urea and triglycerides values and increased TP levels and HDL. A significant increase of malondialdehyde (MDA) activities and decrease of superoxide dismutase (SOD), glutathione (GSH) and cholinesterase (ChE) were observed in fish exposed to metals, NAC displayed repairing of these antioxidants to levels near to control group. Challenge with metals displayed no fold-change in liver glutathione peroxidase (*GPx)* gene expression and up-regular effect of liver cytochrome (*CYP*) gene expression but NAC abolished this effect by achieving the balance of cell proliferation and apoptosis.

Keywords: N-acetylcysteine, Oreochromis niloticus, heavy metals, oxidative status.

Introduction

Aquaculture is one of the fastest-growing food producing sectors [1, 2, 3]. Today, It presents about the half of the total fish production in the world [4]. It is predicted to be the main source of food from aquatic animals in the upcoming years [5]. Aquaculture chief production commonly comes from freshwater earthen ponds and tilapias are the most widely adopted farmed fish species in this system [6]. Tilapias have been known as one of the most important freshwater fish groups. They can tolerate a wide range of different environmental factors and stress conditions [7, 8]. One of the most important tilapias is Nile tilapia, *Oreochromis niloticus* (L.), which is ranked as the highest among the cultured species in tropical and subtropical countries [9, 10]. It is popular among fish farmers owing to its good growth among different aquatic ecosystems. It can tolerate and adopt in heavy metals' polluted environments [11, 12]. Heavy Metals (HMs) are one of the most dangerous substances released to the aquatic environment [13]. HMs are widespread contaminants and have the ability to bioaccumulate in fish tissues [14]. Lead (Pb) and cadmium (Cd) are classified as non-essential metals have no known biological functions in living organism [15] and hazardous to fish even at low levels [14]. These metals can display growth deceleration, immunity deterioration, histopathological modifications and high mortality [16]. Accumulation of these metals brings behavioral, biochemical, and molecular

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(Received 15/03/2024, accepted 12/05/2024)
DOI: 10.21608/EJVS.2024.277055.1917

modifications via generation of reactive oxygen species which causes oxidative stress and carcinogenesis. [14]. Human health subsequently could deteriorate through consumption of contaminated fish [17].

Global demand for fish is going to be achieved by growing in fish supply from aquaculture industry [18]. Increasing demand for safe and good quality aquaculture fish products free from pathogens, pollutants, and carcinogens appears the importance of products which commonly used to improve fish growth and increase its resistance toward diseases [19]. During the last decade, some studies approved application of N-acetylcysteine (NAC) in different medicinal cases as inhibition of carcinogenesis, tumorigenesis, and mutagenesis, as well as the inhibition of tumor growth and metastasis [20, 21]. NAC is a derived form of the naturally occurring amino acid L-cysteine [20]. It is not present in food and is known as thiolic antioxidant [22]. It is mainly applied as a mucolytic agent for a variety of respiratory illnesses [23]. So, It is included on the list of essential medicines of the World Health Organization (WHO) for humans [24]. Remarkably, its ability to work as an antioxidant as well as a detoxifying agent in the metal-injured cells has been recorded [25, 26, 27]. It has antimicrobial properties and applies for anticarcinogenic and antimutagenic effects[21], antioxidant activities and anti-inflammatory activities, inhibition of development to malignancy and metastasis, and protection from negative consequences of chemopreventive and chemotherapeutic agents [20], enhancing GSH levels [28, 29] improving mitochondrial function to protect against liver injury [29], promoting detoxification [28]. Therefore, NAC is commonly used as antioxidant in experimental cell and animal biology, besides clinical studies, especially in human cells and rates [30, 31, 32, 24].

The up-to-date aquaculture performs are mainly conditional on the intensive and super-intensive culturing systems for fish species [33]. Globally, the aquaculture has the challenge to improve its production with sustainable practices [34]. Assimilation metal ions into reared fish to levels that alter their physiological functions impact negatively on the health of consumers and affect the fish production industry [35]. Nile tilapia can exert metabolic mechanisms for its adaptation, metal-detoxification, and antioxidant protection [7]. So, it is a bio-indicator species in understanding environmental metal-pollution [36]. So, the aim of this study was to evaluate NAC effects on growth performance, haematology, biochemical status, oxidative status, and the health of gills, liver and spleen of Nile tilapia exposed to mixture of Pb and Cd.

Material and Methods

Preparation of synthetic heavy metals

Synthetic mixtures of heavy metals (HMs) in the present study, prepared as the same concentrations of drainage water samples determined by Abdel-Tawwab et al. [7] 1.07 mg/L and 61.2 µg/L for Cd and Pb, respectively. Lead nitrate Pb $(NO_3)_2$ and cadmium chloride ($CdCl_2.2.5H_2O$) (Sigma-Aldrich Chemicals, USA) used to prepare stock solutions through dissolving their required concentrations in distilled water. During the test, experimental solutions were added during aquarium water exchange once every 2 days from recently prepared stock solutions with dechlorinated water for prevention of concentration changes caused by adsorption and evaporation processes [36]. All the other chemicals were of analytical grade.

Diets preparation, fish management, and study design

Nile tilapia, *O. niloticus* (L.), were obtained from private fish farm (Kafr El-Sheikh governorate, Egypt). Fish were maintained in an indoor glass tank for two weeks for adaptation to the laboratory condition as light-dark photoperiod cycle (12 – 12h). Fish (68 ± 3.26 g) were randomly distributed at a rate of 6 fish per 60-L aquarium (60 x 40 cm) in triplicates. Each aquarium was supplied with compressed air via air-stones using the aquarium's air pump. The aquarium was also provided with a mechanical filter to collect the fish wastes which were cleaned daily. Fish were fed diets twice daily at 8:00 and 14:00 h for 40 days. Daily fish mortalities were recorded for each aquarium. The tested experimental groups were (1) water-free metals and fish fed on the basal diet (C), (2) heavy metals (Cd and Pb) mixture added into the water and fish received a basal diet (HM), (3) Cd and Pb containing water and fish were fed on the basal diet containing 200 mg/kg fish diet N- acetylcysteine (NAC) powder (ADVENT CHEMBIO PVT.LTD, Navi Mumbai-400 701, India) [37], (NAC). Through feed manufacturing, N- acetylcysteine powder was carefully added to the basal diet after the diet components were minced to obtain a homogenous mixture. The combination was then ground into pellets using a meat grinder after being turned into a paste by the addition of water. After drying the pellets at room temperature for 24 hours, they were stored at -20 C in opaque plastic containers. The basal diet was 30/6, protein/lipids ratio, with 18.73 MJ kg1 gross energy. Water quality parameters were measured daily in each aquarium [total ammonia nitrogen (TAN) using a portable colorimeter (Milwaukee-Mi 405), Dissolved oxygen (DO) using OxyGuard handy Polaris dissolved oxygen, pH and

temperature using pH meter (HANNA- HI98191- PH meter)]. The experimental parameters were under natural conditions. The following parameters (mean ± SEM) were: the water temperature was 27.02 ± 1.3 °C, pH 7.58 ± 0.34, TAN 0.04 ± 0.01 mg/L and DO 5.024 ± 1.04 mg/L [38]. The protocol of this study is reviewed by the institutional committee for the use of aquatic animals in research, faculty of Aquatic and Fisheries Sciences, University of Kafrelsheikh.

Fish growth performance and feed utilization efficiency.

At the end of the experiment, fish were collected and anesthetized using pre-prepared clove oil (50 ml/L of water, Merck, Germany). Each fish was weighed separately to get the final weight. The total length (L) and width of the harvested fish was determined using a measuring board. Fish feed utilization and growth performance were evaluated as described by [39] and [38] as follows:

- Body weight gain (BWG) = final body weight (W1) – initial body weight (W0)
- weight gain rate (%) = (Wt − W0)/W0 × 100
- Specific growth rate (SGR % /day) = 100× (lnW1−lnW0) / t where, "t" is the experimental period (days)
- Feed conversion ratio (FCR) = feed intake (g)/BWG (g)
- Survival rate (SR %) = (total number of fish at the end of the experiment /total number of fish at the start of the experiment) × 100

Haematological analysis

After the exposure of fish to HMs' mixture, feeding was stopped during the 24 h immediately prior to blood sampling. From the caudal vasculature, blood was taken from via heparinized syringe and collected in vacuum tubes (3 replicate/group). EDTA (Ethylenediamine tetra acetic acid) was added to the blood sample as anticoagulant (1 mg/ml) for assessment of Red blood cells (RBCs) count, hemoglobin content (Hb), hematocrit (HCT), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and white blood cell (WBCs) count using an automatic blood cell counter (Exigo- Vet., Boule Medical AB Inc., Stockholm, Sweden) [40]. For the other leucocytes count examination, from each blood sample, two thin films of blood were adjusted on pre-cleaned microscope slide which were placed to dry for staining with a modified Wright's stain. From the stained slide, a total of 100 cells were counted using ×100 oil immersion lens for examination of lymphocytes, monocytes, Neutrophils and Eosinophils percentages (Exigo- Vet., Boule Medical AB Inc., Stockholm, Sweden) [41].

Biochemical measurements

The assembled blood was centrifuged at the room temperature at 5000 x g for 15 min and the supernatant serum was kept in plastic Eppendorf tubes at –20 ^{0}C [7]. Aspartate aminotransferase (AST), alkaline phosphatase (ALP) and alanine aminotransferase (ALT) activities were assessed colorimetrically at 540 nm wavelength according to Reitman & Frankel, [42]. Serum total proteins (TP) were estimated at the wavelength of 540 nm [43]. Serum albumin and bilirubin was estimated colorimetrically at a wavelength 550 nm as described by Dumas and Biggs, [44]. The globulin content was mathematically determined through subtracting albumin from TP. Total lipids were assessed according to Zöllner, N. and Kirsch, [45]. Serum triglyceride (TG) were estimated using GPO- PAP and the total cholesterol (TC) using CHOD- PAP (commercial clinical kit) [46]. Serum creatinine was calorimetrically estimated according to Heinegård and Tiderström, [47]. Urea nitrogen and glucose were determined using Bio-Diagnostic Company kits (Bio-Diagnostic). High density lipoprotein (HDL-c) was measured using commercial kits (Biodiagnostic Co., Egypt) [48].

Oxidative stress assay

Serum samples were utilized to conduct the oxidative stress assay in which Malondialdehyde (MDA), The activities of enzymes catalase (CAT) and superoxide dismutase (SOD) were evaluated using ELISA kits (Inova Biotechnology, China) at the wavelength 450 nm using the microplate ELISA reader [49]. Glutathione (GSH) activity was determined colorimetrically, using commercial kits (Biodiagnostic Co., Cairo, Egypt), in accordance with the method described by Habig et al., [50]. Cholinesterase (ChE) activity were determined according to Brodeur et al., [51].

Total RNA extraction, cDNA synthesis and real-time quantitative PCR assay

Total RNA was extracted from 50 mg of livers of *Oreochromis niloticus* using Trizol (iNtRON Biotechnology) following the manufacturer's instructions. The integrity of RNA was confirmed by ethidium bromide stained 2% agarose gel electrophoresis. The concentration and purity of RNA were determined using a Nanodrop BioDrop spectrophotometer (Biochrom Ltd, Cambridge CB23 6DW, UK) based on the A260/A280 nm ratio. Two µg of RNA sample were reverse transcribed using ABT 2X RT Mix cDNA synthesis kit according to manufacturer's Protocol. Gene expression profiling was performed in Rotor Gene-Q (Qiagen-Germany)

using gene-specific primer sequences for the amplification of Cytochrome (*CYP19*) and glutathione peroxidase (*GPx*) (Table 1). The amplification reaction was done using ABT 2X qPCR Mix (SYBR) kit. The reaction volume was 20 µl consisting of 10µL SYBR Green, 0.6 µL of forward and reverse specific primer, 1 µL of cDNA template, and nuclease-free water to make the final volume 20 µL. The PCR program was carried out with the following conditions: activation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at the primer-specific temperature for 15 seconds, and extension at 72°C for 25 seconds. This was followed by a melt curve analysis to assess the specificity of amplification at 72°C to 95°C. All genes were tested in triplicates. CT values for each sample were determined and incorporated in "fold change" ($2^{-\Delta\Delta CT}$), calculation based on Livak and Schmittgen, [52], and mRNA expressions for each sample were normalized against the beta actin as a housekeeping gene.

Histopathological procedures

Samples for histomorphological check were obtained from dissected fish. Gills, liver and spleen tissues were taken and immersed in 10% pre-prepared formalin for fixation. Specimens were dehydrated and bathed in absolute alcohol, next embedded in paraffin. Sections (3-4 µm) were censored on Leica Rotary Microtome (RM 2145, Leica Microsystems, Wetzlar, Germany). Resulted slides were deparaffinized through administration in xylene to alcohol to water to stained using haematoxylin and eosin (H&E) according to Suvarna and Layton, [55]. Investigation using Leica ICC50W Image Analyzer was applied on each slide, microscope at 10X, 20X and 40X magnification for observation of the spreading, occurrence, and invasion of the inflammatory cells.

Statistical Analysis

Data were tested for distribution normality and homogeneity, and the normality was confirmed by analysis of the residuals. Statistical analysis of data was carried out using Graph Pad Prism 6 (Graph Pad Prism v6.0). One-way ANOVA and Tukey's multiple comparison test were applied for comparison between different treatments. The results were expressed as means ± standard error of the mean (SEM). Comparisons were set at a probability level $P < 0.05$.

Results

Growth performance and feed utilization efficiency

Dietary supplementation with NAC showed significant restoration of samples growth performance (Table 2). Challenge using HMs' mixture reveals significant decrease of WG, WG% and SGR and higher FCR ($P < 0.05$). NAC assimilation showed significant increase of WG, WG% and SGR ($P < 0.05$) and lower FCR to be near to the obtained data of the control group. Survival rate didn't display differences ($P > 0.05$).

Haematological indices

Hematocrit and RBCs are showed significantly lower values ($P<0.05$) in fish exposed to metals in comparing with the control and NAC treatment group. MCHc is significantly lower ($P<0.05$) in fish of the control group. WBCs values were significantly higher in HM challenge group ($P<0.05$). Lymphocytes, Neutrophils, Monocytes and Eosinophils showed nonsignificant changes between groups ($P > 0.05$) as summarized in Table (3).

Serum biochemical indices

Fish fed NAC supplementations displayed significant decline of ALT, AST, ALP, urea, triglycerides and total lipids ($P<0.05$) in comparing with other groups. HM challenge fish displayed significant lower TP and HDL values ($P<0.05$). Bilirubin recorded its higher values in HMs group ($P<0.05$) (TABLE 4).

Oxidative stress assay

The effect of HMs and treatment with NAC supplementations on antioxidant status of Nile tilapia are shown in figure (1). A significant increase in MDA values ($P<0.05$) recorded in fish exposed to metals. In addition, SOD and GSH displayed significantly lower values in HM group. Treatment with NAC showed significant restoration of MDA, SOD and GSH values of fish exposed to metals to be close to the control group. NAC achieved higher significance ($P<0.05$) of fish ChE activity in comparing with other groups.

Gene expression assay

The challenge with Pb and Cd mixture displayed no fold-change in liver *GPx* gene expression. Treatment with NAC showed a 10.7-fold increase in liver GPx gene in comparison with the other groups. On the other hand, metals-exposed fish exhibited up-regular effect of liver *CYP19* gene expression (4.5-fold increase) and treatment with NAC decreased the modification of *CYP19* gene expression to be adjacent to the control fish group (Fig. 2).

Discussion

Using feed additives in the aquaculture field have been found to enhance fish growth performance, improving feed efficiency, optimizing immune response, minimizing antibiotics support [56]. Exposure of fish to heavy metals which bioaccumulate and affect different organs, may impeding growth performance [57]. Previous studies

recorded the deteriorate effect of metals on growth performance and health of Nile tilapia [58, 59]. In contrast, fish treated with NAC supplements showed enhancement of growth performance and feed utilization efficiency. Our results are in harmony with Xie et al., [37] who recorded significant increase in WG and SGR of juvenile Nile tilapia fed with dietary NAC for 8-weeks. This may be due to animals which are exposed to chronic stress having lower ability to provoke a defense response to the stress, causing dysfunctional response, subsequent growth inhibition. Thus, restored food absorption could directly improve FCR, and enhance fish growth and feed efficiency [60].

The hematological indices offer understandings on the fish health [61]. In this concern, there is an observed reduction in HTC and RBCs values of fish exposed to metals. This results match with Hussein et al.,[62] who found that Nile tilapia exposed to Cd demonstrated decrease in RBCs and HCT. Naz et al., [63] revealed that different fish species exposed to metals including Cd and Pb have a significant reduction of RBCs, Hb, and Hct compared with the control group. It could be related to hemolytic crisis caused by HMs mixture [64]. Elevated MCHC amounts may specify the existence of large size red blood cells having fewer hemoglobin [65]. Many researches assumed that exposure to Pb induces disruption of hemoglobin synthesis [66, 67, 68]. Dietary supplementation with NAC achieved higher values of these indices and was considered as a promising therapy for human injured anemia [69, 70]. Ucar et al.,[65] found that application of NAC on Rainbow trout under pesticide stress achieved higher levels of HTC and RBCs. Also Zembron-Lacny et al.,[71] recorded that blood indices of men participate in NAC administration experiment are positively changed compared to the control group. Increase in WBCs in untreated group (HMs) agree with findings of Qiang et al.,[72] who found that Cd stress resulted in a marked increase in WBCs counts in the blood of Nile tilapia. Adhim et al.,[73] found higher levels of WBCs in Nile tilapia exposed to Pb-stress. As the WBCs are accompanying with defense, the change in WBCs values recorded in fish exposed to toxins signifies immuno- modulation of toxicants [65], and/or tissue damage [74]. Lower values of WBCs count of fish fed on NAC supplementations agree with Radan et al., [75]. Also, Ucar et al.,[65] showed positive effect of NAC on Rainbow trout exposed to pesticide toxicity. Kolomaznik et al.,[76] recorded improvement of WBCs counts in male rates received NAC with 10 and 20 mg/kg body weight.

Plasma and serum are the metabolism intermediate products, so they mirror the physiologic state of the fish [77]. ALT, AST and ALP are significant serum biomarkers [77, 78]. In the present study, activity of ALT, AST and ALP are significantly higher in fish exposed to Pb and Cd. This is well-matched with many previous studies that reported increase in the Nile tilapia liver function enzymes exposed to metal pollution [79, 72, 80]. Higher values of these enzymes are biomarkers of liver damage as they are associated with the function of hepatic cells' status [79]. Stress might harm cell structure and elevate the permeability of live cell membranes, causing higher enzymes' activities [72] through leakage into the blood stream [81, 82]. In contrast, NAC treatment group showed decrease in the activities of ALT, AST and ALP and these findings are in agreement with Wang et al.,[32] who revealed that NAC potential in improving the Cd-induced renal tubular toxicity in female rates. Also, Rahmani Talatappeh et al.,[26] found human treated with NAC during busulphan conditioning have a decrease in AST, ALT, and ALP levels. Proteins have effective functions as a source of nitrogenous metabolism and energy (in stress) and manufacture the cell structural components [74]. Many studies demonstrating the impeded effect of HMs' stress on TP levels [83, 84, 74]. This might be related to the damaged or weak protein manufacture under the HMs stress [74].Unlike, NAC dietary supplementations achieved increase in the levels of TP. This result is coincided with Ucar et al.,[65] who stated that cysteine is an important component for protein synthesis. Urea and bilirubin are nitrogenous waste products [85]. Elevations in the levels of serum urea have been used as nephrotoxicity indicators [14]. Fish exposed to toxins recorded higher levels of urea and bilirubin [14]. This might be because metal toxicity induced numerous pathological processes in different fish organs [7]. Dietary supplementation with NAC showed decrease in levels of urea and bilirubin in comparing with HMs- group. Wang et al.,[32] showed significant lower urea and bilirubin levels when treated rates exposed to Cd with NAC supplementations. Ucar et al.,[65] also recorded decrease in bilirubin and urea in Rainbow trout fish fed NAC supplementation. Triglycerides and cholesterol are acknowledged to contribute to the elevation of total lipid [86]. The principal function of triglycerides is storing and providing the cellular energy [87]. Triglycerides are used to estimate growth status and lipid metabolism [87]. In the present study, treatment with NAC achieved the lowest levels of triglycerides. NAC treatment has a significant role in decreasing levels of triglycerides in Rainbow trout fish [65] and rates [88]. Decrease HDL levels in fish exposed to metals could be associated with the increase in the energy requires and the serum carriage of fatty acids by HDLs [57]. Dludla et al.,[89] summarized that NAC mechanistically could block lipid accumulation by

the downregulation of SREBP-1c/SREBP-2 or that of cluster of differentiation 36 (CD36) and PPARγ. Besides, the ability of NAC to inhibit the reactive oxygen species (ROS) production [88, 90].

Antioxidants play a critical role in cellular defense mechanisms against oxidative stress, pretending as the first line of safeguard in supporting cellular health [79] by neutralizing ROS [91]. The antioxidant enzyme system has emerged as a potential biochemical biomarker in environmental biomonitoring especially evaluating the response to heavy metals exposure [74]. According to our findings, Cd and Pb deteriorate the activities of antioxidants. Antioxidant enzymes are potential objectives for Pb. Since most of antioxidants' active sites have thiol groups and Pb has a good affinity for these thiol groups. By this means, Pb can bind causing deactivating the antioxidant capacity [27]. Jan et al.,[92] explained under influence of Pb, ROS values improved while that of GSH, CAT, and SOD decreased. Rahmani Talatappeh et al.,[26] found continuous exposure to Cd significantly accompanied with decrease of antioxidants and increase of MDA levels in the rates liver tissue. Our findings also are in harmony with Rajeshkumar et al.,[93] who tested the effect of Cd and Pb on common carp antioxidants for 30 days. Pal.,[94] found that *Mastacembelus armatus* fish samples collected from two different rivers in Tripura, India recorded higher MDA values and lower values of the other antioxidants in relation to HMs' contamination. The risen MDA concentrations could be appropriate to metal-induced ROS production which would have originated LPO and caused loss of cell membrane fluidity in cases of oxidative stress [14]. Given that SOD and CAT enzymes constitute the initial defense against ROS, heightened activities of these liver and kidney antioxidants are likely a species-specific response to counter the elevated ROS levels induced by toxicity [78]. As part of the antioxidant system, SOD converts superoxide anions (O_2) into less toxic products, namely H_2O_2 and O_2. Subsequently, CAT catalyzes the elimination of H_2O_2, transforming it into water and molecular oxygen [79, 95, 61]. GSH, non-enzymatic antioxidant, acts as a free radical scavenger, assists in stimulating other antioxidants, likewise performances as an electron donor within the peroxides reduction process managed by GPx [22]. Environmental stress can influence the activity of these antioxidants leading to oxidative stress [72]. The combined action of CAT, SOD, and GSH aids in eliminating free radicals, and under normal physiological conditions, a delicate equilibrium exists between these factors. However, when toxin exposure leads to the excessive production of free radicals, this balance is disrupted, resulting in oxidative insult [79]. Treatment using NAC showed lower MDA values and higher SOD and GSH in comparison with HMs fish group. NAC is recognized as a vital building block for antioxidants inside the body [88, 65]. It has been shown to exceed other antioxidants effectiveness when it comes to justifying mitochondrial reactive ROS production, restoring mitochondrial function, and enhancing animal survival [96]. In metal induced experiments, NAC confirms its usefulness in modifying GSH condition in fish [97, 98]. The antioxidative actions of NAC are twofold: direct scavenging of ROS as a disulfide reductant [88, 90] and indirect antioxidation via enhancing intracellular GSH content [24]. Improvement of GSH and indorsing its synthesis arises by providing cysteine and stimulating cytosolic enzyme activities, with glutathione reductase (GR), thus rushing GSH generation, presenting a multifaceted and powerful antioxidant of NAC in various physiological contexts [22]. These mechanisms act to enhance GSH in different animal tissues [23, 99, 21, 29]. NAC inhibited LPO metal exposed- Wistar rats and reduced membrane permeability resulting from oxidative injury in the liver resulting decline in MDA and enhancement in GSH content [88]. Aykin-Burns et al.,[27] informed that NAC may act as a metal chelator where it has the potent of reversing Pb-induced damage in PC-12 cells trial, reducing the prevalence of MDA in the Pb- treated groups to a degree nearly as down as those of the control group. Also, other studies remarked the reduction of MDA formation gradually in lead nitrate-exposed HepG2 cells, Cd- exposed rate when treated with NAC Yedjou et al.,[20] and Wang et al.,[32], respectively. In this concern, Rahmani Talatappeh et al.,[26] documented the protective effect of NAC administration against Cd and supposed it's owing to improving antioxidants measurements, modifying oxidative stress, accompanied by down-adaptable of apoptotic aspects.

Cholinesterase (ChE) is a prominent controlling enzyme that regulates nerve impulses transmission within cholinergic synapses [100, 101]. It has been known as a worthy biomarker of neurotoxic injures in aquatic pollution studies [102]. Exposure to HMs were recorded to drop the ChE activity in different fish species [103, 101, 104]. Water pollution induces possible toxicity reducing ChE enzyme described as "anticholinesterase agents", which is a key factor on the neurotransmitter acetylcholine [104]. Chandrasekera et al.,[105] reported that ChE levels in brain and muscle of *Oreochromis niloticus* exposed to Cd^{2+} (1 mg/L) were lowered to 24-32% and 33-35% respectively. Cogun et al.,[106] observed a drop in ChE synthesis beside hepatocyte dysfunction after fish exposed to mercury and assumed serum ChE activity could be a more liver

dysfunction specific indicator. NAC is recommended to be a promising drug to humans in cases of memory deficits after successful trial on rates which results in restoration of cerebral acetylcholinesterase activity and modulating cholinergic neurotransmission besides improving cognition (learning and memory) [107].

Cytochrome CYP P450, a hepatic protein indicative of natural detoxification mechanisms [108], is supported monooxygenase induction which has been broadly used as a bioindicator of many toxicants exposure as polynuclear aromatic hydrocarbons and polychlorinated biphenyls [105] and [109], pesticides [110]. Gold-Bouchot et al., [102] assumed that CYP P450 has been used as a sensitive indicator for monitoring the exposure of fish to environmental pollutants. It plays an important role in the free radicals generation as the by-products during toxins metabolism and consequently contribute to oxidative stress [110]. *Cyp19* is known to up-regular activate in cases of deterioration of the animal tissue [111]. Unlike, one of the major antioxidative element, *GPx*, which is identified for its role in reducing the oxidative stress inside the body by degenerating the free radicals, causing high antioxidative and immune responses [112]. Fadl et al.,[113] recorded down regulation of *GPx* gene expression as a result of oxidative stress. NAC supplementations were recorded to restore *CYP* and *GPx* in rates [110, 114]. Our results also concord with Xie et al.,[37] who found a significant up-regulation of *GPx* gene expression in fish fed with NAC. NAC achieved the balance of cell proliferation and apoptosis via up-regulation of *GPx* simultaneously with down-regulation of *Cyp19* to impede tumor initiation and progression.

Gills are considered the primary location of toxic response and the main objective of pollutants in aquarium water [115]. Deterioration effect of metals on gills as observation of atrophy and necrosis of gill lamellae has been previously recorded [62, 116]. This may be accredited to the increased permeability of fish gill capillary walls next vessel dilatation by the side of toxic damage [117]. NAC is known for its role in improvement bronchial and lung function and oxygen saturation in the blood and its help in all respiratory disorders [118]. So, in the present study, NAC showed a decrease of secondary gill lamella adhesion with eosinophilic granular cells decrease. Liver is responsible for pollutant detoxification and play a major rule in metal metabolism and elimination [119]. Hepatic steatosis was observed and accompanied with presence of Cd and Pb in the aquarium water. As a general role, exposure to metals causes hepatic fibrosis. Many previous studies indicated that hepatic metals overload does not succeed to initiate the normal a fibrogenic response [120, 93, 63]. Spleen is one of the severely affected fish organs in presence of metals [121, 122, 123] [36]. This was declined, totally or partially, with NAC assimilation. This may be attributed to the ability of NAC in chelation of metals [124]. In addition, NAC showed significant ability to enhance the antioxidant activities.

Conclusion

This experimental work has shown that NAC has valuable actions against Cd and Pb negative effects. NAC succeeded in restoring fish growth performance and impeded the deterioration effect of HMs on oxidative status parameters. It also achieved, totally or partially, cell balance of proliferation and apoptosis. Therefore, we can recommend NAC as a promising prescription which had better study in future to improve therapeutic substitutes caused by metals stress. But it is important to handle the applications of pharmaceuticals in aquatic environments. The prospective ecological impacts of NAC and its long-term effects require further research.

Acknowledgement

The authors would like to thank the Department of Aquaculture, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University, Egypt, for providing facilities to carry out this experiment. Authors are grateful to Dr. Bahaa Abdella, Aquaculture Department, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University his for his valuable help.

Data Availability Statement

All relevant data are available from the authors upon request.

Author contributions

All authors contributed equally to this work (conception, acquisition, samples analysis, statistical analysis, data interpretation, manuscript drafting, and manuscript revision).

Funding Information

This study was not financially supported by any funding organization.

Conflict of interest

The authors have no conflict of interest.

TABLE 1. Primers used for qRT-PCR analysis.

Gene	Primer	Reference
B-actin*	F: CACACAGTGCCCATCTACGA R: CCACGCTCTGTCAGGATCTTCA	Bois et al.[54]
CYP19	F: CGTCATGTTGCTTCTCATCG R: TACCGCAGGCTCTCGTTAAT	Agus et al.[53]
GPx	F: TCGGACATCAGGAGAACTGC R: GCACTGCTCAAAGTTCCAGG	Agus et al.[53]

CYP19: Cytochrome, *GPx*: glutathione peroxidase, *housekeeping gene.

TABLE 2. Growth performance, food utilization and survival of *Oreochromis niloticus* (mean ± SEM) before and after challenge with heavy metals' mixture and NAC treatment

Parameters	C	HM	NAC	p-value
Initial body weight (g)	68.361±3.287	69.071±5.598	66.541±1.555	0.885
Final body weight (g)	109.833±2.827 ^{a}	86.300±3.688 ^{b}	101.270±4.236 ^{a}	0.005
weight gain (g)	41.340±1.095 ^{a}	17.688±0.525 ^{b}	35.657±1.751 ^{a}	0.002
weight gain percent (%)	59.927±1.158 ^{a}	25.751±0.624 ^{b}	53.612±2.632 ^{a}	0.002
FCR	1.611±0.023 ^{a}	2.188±0.065 ^{b}	1.795±0.088 ^{a}	0.017
SGR (%/day)	1.162±0.062 ^{a}	0.559±0.026 ^{b}	1.073±0.043 ^{a}	0.001
Survival rate (%)	100.00±0.000	95.840±4.165	95.840±4.165	0.649

Note: Means within the same row lack common superscripts are significantly different at $p < 0.05$.

FCR: Feed conversion ratio, SGR: Specific growth rate

TABLE 3. Hematological indices (mean ± SEM) of *Oreochromis niloticus* before and after challenge with heavy metals' mixture and NAC treatment

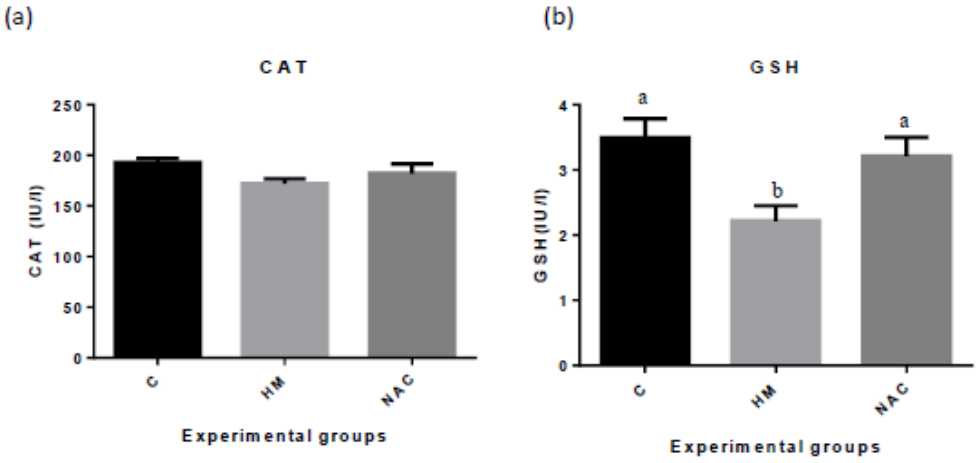
Indices	C	HM	NAC	p-value
Hb (g/100l)	11.770±0.233	8.367±1.629	9.867±0.669	0.141
HCT (%)	30.572±0.5207^{a}	20.071±3.148 ^{b}	25.070±1.415 ^{c}	0.029
RBCs (x 10^6/mm^3)	1.880±0.033 ^{a}	1.267±0.167 ^{b}	1.531±0.088 ^{ab}	0.024
MCH (pg)	62.333±0.670	58.677±2.191	57.000±4.040	0.411
MCHC (%)	39.677±0.333 ^{a}	41.677±0.333 ^{b}	41.001±0.582 ^{ab}	0.042
WBCs (x10^3/ µl)	69.800±5.934 ^{a}	89.900±0.814 ^{b}	69.571±4.599 ^{a}	0.026
Lymphocytes (×10^3/µl)	73.000±3.512	81.000±0.577	74.000±2.517	0.125
Neutrophils (×10^3/µl)	8.001±0.580	9.333±0.333	9.688±0.333	0.072
Monocytes (×10^3/µl)	13.677±1.201	9.688±2.186	13.000±1.533	0.275
Eosinophils (×10^3/µl)	5.000±0.583	3.000±1.000	5.000±1.000	0.257

Note: Means within the same row lack common superscripts are significantly different at $p < 0.05$. WBCs: white blood cell count, RBCs: Red blood cells count, Hb: hemoglobin content, HCT: hematocrit, MCH: mean corpuscular haemoglobin and MCHC: mean corpuscular haemoglobin concentration.

TABLE 4. Blood biochemical (mean ± SEM) of *Oreochromis niloticus* before and after challenge with heavy metals' mixture and NAC treatment

Parameters	C	HM	NAC	p-value
ALT (U/L)	37.001±7.024 [a]	61.011±2.309 [b]	15.333±2.603 [c]	0.012
AST (U/L)	62.000±6.112 [a]	297.717±7.312 [b]	31.000±6.658 [c]	< 0.001
Albumin (g/dl)	1.233±0.099	1.067±0.071	1.233±0.133	0.446
TP (g/dl)	3.367±0.091 [a]	2.477±0.221 [b]	3.67±0.221 [a]	0.009
Globulin (g/dl)	2.133±0.177	1.406±0.267	2.433±0.322	0.070
Urea (mg/dl)	20.337±1.155 [a]	25.026±0.577 [b]	19.333±2.186 [c]	0.047
Creatinine (mg/dl)	0.277±0.003	0.377±0.003	0.243±0.033	0.068
ALP (mg/dl)	121.707±6.577 [a]	160.713±6.988 [b]	117.337±2.192 [a]	0.003
Triglycerides (mg/dl)	248.077±9.177 [a]	236.022±39.597 [a]	170.300±5.881 [b]	0.017
Total lipid (mg/dl)	497.002±16.177 [a]	599.733±23.247 [b]	309.720±8.066 [c]	< 0.000
HDL (mg/dl)	29.231±0.586 [a]	21.033±0.887 [b]	35.677±2.316 [a]	0.002
Glucose (mg/dl)	30.677±2.082	34.677±0.882	34.333±1.667	0.156
Cholesterols (mg/dl)	175.677±18.980	183.033±8.413	193.333±3.512	0.624
Bilirubin (mg/dl)	0.013±0.003 [a]	0.050±0.006 [b]	0.023±0.003 [c]	0.002

Note: Means within the same row lack common superscripts are significantly different at $p < 0.05$. AST: aspartate aminotransferase, ALT: alanine aminotransferase, TP: total protein, ALP: alkaline triphosphatase and HDL: High density lipoprotein



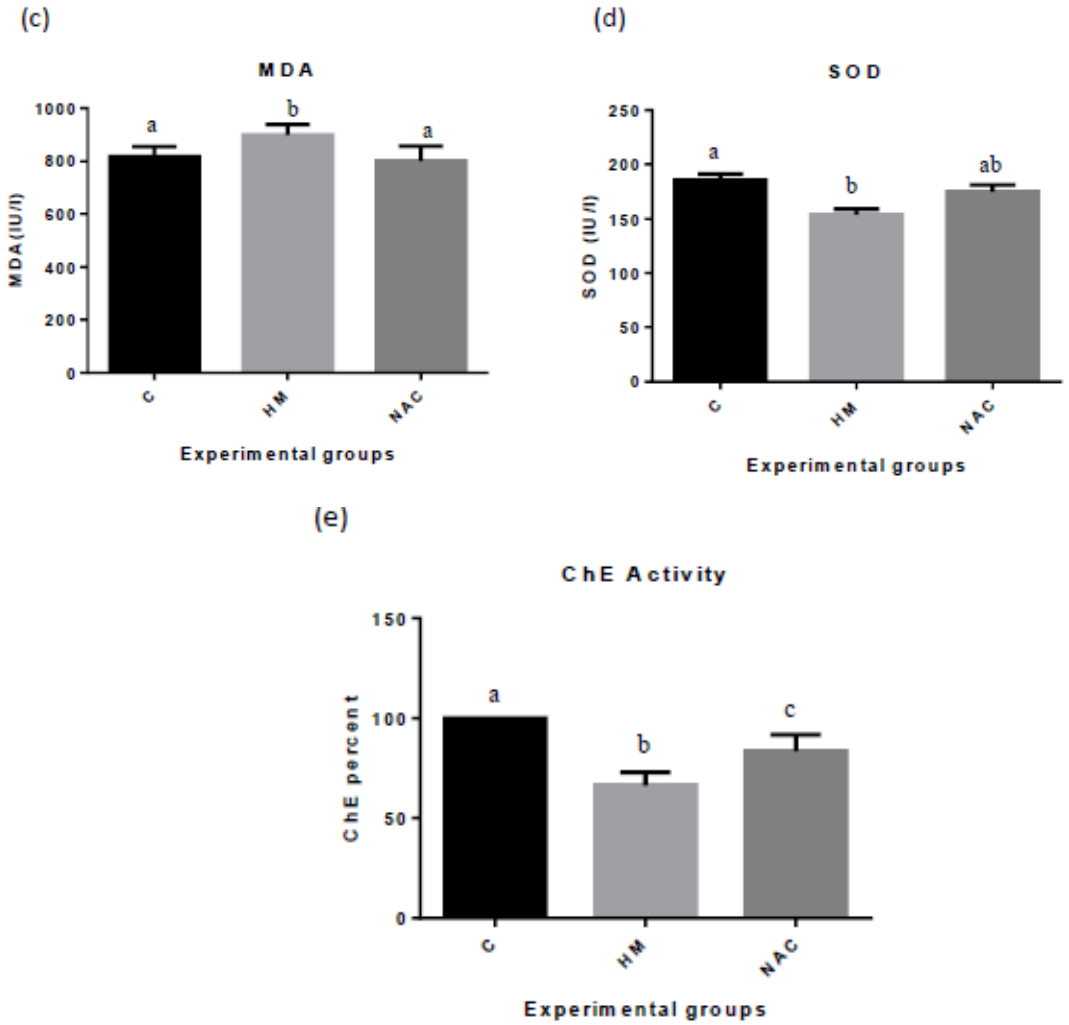


Fig. 1 Oxidative stress assay parameters: (a): CAT (catalase), (b): GSH (glutathione), (c): MDA (malondialdehyde), (d) SOD (superoxide dismutase), (e) ChE % (cholinesterase percent). C: fish of control group, HM: fish exposed to Pb and Cd mixture, NAC: fish fed N-acetylcysteine additives. values are expressed on columns (mean ± SEM) and different superscripts are significantly different ($p < 0.05$) using one- way ANOVA analysis.

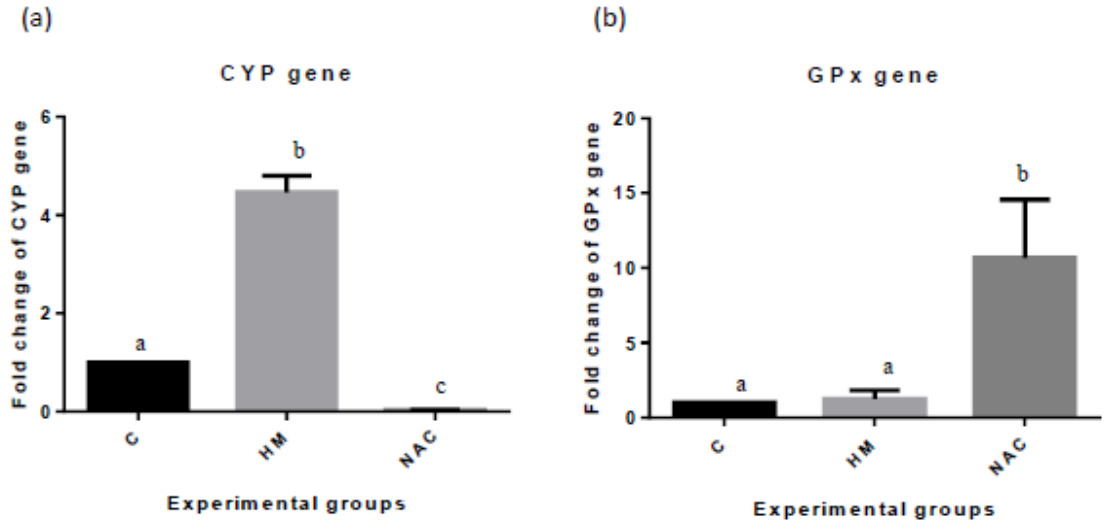
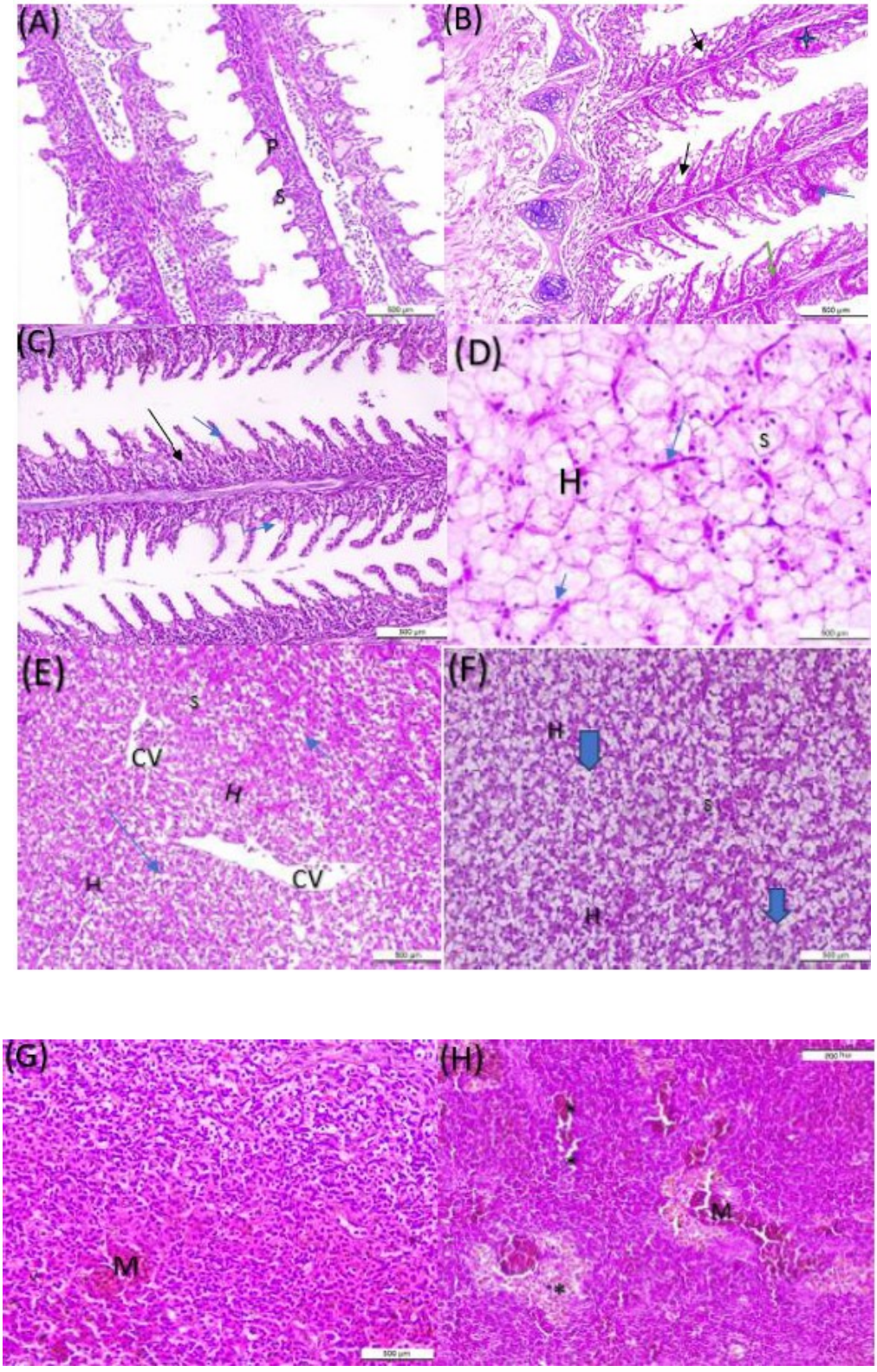


Fig. 2. Relative mRNA expressions of (a) *CYP* (cytochrome) gene and (b) *GPx* (glutathione peroxidase) gene in Nile tilapia liver. C: fish of control group, HM: fish exposed to Pb and Cd mixture, NAC: fish fed N-acetylcysteine additives. Transcriptomic levels are expressed on columns (mean ± SEM) and different superscripts are significantly different ($p < 0.05$) using one- way ANOVA analysis.


(A)
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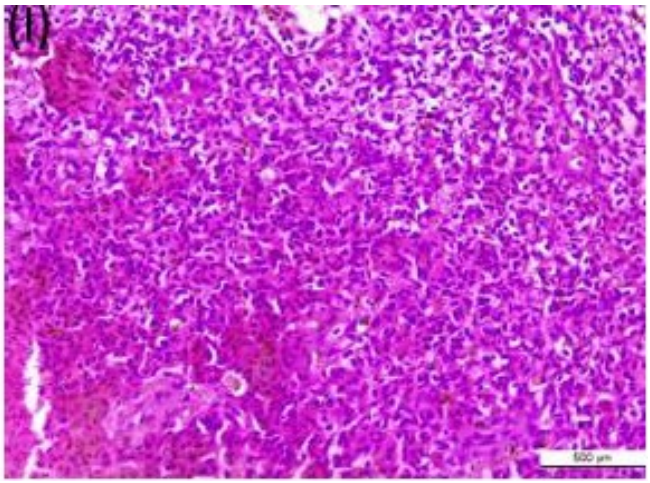

Fig. 3. (A), (B), (C) Photomicrograph of a transverse section of a gill filament of Nile tilapia gills (H & E stain, scale bar = 500 µm. (A) of the control group showing the normal appearance of the primary lamella(p) and secondary lamella (s). (B) Gills of fish exposed to metals showing eosinophilic infiltrate (green arrow), interlamellar hyperplasia (blue arrow), detachment of epitilum layer in secondary lamella and congested blood vessels (*), ypertropy of chloride cells (black arrow). (CI) Gills of fish treated with NAC show decreased adhesion of secondary gill lamella (blue arrow) with decreased eosinophilic granular cells (black arrow). (D), (E), (F) (A) Microscopic examination of Nile tilapia liver sections with representative microphotographs (H & E stain, scale bar = 500 µm). (D)The control group exhibits a typical hepatic structure characterized by well-arranged cords of normal hepatocytes (H). These hepatocytes display central rounded vesicular nuclei and prominent nucleoli. Blood sinusoids (S) separate the hepatic cords, and they are lined with endothelium and von Kupffer cells (indicated by a blue arrow). (E) The metal-exposed group displays a dilated and congested central vein (CV) accompanied by congested blood sinusoids (S). There is a significant presence of massive fatty infiltration in hepatocytes (H), with some hepatocytes exhibiting a signet ring appearance. (F) The hepatic lobules demonstrate good organizational architecture, with only a few congested sinusoids and blood vessels. Additionally, there is a minimal presence of inflammatory cell infiltration, and sporadic vacuolar degenerative changes (indicated by an arrow) are observed in hepatocytes and normal pancreas architecture. (G), (H), (I) Microscopic examination of H and E-stained Nile tilapia spleen sections with representative microphotographs. (G)photomicrographs of splenic tissues of Nile tilapia in the control group showed normal splenic structures, mixed red and white pulp and melanomacrophage aggregation. (H) Fish with metals mixture showed necrosis (*), melanomacrophaes. (I) Fish treated with NAC showed nearly normal splenic tissues with mild individual melanomacrophages (M) normal white pulp surrounded by blood vessels.

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دراسة تناول اسيتايل سيستين على أداء وصحة سمكة البلطي النيلي أثناء تعرضها لضغط ناتج عن وجود العناصر الثقيلة

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المستخلص

تعتبر مادة الاسيتايل سيستين مستخلص طبيعي من الحمض الاميني سيستين. في هذه الدراسة تم بحث تأثير تقديم السيستين إلى سمكة بلطي نيلي معرضه للعناصر الثقيلة لمدة 40 يوما. العناصر الثقيلة أخرت نمو سمكة البلطي ولكن الاسماك التي تم اضافه السيستين لها وجد ان نموها لم يتأثر كثيرا بوجود العناصر الثقيلة. وايضا استطاع السيستين التقليل من التأثير السلبي للعناصر الثقيلة على قيم الدم والسيرم والانزيمات. كذلك نجح السيستين في الحد من تأثير العناصر الثقيلة على الجينات الوراثية

الكلمات الدالة: أسيتايل سيستين- البلطي النيلي- العناصر الثقيلة.