RESEARCH ARTICLE

Alteration in Biochemical Levels and mRNA Expression of Specific Antioxidant Enzymes in Pb-exposed *Cyprinus carpio L.* Liver

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ABSTRACT

Considering the toxicity and possible danger to humans and aquatic environments posed by heavy metals. The study examines adverse changes in glycogen and glucose reserves from sublethal concentrations of lead (Pb), and mRNA control of antioxidant enzymes in fish liver, *Cyprinus carpio L.* Pb's LC_{50} was measured for 96 hours as 8.98 ppm with the confidence limits of 95 %. The fish expose to (T1:0.599, T2: 0.898, and T3: 1.797) ppm for 1, 7, and 14 days. The biochemical analysis revealed that the activity of superoxide dismutase (SOD) and catalase (CAT) in the liver increased significantly comparable to control ($P < 0.0001$). Whereas the levels of glycogen and glucose measured in the fish liver homogenates decreased gradually ($P < 0.05$) over the experimental duration and substantial differences ($P < 0.05$) occurred between the days of exposure. Finally, using Pb-exposed fish liver tissue, comparable gene expression evaluated using the real-time quantitative polymerase chain reaction. The findings show that exposure to this metal resulted in significant changes in gene expression, and CYP1A enhanced in response to treatment with Pb-T3. Expression of genes for the Cu-Zn SOD, Mn-SOD, and GPx genes, inhibited with exposure to T3-Pb, may result from harmful effects and metal toxicity, which increased reactive oxygen species (ROS) and oxidative stress rates. The results of this study offer basic evidence on the impacts of metal emissions on aquatic environments for future studies, which will be a significant step towards a systematic risk evaluation of environmental stressors on aquatic organisms.

Keywords: Antioxidative defence, Biochemical changes, Pb-toxicant, RT-qPCR.

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INTRODUCTION

Metal contamination is a great global concern which is increasing at an alarming rate.[1] For rising industrial activity, heavy metals enter the marine environment through the intensive use of metals that turn it into a worldwide problem.[2] Due to their non-biodegradable, cannot be formed or destroyed,[3] their toxicity, stability and bioaccumulation in food and then transmitted through the food chain to humans.[1] As a consequence of the health hazards correlated fish eating, heavy metal loads in fish have become a significant global threat, not just for fish but also for all people.[4]

Lead is a naturally occurring omnipresent poisonous metal whose unregulated usage in many manufacturing, domestic, agricultural, medical and technical applications have led to its broad environmental spread, widespread human exposure and consequent serious health and intellectual issue.[5] Pb is a possible oxidizing agent in the chemical compound of lead nitrate, and regarded as a carcinogenic for beings.[6,7] Pb, do not play important biological roles in vertebrate animals and can cause highly poisonous events at low concentrations.[8,9] Their negative impacts on physiological, biochemical, and behavioral functions have recorded in living things.[10-13]

Several mechanisms are responsible for shielding beings from oxidative harm impact induced by metal exposure. Therefore, antioxidant enzymes are affecting reactive oxygen species (ROS) molecules directly or indirectly. To decide the fate of the organism, controlling the antioxidant defense mechanism that regulates oxidative and antioxidant reactions is important. Because antioxidant systems describe the mechanism of cell defense and the possible threats to heavy metals, their research is critical in explaining the metal exposure influence. Enzymatic and non-enzymatic antioxidants that allow free radicals to secure and by ROS offer the primary protection. Antioxidant enzymes with at least three distinct superoxide dismutase (SOD) isoenzymes, including one manganese type (Mn-SOD) in the mitochondrial matrix and two forms of copper and zinc (Cu, Zn-SOD), one of which is found mostly in the cytosol and another in different extracellular fluids.[14] SOD plays an important role in catalyzing $O_2^{\bullet-}$ to O_2 and H_2O_2 dismutation. Glutathione peroxidase (GPx)

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and CAT, hydrogen peroxide removal.[15] Nevertheless, through certain catalyzing reactions by GPx, metabolizing water and corresponding alcohols (ROH) need to reduce H_2O_2 and a large array of organic hydroperoxides (ROOH). Such enzymes used in animal and human studies to assess Pb -induced oxidative damage and may be used as a water contamination biomarker. Where aquatic beings subjected to a possible contaminant, such enzymes are typically influenced by activation or inactivation; several studies have documented these effects on fish.[16,17] Stress can be described as a collection of acts undertaken by an organism to transfer into a natural physiological state; this stress can affect biochemical changes that damage the fish's health.[6]

And it shows that non-enzymatic antioxidants like GSH are much influenced by contaminant exposure. Non- enzymatic antioxidants may discourage the unregulated development of free radicals or impede their transfer to biological sites, and most free radicals degradation relies mainly by oxidizing endogenous antioxidants through scavenging and removal of molecules.[18] Biochemical biomarkers used in fish to quantify modifications due to toxicant exposure show that some genes expressed significantly following exposure to these compounds.[19]

Metal exposure has a genotoxic impact on aquatic animals through triggering ROS development, causing oxidative stress and harm to DNA.,[20] that could be passed on to future generations.[21] Studying the molecular mechanisms through which environmental toxins exert their impact can adequately describe how pollutant concentration, exposure length, and gene regulation correlated with detrimental physiological consequences at beings and ecosystem levels. Moreover, this info can possibly improve risk analysis and can even aid with regulatory processes. Transcriptomics can give key information on signatures of environmental toxins in global gene expression and comprehend the molecular mechanisms fundamental the effect of Pb on aquatic vertebrates.[21]

The common carp (*Cyprinus carpio*), a member of Cyprinidae, is a freshwater organism, common to Asia and Europe but released worldwide.[22] Farmed for nutrition, ornamental or leisure purposes, *C. Carpio* is of great economic significance in Iraq. It used widely in scientific studies.[23] In the light of the above, this study intended to assess the different anti-oxidant enzymes functions, the control of mRNA and certain biochemical parameters including glycogen, glucose and induced by Pb in liver of *Cyprinus carpio L.*

MATERIALS AND METHODS

Experimental Design and Acute Toxicity Determination

Sexually mature *Cyprinus carpio L.* acclimatized in aquariums at 25 ± 1°C under a regulated natural photo-regime (14/10hours, light/dark) environment before the experiments, after being moved from the polyethylene bag to a continuously aerated glass aquarium. The fish is given commercial feed twice a day.

A standardized acute toxicity bioassay was conducted to prove Pb's lethal concentration levels of 24, 48, 72, and 96 h (LC_{50}) for experimental fish. The various concentrations (3, 6, 9, and 13 ppm) of Pb, along with three replications, were added, and the concentration of Pb was 0 ppm in control units. Mortality evaluated after 24, 48, 72, and 96 hours, and dead fish are quickly extracted.

Experimental Design, Sampling Preparation and Measurement of Enzymes and Biochemical Assay

In the sub-lethal test experiments, healthy and uniform-sized fish were collected and distributed equally into four groups, the first group used as control (0 ppm) and three treatments. Pb, 96 hour LC_{50} was 8.98 ppm. A nonlethal level of (T1:0.599, T2:0.898, and T3: 1.797) ppm, subjected to 1, 7, and 14 days, identified. The random fish from the control and treatment groups were picked after the specified period ends.

The liver tissue was homogenized in a phosphate-buffered saline (PBS) solution (50 mM and pH 7.0) and centrifuged for 10 min in 4°C at 8000 r/min. The supernatant is used to test the activity of enzymes, to assess glucose and glycogen. The sugar content measured using Miller.[24] method, using the DNS (Dinitrosalicylic Acid Reagent). Glycogen concentrations were assessed in the Carroll *et al*.[25] procedure using anthrone reagent. Superoxide dismutase (SOD) uses the Superoxide Dismutase Kit and CAT in liver tissue as demonstrated by the supplier (Human SODI and CAT (ELIZA), USA).

In each supernatant, the total protein amount measured using the Bradford test,[26] utilizing bovine serum albumin as standards.

Expression of Antioxidant Genes in the Liver Tissues of Fish

Total RNA from frozen Pb- treated *C. carpio L.* liver was isolated using Total RNA Reagent (TRIzol® Reagen, ambino/ RNA(Invitrogen)) isolator. The prepared RNA can represent as a reverse transcriptase template. Total RNA (1µg) reversed to produce the first-strand cDNA using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific Kit), as directed by the supplier.

The amplification efficiencies of the primers and the transcriptional integrity of candidate genes were evaluated before the mRNA expression test. The empirical findings revealed that the β-actin was the most stable gene for the Pb treatment. Increasing target gene's mRNA expression is standardized to β-actin.

The quality of RNA was tested for the 1% agarose-formaldehyde gel by electrophoresis. While the purity and quantity of the RNA were examined using a NanoDrop 2000 spectrophotometer.

The design of the primers based on knowledge obtained from GenBank's NCBI BLAST quest for superoxide dismutase (Cu/Zn-SOD), Mn-superoxide dismutase (Mn-SOD), Catalase (CAT), Cytochrome c oxidase (COX-17), Cytochrome P450 1A (CYP1A), Glutathione peroxidase (GPx), Glutathione (GSH) and β-actin used in the production of the corresponding primers.

qRT-PCR conducted using SuperReal PreMix Plus (SYBR Green) with roxatidine (ROX) as reference dye according to the manufacturer's instructions in the Applied Biosystems AB7900HT Fast Real-Time PCR System instrument with (SDS v2.3 Software). The relative rates of gene expression in mRNA examined using the $2^{-\Delta\Delta Ct}$ approach.[27] to assess the degree of expression in common carp following Pb treatment.

RESULT

At various times, fish are subjected to three levels of Pb, depending on the toxicity tests. There was no mortality found at the experiment. In liver, at the initial dose of Pb-T1, there was a significant rise in SOD rates ($p < 0.0001$) between the 7th, 14th days (165.69 and 151.07)% respectively. In comparison, in T2 and T3, dose and time-dependent enzyme activity ($P < 0.001$) in liver tissues improved at (170.78, 199.92, and 208.9) % respectively, after the 1, 7, and 14 days, in fish (Figure 1 A), higher than that found in controls.

As seen in Figure 1B, during the experiment development, dose-dependent antioxidant enzymes CAT ($P < 0.0001$) improved in liver tissue after 14 days in all cases of Pb treatment (618.58, 237.67, and 491.60) % respectively, over control values (Figure 1 B).

The glucose rates in experimental fish liver was significantly increased ($P < 0.001$) with early exposure to Pb toxicity throughout both doses (129.09, 193.47, and 198.64) % respectively, relative to control (Figure 2 A).

Whereas glucose values were an aggressive decrease at doses (Pb-T1, T2, and T3) (58.06, 58.30, and 57.37) %, respectively, compared to control fish after 14 days (Figure 2A) ($P < 0.01$). Glycogen concentrations in the first-day exposure (Pb-T1, T2, and T3) were higher than in the later-day exposure (191.58, 151.23 and 261.92) %, respectively. After 7 and 14 days, Pb-T3 doses displayed a gradual decrease ($P < 0.0001$) by (27.93 and 74.09) %, respectively, according to fish controls (Figure 2 B).

Expression of *Cyprinus carpio L.* genes in liver

Table 1 revealed the mRNA expression levels of (Cu / Zn-SOD, Mn-SOD, CAT, COX-17, GPx, CYP1A and GSH genes

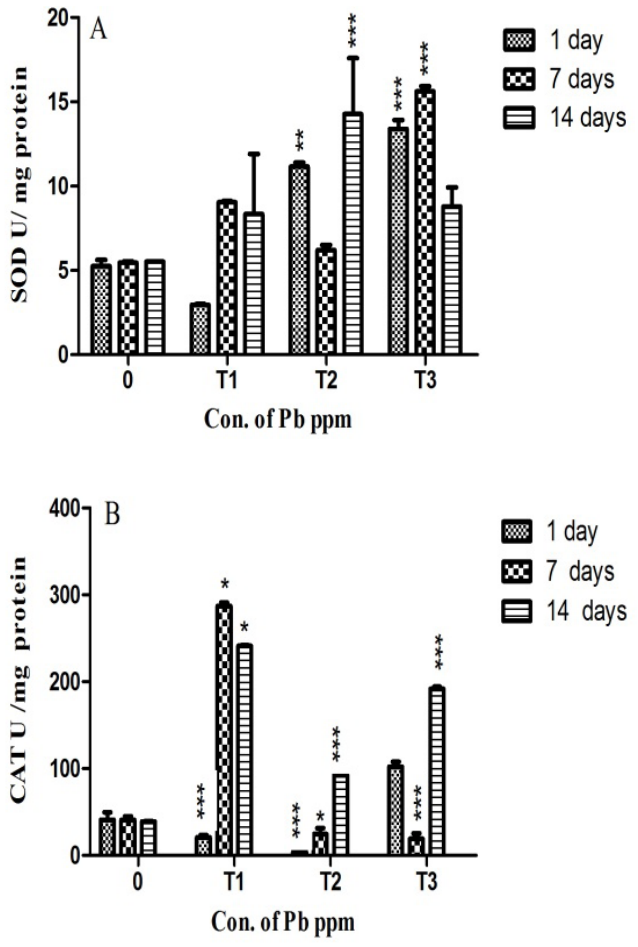

Figure 1: (Mean values ± SD) of SOD (A) and CAT (B) activities in *Cyprinus carpio L.* liver subjected to varying levels of Pb for 1, 7, 14 days. As compared with the controls the asterisk indicates a statistically important variation.

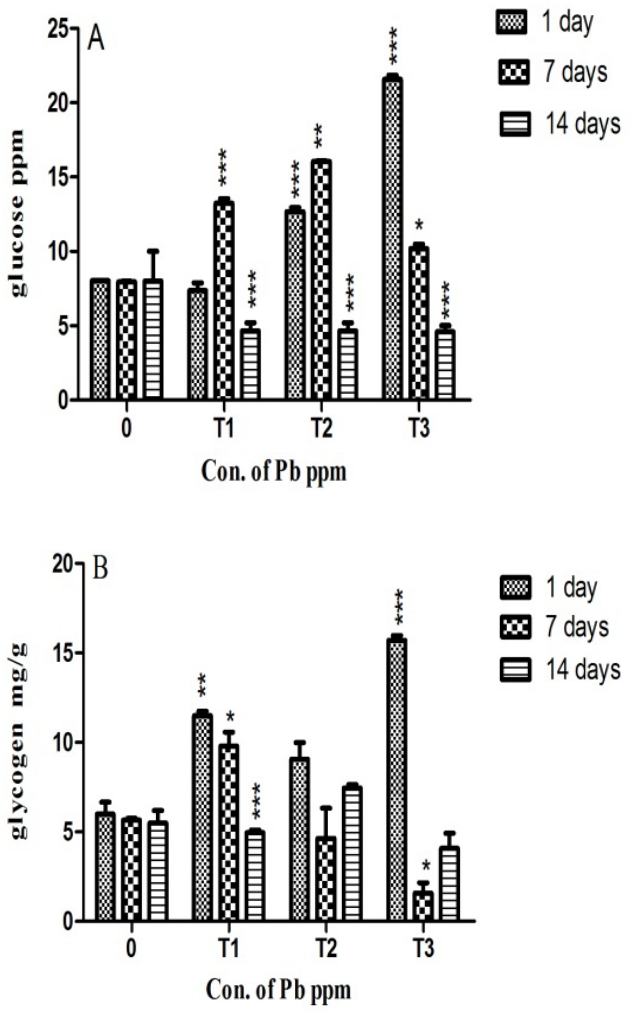

Figure 2: (Mean values ± SD) of glucose (A) and glycogen (B) in the *Cyprinus carpio L.* liver subjected to varying levels of Pb for 1, 7, 14 days. Comparative with the controls the asterisk indicates a statistically significant variation.

Table 1: The variability expression of common carp genes in liver

Pb treatment dose in adult fish (ppm)	*Cu/Zn-SOD ± SD*	*Mn-SOD ± SD*	*CAT ± SD*	*COX-17 ± SD*	*GPx ± SD*	*CYP1A ± SD*	*GSH ± SD*
0	1.00 ± 0.1	1.00 ± 0.70	1.02 ± 1.0	1.08 ± .90	1.00 ± 0.08	1.08 ± 0.09	1.0 ± .0.01
T1	0.29 ± 0.13	4.40 ± 0.28	0.47 ± 0.09	10.29 ± 4.99	8.04 ± .05	0.19 ± 0.14	6.88 ± 4.79
T3	0.95 ± .07	0.10 ± 0.01	4.07 ± 0.09	2.02 ± .03	0.69 ± .13	1.24 ± .34	1.49 ± 0.15

The gene-level results are seen as mean ± SD (n = 3)

compared to β-actin in fish's liver after 7 days of subjected to Pb treatments.

The expression Mn-SOD and GPx mRNA displayed a negative dose-reaction in the common liver carp, which handle with T3 Pb treatment, but this was not observed in their mRNA expression after T1 Pb treatment (Table 1). By comparison, all (COX-17 and GSH) mRNA levels demonstrated a positive dose response (6.16 and 4.19) fold in the liver fish handled with T1 and T3- Pb. CAT and CYP1A mRNA expression in fish improved in the T3 Pb treatment group (4.07 and 1.24) fold, respectively, relative to the control group treated to 0 ppm Pb (Table 1).

Statistical Analysis

Values were described as the mean ± SD, and all evidence was statistically dependent on the model designs that were evaluated using a two-way variance test (ANOVA). GraphPad Prism statistical software (GraphPad Software Inc., version 5.01; La Jolla, CA, USA) carried out the statistical evaluation. A p-value<0.05 has been accepted as being statistically important.

DISCUSSION

Specific anthropogenic pollutants are released into water bodies which particularly affect fish aquatic life. Biochemical and enzyme levels are beneficial in assessing the impact of anthropogenic contaminants.[28] The enzymatic anti-oxidant mechanism defends cells from the harmful impact of the active oxygen molecule and aims to maintain cellular homeostasis by eliminating ROS.[17,29] The level of certain oxidative stress biomarkers in common carp liver subjected to sublethal treatments of pb evaluated (Figure 1). Fish produce reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}HO$) upon subject to contaminants.[30-32] Oxidative stress is an inconsistency between the development of free radicals and the capacity of the biological system to detoxify the reacting intermediates readily or restore the resultant harm.[33] As the rates of ROS rise, the biological system produces a first-line protection mechanism by modulating anti-oxidant behaviors such as CAT and SOD.[34-36] and used as a biomarker representing ROS generation. SOD catalyzes the dismutation of H_2O and H_2O_2-radical superoxide anion.[32,37-38] CAT behaves as a hydrogen peroxide scavenger.[17,39] Consequently, SOD and CAT change identified as indicators of lead toxicity in fish.[40] Since the fish are the important species that show the toxic impact of pollutants in bodies of water.[41] A distinct pattern was found in the Pb treated types, distinguished by a substantial dose and time-dependent rise in SOD activity ($P < 0.0001$) in the *C. carpio L.* liver (Figure 1 A). Such changes may be attributed to the transformation of superoxide radicals into hydrogen peroxide, which is then converted into oxygen and water by CAT.[41] Thanks to its inhibitory impact on oxyradical creation, SOD is regarded as the first defense line against its oxygen toxicity.[39] Kumar *et al*.[42] have observed a real increase in SOD activity in chlorpyrifos-subjected fish. Likewise, the oxidative stress of a sub-lethal dosage (1/10 LD_{50}) of imidacloprid has demonstrated a rise in liver SOD in male mice.[18] In comparison, Muthappa *et al*.[34] proposed greatly enhanced production in liver SOD and muscle catalase to scavenge superoxide anion overproduction under the oxidative stress caused by low-dose endosulfan as used in this research. In contrast, these findings conflict with a research performed by Han *et al*.,[35] in which, after azoxystrobin was introduced to zebrafish, SOD behaviors in the liver reduced dramatically compared to control. As shown by Min *et al*.,[43] after a 2–4 weeks exposure to Cr^{6+}, SOD production decreased in mullet *Mugil cephalus*' liver. An essential characteristic of anti-oxidant enzymes is their changed activities under oxidative stress, and such a transition may involve a significant adaptation to the tension induced by contaminants.[44] The substantial increase in CAT behavior ($P < 0.0001$) for three treatments (618.58, 237.67, and 491.60) %, respectively after 14 days of *C. carpio L.* liver exposure (Figure 1 B), is possibly a reaction to pb-caused harmful stress and helps to neutralize the effect of elevating ROS production.41 The fact that lead-containing solutions stimulated CAT behavior emphasizes the significance of enzyme as key anti-oxidant protection in common carp liver. As an intracellular enzyme, CAT reveals highly efficient, and selectivity in the H_2O_2 stimulate.[31] and reflected the fish body's tolerance to toxicant pressure.[17] Similar discovery in tissues of *Labeo rohita* subjected to sublethal chromium level reported by Kumari *et al*.[30] And Salvo *et al*.[45], in the presence of endosulfan recorded substantially increased CAT activity in the common carp liver. Both in the liver and kidney of African Catfish (*Clarias gariepinus*) of Cd-subjected and the kidney of the Pb- subjected group, the rising of CAT level was greatly increased.[40] Accordingly, an increase in CAT activity ($P < 0.0001$) (698.37 and 183.83) % respectively, after 7 and 14 days of treatment at low doses of pb-T1 (Figure 1 B) that constituted a compensatory mechanism for oxidative stress. Similar findings were recorded in research conducted by Muazzam *et al*.,[39] in which, after treatment to endosulfan and imidacloprid to zebrafish, CAT behaviors increased dramatically at low concentration in the liver in contrast with controls. In this study, glycogen reserves and glucose in fish liver tissues subjected to three Pb-treated reduced substantially

after 14 days ($P < 0.001$) on the glycogen reserves and glucose estimated in control groups (Figure 2 A, B). This reduction may be the exposure of Pb stimulating the function of enzymes that use in glycogenolysis and glycolysis.[46] Carbohydrates preserved in fish tissue as glycogen and organs such as the liver provide nutritional requirements if hypoxic conditions. It has also been observed that heavy metals can generate stress in fish and that Pb can reduce glycogen reserves. Obiakor and Ezeonyejiaku.[46] referred that the source of carbohydrate retained as a reserve fuel in fish's liver and muscle tissues for endogenous energy derivation during acute and chronic stress and that the reduced glycogen content as a consequence of hypoxic or anoxic state activates the glycolytic enzymes via catecholamines that initially enhance glycogen content. Nonetheless, chronic tension after metal toxicity exerts a weakening and hypoxic state with hepatocyte failure to spread the regular metabolism of the cells. The liver is a significant detoxification site and the first target of ingested oxidants.[47,48] and may also clarify the steady decline in glycogen concentrations via the development of glucose energy in response to the stress caused by metal. Similar declines were found in edible carp Catla catla's liver and other tissues subjected to cadmium chloride.[49] Mattioli *et al*.,[50] glucose are the more important measurement criteria for the stress in fish. Improve amounts of glucose contributes to increased energy metabolism.[51] As the fish are exposed to stressors, cortisol is emitted into circulation directly, at which it induces glycogenolysis and hepatic gluconeogenesis.,[52,53] all of which give to enhanced amounts of glucose.[38] As seen in this research, earlier exposures demonstrated important rises ($P < 0.0001$) in glucose concentrations at low and mid (T1, T2) concentrations of Pb between 1st and 7th days (129.09 and 193.47) % respectively, followed by a substantial increase in T3-Pb (198.64) % during early exposures (Figure 2 A). This increase may contribute to improved gluconeogenesis of stressed fish in their attempts to fulfill their new energy requirements.[23] Relative to control increased exposure time (14 days) demonstrated substantial reduction of glucose rates for both treatments (58.06, 58.30, and 57.37) %, respectively (Figure 2 A). This is possibly attributed to the fast usage of glucose during extreme excitability, shocks, and tremors, common metal toxicity in fish.[54] Similar variations in blood glucose rates recorded in common carp (*Cyprinus carpio*), which subjected to Sumithion sub-lethal levels.[23] Likewise, catfish *Lophiosilurus alexandri* subjected to various water salinities.[50] And in the catfish *Ictalurus punctatus*.[53] the Curimbatá (*Prochilodus lineatus).* were exposure to transport stress. An inherent characteristic of contaminants-exposed species is their capacity to respond to such pollutant concentrations through moving on a range of compensatory or detoxifying pathways or changes in the expression and functioning of several enzymes.[37] Seven genes in adult *Cyprenus carpio L.* have been studied using real-time qPCR to assess improvements in gene expression rates following treatment with Pb (Table 1). Between them, expression of COX-17 and GSH in Pb-exposed fish was over-regulated (6.16 and 4.19) fold, compared to control expression (Table 1).

The enzyme superfamily 'Phase I' cytochrome P450 (CYP450) is usually the cell's initial response to biotransform bio-xenobiotics.[19] The CYP1A genes were substantially up-regulated at concentrations of Pb-T3 (Table 1); however, metal ion's effect on the expression of CYP genes was also documented.[31] Cytochrome P450-dependent monooxygenases are the most significant protein groups in reducing and oxidizing reactions, particularly drug metabolization and endogenous material metabolization.[55] Metals can metabolize by CYP450 enzymes to produce fewer harmful materials. Nevertheless, they can also impair CYP450 production by increasing the rates of active compounds with the potential to trigger an adverse reaction because they are not metabolized. These findings have also been documented in fish exposed to certain toxins, including the common carp liver (*C. carpio L.*) exposed to chlorpyrifos.[56] in zebrafish (*Danio rerio*) embryos subjected to endosulfan.[57] Therefore, induction of both Mn-SOD and GPx in fish at T1 doses may be an example of the decrease in the amount of ROS provided by Pb, which was necessary to trigger and induce Mn-SOD and GPx both. However, following exposure to T3 levels, Pb treatment inhibited specific genes, including the Cu-Zn SOD, Mn-SOD, and GPx genes, in liver fish. (Table 1). If fish are exposed to metal, the decline may result from harmful impacts and metal toxicity that higher rates of ROS resulting in oxidative stress.[58] Further studies needed to test and confirmed this hypothesis.

CONCLUSION

The research specifically demonstrates the biochemical consequences of heavy metal Pb in fish as an aquatic risk factor across a length of exposure duration. This research found that Pb changed the metabolism of carbohydrates in common carp by altering glucose levels and the glycogen reserves in liver tissues. Such variations in the biochemistry of liver tissues under the Pb influence may give to energy impairments involving essential processes, and thus indicate the health state of the fish community. Nevertheless, the study found that Pb exposure would lead to major changes in anti-oxidant enzymes in fish, along with alterations in gene expression. There were many differences between the control and treatment groups. Analyses of molecular and biochemical criteria of the fish's metal levels would offer a valuable monitoring tool for the hazard evaluation of metal contamination where such toxicants may have many and vary types of impacts on non-target organisms. In summary, this study may include knowledge for metal risk control, eco-toxic, and health security of aquatic.

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