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Toxic Effects Assessment of Acute Cobalt Exposure on Stress Biomarkers Responses of *Cyprinus carpio* Intestine

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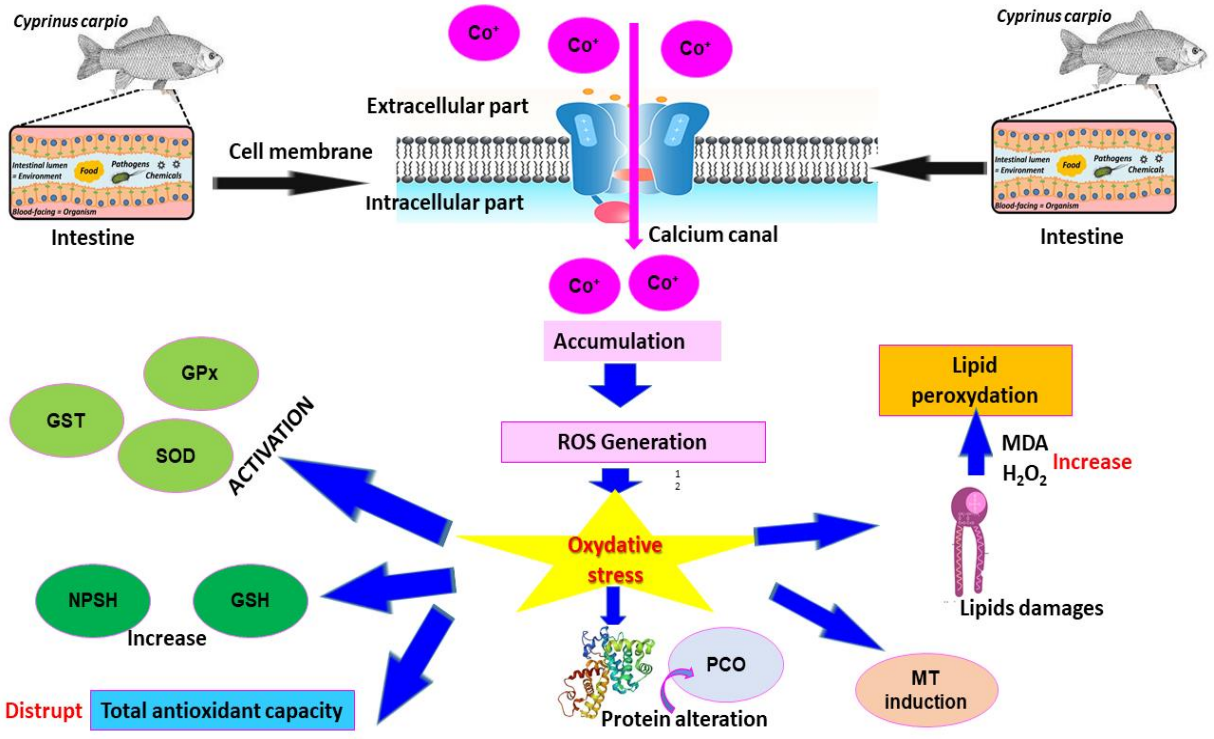
ABSTRACT

The aim of the current study is to assess in the first time the toxic effects of acute chloride cobalt concentrations exposure (0(CT), 50 (D1), 500 (D2) $\mu gL^{-1}CoCl_2$) on stress biomarkers responses of *Cyprinus carpio* intestine during 3 days. Cobalt accumulation alters the lipids of intestine cells causing therefore lipid peroxidation objectified by significant increases of H_2O_2 and MDA levels in all treated groups. The cobalt toxicity induced the disruption of the total antioxidant capacity confirmed by decrease of FRAP levels. The activation of enzymatic and no-enzymatic antioxidants defense system was determined by increases of SOD, GPx activities and GST levels (1) and GSH and NPSH levels (2) in all carp treated groups. Similar MT's induction recorded in our treatment suggested the better response of intestine cell against the cobalt toxicity.

Keywords: *Cyprinus carpio*, cobalt, intestine, stress biomarkers

GRAPHICAL ABSTRACT



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INTRODUCTION

It is known that trace elements/metals with higher concentration cause serious injurious impacts on aquatic ecosystem and human health (Ogunwole et al., 2021). Due to their persistence, toxicities, and non-biodegradable features, trace elements are considered as significant pollutants (Hasanein et al., 2022).

Cobalt (Co) is an essential element for animals regarding its principal function in the synthesis of vitamin B12 (Garoui et al., 2011). The unnatural origin of cobalt was from alloy metals, as colorant for paints, glass and ceramics industries, as additives in agricultural and medical products (ATSDR, 2004). Co becomes a toxic element when its concentration exceeds the permissible limits that signaled in surface, irrigation and livestock waste waters with 1 ppm, 0.05 ppm and 1.0 ppm, respectively (Comhaire et al., 1998). Moreover, aquatic ecosystems are directly or indirectly affected by many metals including cobalt through different sources. In fact, the common carp (*Cyprinus carpio*, Linnaeus 1758) is advised as the most abundant cyprinid fish species in the world's aquaculture with 54% of total world fish production (Takeuchi et al., 2002; FAO, 2009). The common carp is mediated as beneficial seafood products to human health regarding its high and health nutritional quality supported by the important quantity of protein, fat, carbohydrates and by the main source of (n-3) polyunsaturated fatty acid especially eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA (Ćirković et al., 2002). Nonetheless, *C. carpio* with high capacity to accumulate different metals, great resistance to diseases and good vulnerability to environmental conditions, so it well be used as a suitable bioindicators for water pollution and reflect the environment status (Ahmed et al., 2016; Sadeghi et al., 2020). It is known that metals penetrate in fish either by ingestion, gills and skin or by digestive tract (Pourang, 1995). For several studies, gills, muscle and detoxification organs of fish were considered as the better target for assessing the aquatic contaminants effects (Pourang, 1995). However, subjects focused on the evaluation of metals impacts on fish intestines appear to be rare despite their crucial roles in digestion and absorption of nutrients (Clearwater et al., 2000).

Once the cobalt (Co) enters cell, its excessive accumulation induced an oxidative stress through the overproduction of reactive oxygen species (ROS) and affecting the cell functions and causing lipid peroxidation, protein denaturation and pathologic irritations (Shacter, 2000; Hassoun et al., 2001). According to the literature, cobalt is considered as genotoxic and carcinogenic (De Boeck et al., 2003) and its toxicity produced cell death via apoptosis and necrosis inflammations (Petit et al., 2004; Catelas et al., 2005). Thus, Suganthi et al. (2015) showed that fish exposed to cobalt revealed an alteration in biochemical composition and change haematology aspect.

Furthermore, in order to protect against the toxic effects of metals and to overcome the over-production of ROS, the aquatic organism advanced a wide variety of detoxification mechanisms including the antioxidant defense system, metallothionein. These defense systems play a key role in remove of the oxygen reactive species (ROS) and in maintain of the cell homeostasis in order to protect organisms from oxidative stress (Cotou et al., 2013). According to our knowledge, there are lack information about the effect of cobalt on redox status of *C. carpio* intestine. Our study aimed to assess the effects of cobalt toxicity on the stress biomarker responses of *Cyprinus carpio* intestine following acute $CoCl_2$ concentrations exposure during 3 days.

MATERIALS AND METHODS

Fish and Experimental Design

Acclimation

Samples of *Cyprinus carpio* with body weight (4.59g±0.05) and length (3.88±0.11cm) was obtained from private fish society and transported to the Laboratory of Ecology, Biology and Physiology of Aquatic Organisms on the Faculty of Sciences of Tunisia, University of Tunis El Manar. Fish were acclimated in experimental aquariums (80 L) for 5 days (Figure 1). The acclimation parameters were fixed by constant photoperiod (12h/12h) light–dark cycle and by temperature estimated at 28 ± 2 °C. Animals were feed with commercial basal diet three times daily at 8:30, 13:30 and 15:30, respectively. The dissolved oxygen levels is maintained near the saturation using a supplemental aeration. For all aquaria, 50% water was refreshed regularly every 24h.

Cobalt exposure

After acclimation, fish with similar initial weight body were distributed randomly into three groups (aquaria 80 L for each). Each group is represented in duplicate (n=8 fish per replicate). Fish were exposed to filtered water (control) or unmixed $CoCl_2$ metal (Cobalt chloride; $CoCl_2$; Sigma-Aldrich; powder 98%) which was dissolved in pure water. The animals were exposed to acute Co concentrations during 3 days as following:

Group I(CT): Carps unexposed to $CoCl_2$;

Group II (D1): Carps exposed to $50\mu gL^{-1}CoCl_2$;

Group III(D2): Carps exposed to $500\mu gL^{-1}CoCl_2$.

The selected Co concentrations in our experiment were carried out using preliminary trials focused on other fish. These concentrations were determined according to previous described investigations and slightly modified (Suganthi et al., 2015). The exposure doses were selected based on the published data on cobalt levels in freshwater bodies (ranged from 50 to 110µg/L) (Beyene & Berhe, 2015).

Tissue Preparation

After 3 days of $CoCl_2$ treatment, no mortality was recorded.

Fish were sacrificed on ice and the intestine were quickly removed. For each experimental group, intestines were homogenized in Tris-HCl buffer (20mM; pH=7.4) at cold, then, centrifuged at 10.000 × g for 20 min (4°C). Intestine supernatants were stored in Eppendorf tubes at -80°C for stress biomarkers analyses. 8 replicate were used for biochemical analyses (2 fish for each replicate).

Biochemical Assays

Reduced glutathione (GSH), 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB), 2-thiobarbituric acid) (TBA) were purchased from Sigma chemical Co (Saint Louis, MO63103, USA). All other chemicals were purchased from standard commercials suppliers.

Determination of Protein contents

The intestine protein content was estimated using Folin Reagent and Bovine serum albumin (BSA) as a standard range according to Lowry et al., (1951) method.

Determination of Malondialdehyde (MDA) levels

The malondialdehyde (MDA) levels was carried out according to Draper and Hadley (1990). It was estimated using to thiobarbituric acid (TBA 0.67%) and was measured by spectrophotometer method at 532 nm. Results were expressed as nmol /mg protein.

Determination of Hydrogen peroxide (H_2O_2)

According to method of Ou and Wolff (1996), the hydrogen peroxide (H_2O_2) was determined through the ferrous ion oxidation xylenol orange (FOX1). The H_2O_2 levels was measured at absorbance (560 nm). Values were expressed as mmol/mg protein.

Determination of Protein carbonyl (PCO) levels

According to Reznick and Packer (1994) method, protein carbonyl levels were determined. PCO amount expressed as nmol/mg protein.

Determination of Metallothionein (MTs) levels

Metallothionein levels was carried out through Viarengo et al. (1997) method modified by Petrovic et al. (2001). Following DTNB addition, MTs reaction absorbance was measured at 412nm. MTs levels were expressed as nmol GSH/mg protein.

Determination of Glutathione (GSH) levels

Using to Ellman (1959) method, glutathione level was determined at 412nm following 5-dithio-bis (2-nitrobenzoic acid) (DTNB) addition. The GSH concentration was calculated by standard concentration and was expressed as µg/mg protein.

Determination of Non protein –SH (NPSH) levels

According to Ellman (1959) method, Non protein–SH levels were carried out. Through potassium phosphate buffer and DTNB addition, the SH groups were determined in a pure supernatant. The colorimetric reaction absorbance was measured at 412 nm and NPSH level was expressed as µmol/mg protein.

Determination of* Ferric Reducing Ability of Plasma *(FRAP) levels

According to Benzie and Strain (1996) method, FRAP levels were determined through FRAP reagent (mix of acetate buffer (2,3,5-Triphenyltetrazolium chloride (10mM) and Ferric chloride). The FRAP levels was measured using spectrophotometry assays at the absorbance 593 nm. The values obtained were referred to a calibration curve from a 0.001 M ferrous sulfate heptahydrate ($S_2SO_47H_2O$) standard solution. Results were expressed as µM/mg protein.

Determination of superoxide dismutase (SOD) activity

According to Beauchamp and Fridovich (1971) method, superoxide dismutase activity (SOD) was accomplished. SOD activity was determined using the enzyme amount appropriate to inhibit the reduction of Nitroblue tetrazolium (NBT, 50 %). Under light condition, the SOD activity was measured at 560 nm after incubation. Levels were expressed as µmol/mg protein.

Determination of glutathione peroxidase (GPx) activity

Using Flohe and Gunzler (1984) method, glutathione peroxidase activity (GPx) was carried out by spectrophotometrically essays at 340. GPx levels was expressed as nmol GSH/min/mg protein.

Determination of glutathione S-transferase (GST) activity

Using to Habig et al. (1974) method, glutathione S-transferase activity (GST) was carried out by quantifying the conjugation of CDNB (1-cloro-2,4-dinitrobenzene) with GSH (glutathione). The GST activity was measured spectrophotometrically assays at the absorbance (340 nm) every minute for 6 min (with 3 replicas per sample). The GST activity was expressed by nmol/min/mg protein.

Statistical Analyses

Statistical data were performed with Statistica v. 5.0. Results were expressed as means ± SE (standard error) for each analysis. Normality was assessed for all datasets using the Shapiro-Wilcoxon test. The significant differences between variables were analyzed using one-way analysis of variance (ANOVA) followed by a post hoc Tukey's test ($p<0.05$). When the conditions for ANOVA were not satisfied, nonparametric Kruskal–Wallis's test was used ($p<0.05$). The differences between samples were deemed to be significant at $p<0.05$.

RESULTS

Assessment of MDA levels in intestine C. carpio treated by acute $CoCl_2$ concentrations

An increase of MDA levels were recorded in all treated carp intestine (*P≤0.001*) during 72h of acute $CoCl_2$ exposure (50 and 500µg$L^{-1}CoCl_2$) with (+6025 and +9205%) when compared to controls carps (Table 1).

Assessment of H_2O_2 levels in intestine C. carpio treated by acute $CoCl_2$ concentrations

Significant induction of H_2O_2 levels were observed in the intestine of all treated groups (50 and 500 µgL^{-1} $CoCl_2$) with dose dependent manner (+151% and 6917%, respectively) (Table 1).

Assessment of PCO levels in intestine C. carpio treated by acute $CoCl_2$ concentrations.

By compared to the carps controls, the intestine PCO levels were significantly increased by (+209 and +700%) after exposure to acute $CoCl_2$ concentrations (50 and 500µgL^{-1} $CoCl_2$, respectively) for 3 days (Table 1).

Assessment of MT's in intestine C. carpio treated by acute $CoCl_2$ concentrations

Our experiment revealed a significant increase in intestine MT's levels in all treated groups by acute $CoCl_2$ concentrations (50 and 500µg$L^{-1}CoCl_2$) with (+173 and +221% respectively) by comparing to control carps (Table 1).

Table 1. MDA, H_2O_2, PCO and MT's levels revealed in control and treated *C. carpio* intestine with acute concentrations of $CoCl_2$ during 3 days

Biochemical biomarkers	***CT***	***D1***	***D2***
MDA[a]	1,37±0,18	72,3±8,8***	91,06±12,4^{***+++}
H_2O_2[a]	0,18±0,13	8,76±0,1***	12,42±0,28^{***+++}
PCO[b]	2,06±0,29	6,37±1,2***	16,45±3,93^{***+++}
MTs[c]	0,5±0,09	1,35±0,2***	1,592±0,28^{***+++}

Results are expressed as means ± SD (n=9)

CT: 0µg/L $CoCl_2$; D1: 50µg$L^{-1}CoCl_2$; D2: 50µg$L^{1}CoCl_2$ $CoCl_2$

$CoCl_2$ groups VS controls: *p < 0.05; ** p < 0.01; *** p < 0.001.

D2$CoCl_2$ VS D1$CoCl_2$: +p < 0.05; ++p < 0.01; +++ p < 0.001.

[a] nmol/mg protein; [b] mol/mg protein; [c] µmol GSH/mg protein.

Assessment of NPSH and GSH levels in intestine C. carpio treated by acute $CoCl_2$ concentrations

High increases *(P≤0.001)* in NPSH levels were observed in the *C. carpio* intestine exposed to acute $CoCl_2$ concentrations (50 and 500µgL^{-1} $CoCl_2$) with (+38 and +51% respectively) when compared to controls (Figure 1a).

After 3 days of acute $CoCl_2$ treatment, the intestine GSH levels increased significantly only in fish exposed to 500 µg/L$CoCl_2$ (+50%) when compared to control carps (Figure 1b).

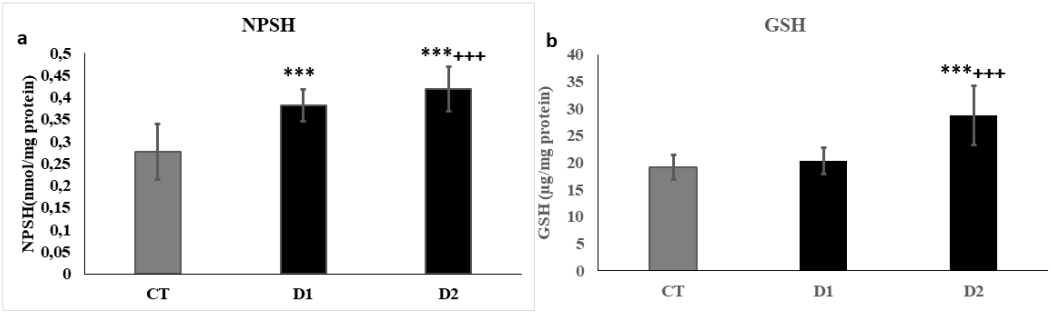

Figure 1. NPSH (a) and GSH (b) levels revealed in control and treated *C. carpio* intestine with acute concentrations of $CoCl_2$ during 3 days

Results are expressed as means ± SD (n=9 fish)
CT: 0µg/L $CoCl_2$; D1: 50µg$L^{-1}$$CoCl_2$; D2: 50µg$L^{1}$$CoCl_2$ $CoCl_2$
$CoCl_2$ groups VS controls: *$p < 0.05$;**$p < 0.01$; *** $p < 0.001$.
D2$CoCl_2$ groups VS D2$CoCl_2$ groups: +$p < 0.05$; ++$p < 0.01$; +++ $p < 0.001$.

Assessment of FRAP levels in intestine C. carpio treated by acute $CoCl_2$ concentrations

The intestine FRAP levels have declined significantly after 3 days of acute $CoCl_2$ exposure in a concentration-dependent manner (50 and 500µgL^{-1} $CoCl_2$) with (-82% and -94%) when compared to controls fish (Figure 2a).

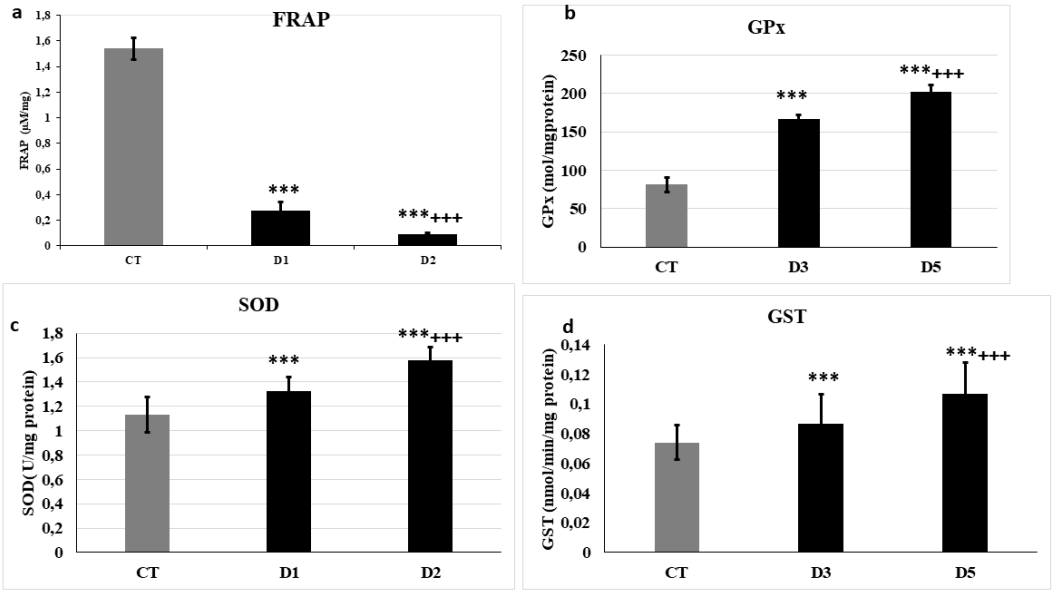

Figure 2. FRAP (a), GPx (b), SOD (c) and GST (d) levels revealed in control and treated *C. carpio* intestine with acute concentrations of $CoCl_2$ during 3 days

Results are expressed as means ± SD (n=9 fish)
CT: 0µg/L $CoCl_2$; D1: 50µg$L^{-1}$$CoCl_2$; D2: 50µg$L^{1}$$CoCl_2$ $CoCl_2$
$CoCl_2$ groups VS controls: *$p < 0.05$;**$p < 0.01$; *** $p < 0.001$.
D2$CoCl_2$ groups VS D2$CoCl_2$ groups: +$p < 0.05$; ++$p < 0.01$; +++ $p < 0.001$.

Assessment of GPx activity in intestine C. carpio treated by acute $CoCl_2$ concentrations

Our experiment has shown significant increases in GPx activity for all cobalt treated carps (50 and 500µgL^{-1} $CoCL_2$) with (+105 and +148%, respectively) when compared to control fish (Figure 2b).

Assessment of SOD activity in intestine C. carpio treated by acute $CoCl_2$ concentrations

Significant elevation in SOD activity was recorder for carps treated by ($50\mu gL^{-1}CoCL_2$, (+ 17%)) and by ($500\mu gL^{-1}CoCL_2$, (+40%)) when compared to control animals (Figure 2c).

Assessment of GST activity in intestine C. carpio treated by acute $CoCl_2$ concentrations

Our result showed significant increases in GST levels for all treated animals with doses dependent manner (50 and $500\mu gL^{-1}CoCL_2$) expressed by (+17 and+44% respectively) when compared to carp controls (Figure 2d).

DISCUSSION

Freshwater as *Cyprinus carpio* was characterized by a great capacity to accumulate an important amounts of trace elements from different ways such as water, sediment, and food chain organisms (Eimers et al., 2001). Among these trace element, we cited cobalt which represent an essential nutrient for animals functions. However, when cobalt exceed the permissible limit concentration, it became toxic for several organisms. Cobalt can be infiltrated into the body through gills, and digestive tract (Dallinger et al., 1987; Pourang, 1995). Our study is the first investigation assessing the *Cyprinus carpio* intestine responses following exposure to acute concentrations of chloride cobalt (0(CT), 50(D1) and 500(D2) $\mu g/LCoCl_2$) during 3 days. Using calcium ion channels, cobalt enter in intracellular part. Its excessive accumulation generate an over-production of ROS inducing therefore disruption of multiple cellular functions (Lushchak, 2008). Our experiment revealed that the toxicity from acute $CoCl_2$ exposure is objectified by installation of oxidative stress in intestine cell of common carp. That suggestion was confirmed in current work by the increases of hydrogen peroxide H_2O_2 in D1 and D2 Co-treated groups which advise a dysfunction of the mitochondrion respiration chain. According to the literature, and through the Fenton/Haber-Weiss pathway, H_2O_2 can react with free iron ($Fe2^{+}$) and generate hydroxyl ($HO^{\bullet}$). The previous compounds constitute key elements in the enhancement of the lipid peroxidation process leading to the initiation of the oxidation polyunsaturated chain (Gueraud et al., 2010; Ayala et al., 2014). Malondialdehyde (MDA) was considered as better biomarker of lipid damage in aquatic organisms, represented the final products of lipid peroxidation chain and provided a good result of the lipid degradation. In fact, the lipid peroxidation reactions developed through free radicals' attack, induced an alteration of intestine membrane integrity (Yin et al., 2011). Thus, a significant increase of MDA levels in all carps intestine treated by acute $CoCl_2$ concentrations confirmed therefore the previous suggestions. Our results are in agreement with those recoded in fish intestine from contaminated locations (Mijosek et al., 2021) and in liver of goldfish exposed to high doses of cobalt (Kubrak et al., 2011).

Furthermore, the generation of ROS following cobalt toxicity constituted a principal consequence of protein damage. In biological cell, protein carbonyls represented the final product of ROS-induced protein oxidation (Shacter, 2000). Our treatment showed significant raise in intestine PCO levels for all $CoCl_2$ treated groups pointing the disruption of protein structure, function and therefore change in natural activity of carp intestine. Like increases were revealed only in kidney of goldfish exposed to the highest $CoCl_2$ concentration, while, no change observed in brain and liver (Kubrak et al., 2011).

Following acute $CoCl_2$ exposure, CO^{2+} reacts directly with H_2O_2 in a Fenton-type reaction which generate excessively ROS production causing accordingly probable perturbations of the total antioxidant power (Ferric Reducing Power (FRAP) (Valko et al., 2005; Lushchak, 2011b). FRAP constitute a novel method to assess the "antioxidants power". It able to measure the antioxidant potential reaction when the ferric tripyridyltriazine [Fe^{3+}-TPTZ] complex is reduced to the ferrous (Fe^{II}) form (Benzie and Strain 1996). In our study, the intestine FRAP levels in *C. carpio* treated with acute $CoCl_2$ concentrations decreased considerably. The disorder of total antioxidant capacity in our experiment constitute among

result of harmful effects of cobalt toxicity. Similar results were recorded by Banaee et al. (2013) and Mozhdeganloo et al. (2015).

Aquatic organisms involved many intracellular antioxidant systems in order to overcome the overproduction of ROS and the disruption of redox homeostasis and therefore to restore normal metabolism and functions of cells. Among these antioxidant system, we cited SOD, GPx and GST assays which were assessment in our experiment (Matés et al., 1999). Superoxide dismutase (SOD) as the better enzymes in the antioxidant defense system, which was involved in the protection of organisms from the oxidative stress through the detoxification mechanism in cytosilic cell part (Matés et al., 1999). The Glutathione peroxidase activity (GPx) is considered as a selenium-rich enzyme participate in the overcome of the excessive production of free radicals. Via glutathione (GSH) as a cofactor, the GPx contributed in cells protection against oxidative stress and lipid peroxidation (41). Glutathione S-transferase (GST) enzymes constituted a better biomarker in assessment of xenobiotics impacts and in highlight of the potential of cell detoxification. Our result revealed significant increases in all antioxidants enzyme system for all $CoCL_2$ treated groups. In fact, that activation of antioxidant systems seems to be a normal cell reaction in order to repair a normal function of carp intestine cells through the reduction of the oxidative damages caused by ROS over-production. Similar results were in agreement with those signaled by Kubrak et al. (2011) and Greani et al. (2017).

Non protein-SH (NPSH) and glutathione (GSH) represented the non-enzymatic antioxidant defense systems, which were determined as the endogenous sulphydryls (SH) compounds. GSH and NPSH are involved by cell mainly in a direct reactive free-radical scavenger (Roméo et al., 2006). Significant increases of NPSH levels recorded in all $CoCl_2$ treatment groups. While, GSH levels increased considerably in intestine treated with $500\mu gL^{-1}$ $CoCl_2$. That activation of non-enzymatic antioxidant system suggested the probably adaptive response of *C. carpio* against the oxidative stress produced by cobalt toxicity. An opposite result was observed in Prussian carp intestine collected from natural contaminated environments (Mijosek et al., 2021).

The metal toxicity caused the MT induction in several marine organisms. It used as appropriate metal-pollution tools (Amiard et al., 2006). Using to its sulfhydryl groups (-SH), the MT is involved in major detoxification processes and play a key role in protective activity (Amiard et al., 2006; Davis & Cousins, 2000). A significant induction of MT's was revealed in all $CoCL_2$ treated groups during 3 days. Furthermore, that activation of MT's was probably elucidate by the high capacity of intestine tissue to accumulate metals and to detoxify the excess of ROS production induced by the harmful effects of acute cobalt exposure (references). Our results are in agreement with other studies focused on carp tissues response following metal exposure (Mijosek et al., 2021).

CONCLUSIONS

Our paper is the first data elucidate the evaluation of the toxic effects of acute cobalt chloride exposure on stress biomarkers response of *Cyprinus carpio* intestine. After 3 days of acute $CoCl_2$ exposure, high H_2O_2 and MDA and PCO levels suggested a great lipid peroxidation and protein oxidation. Co^{2+} pro-oxidant induce the over- production of ROS in intestine cell causing therefore the disruption of the total intestine antioxidant capacity revealed by decrease of FRAP levels. Significant increase of SOD, GPx and GST activities and GSH and NPSH levels were recorded in our treatment, supporting the activation of enzymatic and non-enzymatic defense systems in order to maintain the redox homeostasis of intestine cell. An induction of MT's constitute a good response of carp intestine against cobalt toxicity as a consequence to overcome the excess of ROS production. All response of stress biomarkers of carp intestine are involved in activation of defense systems supporting the adaptation of cell to

oxidative stress. Our paper provides in the first time the use of intestine as good target organs for metal exposure.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

RESEARCH DATA POLICY

No datasets in a repository and no supplement data.

ABBREVIATION

FRAP, Ferric Reducing Power; GPx, Glutathione peroxidase; GSH, Glutathione reduced; MDA, Malondialdehyde; MT, Metallothionein; NPSH, Non-protein SH; PCO, Protein carbonyl; SOD, Superoxide dismutase.

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