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Exposure to Sub-Lethal Concentration of Zinc Sulphate Drastically Affects the Hematological and Serum Biochemical Profile of Grass Carp (*Ctenopharyngodon idella*)

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Authors' Contribution
FI had designed and supervised this study. SA, BA, AA, MI, MS and ZUR conducted the lab experiments and collected blood from fish. MA, AR, NN and SA performed the complete blood count and serum analysis. AA performed statistical analysis. FI edited and finalized the manuscript. All authors contributed to the writing of manuscript and approved the final version for submission.

Key words
***Ctenopharyngodon idella*, $ZnSo_4$, Hematology, Serum biochemistry, LC 50**

ABSTRACT

Zinc Sulphate ($ZnSO_4$) is a common component of pesticides that are extensively used in Pakistan. These pesticides, directly or indirectly, end up in water bodies but their effects on aquatic fauna is least explored. Following the determination of 96h LC_{50} of $ZnSO_4$ for *Ctenopharyngodon idella,* fish were exposed to sub lethal concentration of $ZnSO_4$ (38 mg L^{-1}) under short (2, 4, 6 days) and long term (8, 16, 32 days) experimental conditions to report its effect on the hematological and serum biochemical profile. The acute toxicity test revealed that 96hr LC_{50} of $ZnSO_4$ for *C. Idella* was 75 mg L^{-1}. Data Analysis indicated that $ZnSO_4$ had significantly reduced the red and white blood cell count and mean corpuscular volume in 2 days, packed cell volume in 4 days and white blood cell count in 6 days $ZnSO_4$ treated *C. Idella*. Under long term exposure conditions, packed cell volume, red blood cell count, white blood cell count and mean corpuscular volume of *C. Idella* were significantly reduced upon 16 days treatment with $ZnSO_4$. Severe effects of $ZnSO_4$ exposure on serum of *C. idella* was observed under both short- and long-term experimental conditions. Decreased serum protein and increased aspartate transaminase levels were the hallmark of $ZnSO_4$ exposure to *C. idella* under short- and long-term experimental conditions. In conclusion, this study underscores the profound impact of exposure to sub-lethal concentrations of ZnSO4 on the complete blood count and serum parameters of *C. idella*. Furthermore, the pronounced effects were particularly evident under prolonged experimental conditions. The findings contribute to our understanding of the potential ecological implications of ZnSO4 contamination in aquatic ecosystems, offering valuable insights for both scientific research and environmental management strategies.

INTRODUCTION

Water pollution stands as one of the most pressing environmental challenges of our era. The influx of pollutants originating from diverse origins into aquatic ecosystems has placed numerous freshwater habitats at risk, subjecting them to elevated concentrations of hazardous substances (Misra *et al*., 2005; Chebbi and David, 2010). Pollutants that are mainly released from effluents discharged from industries, sewage treatment plants and drainage from urban and agricultural areas into aquatic environment are prominent source of water pollution. These pollutants cause serious damage to aquatic life (Karbassi *et al*., 2006; Azeem *et al*., 2023).

Industrial development in Pakistan during past few decades produced many industrial zones at major cities producing huge amount of effluents that are drained untreated into nearby rivers like Ravi (Ahmed *et al*., 2011; Riaz ul Haq *et al*., 2018). Another source of environmental deterioration and aquatic pollution in Pakistan is pest control programme which is a routine practice, on large scale, by applying synthetic, organic, non biodegradable

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pesticides. At present, there are more than 200 types of organic pesticides which are available in market containing various heavy metals such as iron, copper, chromium, cadmium, zinc, lead, nickel, and manganese as active ingredients (Malik *et al*., 2010).

Zinc plays a crucial role in supporting the function of structural, regulatory, and catalytic proteins that are essential for the normal physiological processes, growth, and developmental stages across all animal species (Eide, 2006; Maret and Krel, 2007). Elevated levels of zinc in aquatic systems can be due to liquid effluent discharge, atmosphere deposition, the leaching of domestic sewage, metal bearing minerals, insecticides and galvanizing processes (Fiaz *et al*., 2015). As fish are constantly exposed to pollutants in contaminated water, they could be used as excellent biological markers of heavy metals in aquatic ecosystem as they are present at the end of food chain (Qadir *et al*., 2014). Zinc exerts adverse effect in fish such as structural damage which affects the growth, development and survival of fish. Sub lethal levels of zinc adversely affect hatchability, survival and hematological parameters of fish (Eide, 2006).

The current investigation aimed to determine the LC_{50} values of $ZnSO_4$, a prevalent element in pesticides and industrial waste, for the economically significant carp species in the region, *Ctenopharyngodon idella*. Additionally, this study sought to elucidate the impact of sub lethal $ZnSO_4$ concentrations on the hematology and serum biochemical profile of this fish species, considering both short-term and prolonged experimental exposure scenarios.

MATERIALS AND METHODS

Specimen collection

Two hundred and fifty fingerlings of freshwater Cyprinid fish, *Ctenopharyngodon idella* were purchased from Fish Seed Hatchery, Mian Channu (district Khanewal) and transported to Fisheries Laboratory, Institute of Pure and Applied Biology at Bahauddin Zakariya University, Multan, (Punjab), Pakistan and acclimatized for 15 days to laboratory conditions.

LC_{50} determination

For determination of 96 h LC_{50} values, group of 16 juveniles *C. idella* were exposed to one of the five concentrations; 60, 80, 90, 110, 120 mg L^{-1} of $ZnSO_4$. Fish mortality was observed after 24, 48, 72 and 96 h. LC_{50} values were calculated following Iqbal *et al*. (2005).

Experimental design

Experiment was divided into short and long term phases. During short term experiments, three treatment groups (each having 20 fish) were exposed to sub lethal concentration of 38 mg L^{-1} $ZnSo_4$ for 2, 4 and 6 days respectively while in long term experiments *C. idella* were exposed to above mentioned dose for 8, 16 and 32 days. Separate control groups were used for each treatment. All fish were fed with ordinary fish diet used in fish farms (24 % protein). All experiments were carried out in semi-static systems with water renewal after every 24 h. Temperature, pH and oxygen concentration of water were maintained throughout the experimental duration following Ali *et al*. (2006).

At the end of each experiment, 1-2 ml of blood sample was collected from each fish by making a cardiac/ caudal puncture. Part of the blood was directly used to study hematological parameters while remaining blood was preserved in viols containing 0.5M EDTA for the determination of biochemical parameters.

Hematological and serum biochemical analysis

Hematological parameters, blood glucose level, mean corpuscular volume, packed cell volume, total red and white blood cell count and serum biochemical parameters, cholesterol, aspartate transaminase (AST), alanine transaminase (ALT), high density lipoprotein (HDL), total protein (TP) and triglycerides (TG), were determined in treated and untreated fish blood samples by using Hitachi 902 Automatic Analyzer (Japan).

Statistical analysis

All data are presented as Mean ± Standard Deviation. The statistical analysis was performed using the Minitab software (version 19) to analyze the results. A two-sample t-test was employed to assess the differences in various hematology and serum biochemical parameters between ZnSO4-treated and untreated *C. idella*, considering both short-term and long-term experimental conditions.

RESULTS

LC_{50} value of $ZnSO_4$ against Ctenopharyngodon idella

The 96 h LC_{50} value for *Ctenopharyngodon idella* treated with $ZnSO_4$ was 75 mg L^{-1}. All fish survived at 38 mg L^{-1} $ZnSO_4$ while 100% mortality was observed at 120 mg L^{-1} $ZnSO_4$.

Hematological parameters

Analysis of our results indicated that during short term experimental phase, *Ctenopharyngodon idella* treated with $ZnSO_4$ for two days had elevated blood glucose levels while reduced total red blood cells (TRBC), white blood cells (TWBC) and mean cell volume (MCV) than their untreated control group (Table I). In *C. idella* exposed to $ZnSO_4$ for 4 and 6 days, packed cell volume

(PCV) (P= 0.05) and TRBC were the only parameters that were significantly reduced as compared to their respective control groups (Table I).

Table I. Effect of $ZnSO_4$ administered for 2, 4 and 6 days on blood glucose content and hematological and biochemical parameters of *Ctenopharyngodon idella.* Data is expressed as Mean ± Standard deviation.

Parameters	$ZnSO_4$ treatment for 2 days		$ZnSO_4$ treatment for 4 days		$ZnSO_4$ treatment for 6 days	
	Control (n = 10)	Treated (n = 10)	Control (n = 10)	Treated (n = 10)	Control (n = 10)	Treated (n = 10)
Glucose (mg/dl)	139.9 ± 57.2 *	230 ± 125*	145.7 ± 36.7	137.7 ± 36.7	123 ± 43	138 ± 26
Haematological parameters						
PCV (%)	11.43 ± 3.39	11.8 ± 1.72	11.12 ± 2.12	9.34 ± 1.62*	10.8 ± 4.08	10.6 ± 2.8 *
TRBC ($x10^6 \mu l^{-1}$)	1.28±0.67	0.67 ± 0.34*	1.30±0.16	1.43 ± 0.89*	1.30 ± 0.61	1.35±1.09 *
TWBC ($x10^4 \mu l^{-1}$)	41.44 ± 16.53	21.88 ± 4.09 *	50.19 ± 16.23	51.01 ± 14.22	41.29 ± 10.33	28.85 ± 10.39
MCV (fp)	1.3 ± 0.71	0.79 ± 0.45 *	87.8 ± 24.7	91.3 ± 563	1.6 ± 1.18	1.32 ± 0.8
Biochemical parameters						
TP (g/dl)	4.30 ± 5.07	1.57 ± 1.52	4.23 ± 0.56	1.35 ± 0.07 ***	4.84 ± 0.72	1.38 ± 0.65
TG (mg/dl)	4.84 ± 0.72	1.38 ± 0.65	214 ± 60.3	203 ± 49.6	392 ± 85.3	366 ± 164
Cholesterol (mg/dl)	378 ± 131	262 ± 272 **	322 ± 82	157 ± 31.9 *	274 ± 76	106 ± 22 *
HDL (mg/dl)	44.0 ± 13.2	53.50 ± 1.87	44.6 ± 2.5	33.6 ± 3.52	53.33 ± 0.81	44.6 ± 2.9
LDL (mg/dl)	406 ± 135	291 ± 234 *	391.1 ± 68.8	215 ± 4.91 **	392 ± 80	228 ± 17 **
AST (IU L^{-1})	253 ± 87	228 ± 86	225 ± 111	167 ± 82	250 ± 63	165 ± 75 *
ALT (IU L^{-1})	241 ± 141	332 ± 104 **	250 ± 25	176 ± 106 **	211 ± 94	210 ± 67

PCV, packed cell volume; TRBC, total red blood cell count; TWBC, total white blood cell count; MCV, mean corpuscular volume; TP, total protein; TG, triglycerides; HDL, high density lipoproteins; LDL, low density lipoproteins; AST, aspartate transaminase; ALT, alanine transaminase.
P > 0.05 = Non significant; P < 0.05 = least significant (*); P < 0.01 = highly significant (**).

Table II. Effect of $ZnSO_4$ administered for 8, 16 and 32 days on blood glucose level, hematological and biochemical parameters of *C. idella.* Data is expressed as Mean±Standard deviation. P-value indicates statistical results of two sample t - test.

Parameter	$ZnSO_4$ treatment for 8 days		$ZnSO_4$ treatment for 16 days		$ZnSO_4$ treatment for 32 days	
	Control (n= 10)	Treated (n= 10)	Control (n= 10)	Treated (n= 10)	Control (n= 10)	Treated (n= 10)
Glucose (mg/dl)	168 ± 54	152 ± 70	89.6 ± 29.4	108 ± 33	139.8 ± 63.6	142 ± 62.6
Hematological parameters						
PCV (%)	10.7 ± 1.77	10.6 ± 2.6	11.76 ± 1.79	8.48 ± 2.65*	13.4 ± 3.69)	11.4 ± 4.56
TRBC ($x10^6 \mu l^{-1}$)	1.16 ± 0.05	1.43 ± 0.94	1.89± 0.94	0.84 ± 0.37**	1.29± 0.21	1.47± 0.29
TWBC ($x10^4 \mu l^{-1}$)	41.11 ± 9.24	33.99 ± 11.67	56.41±16.11	29.05±8.82***	59.70±26.83	53.81 ± 21.60
MCV (fp)	1.09 ± 0.62	1.33 ± 1.23	2.17 ± 1.05	0.71 ± 0.42**	1.7 ± 0.36	1.69 ± 0.75
Biochemical parameters						
TP (g/dl)	1.4 ± 0.48	1.0 ± 0.83	1.48 ± 0.6	1.16 ± 0.38	0.21± 0.13	0.14± 0.07
TG (mg/dl)	146 ± 4.5	393 ± 205*	146 ± 4.24	256 ± 206	236.5±73	241 ± 64.8
Cholesterol (mg/dl)	373 ± 86.2	569 ± 347	373 ± 86	569 ± 347	441 ± 133	167 ± 27***
HDL (mg/dl)	44.5 ± 13.3	33.5 ± 8.29*	44 ± 13	33.5 ±8.5*	45.5 ± 17.5	33.2 ± 22.8***
LDL (mg/dl)	300 ± 106	420 ± 140**	338 ± 168	434 ± 194	376 ± 144	116 ± 389***
AST (IU L^{-1})	565 ± 215	757 ± 386	558 ± 314	588 ± 211	561 ± 13	650 ± 275***
ALT (IU L^{-1})	190 ± 57	294 ± 150	171 ± 189	361.5 ± 39**	210 ± 44	341.3 ± 54***

For abbreviations see Table I. P > 0.05 = Non significant; P < 0.05 = least significant (*); P < 0.01 = Significant (**); P < 0.001 = Highly significant (***).

In long term experimental phase, fish exposed to $ZnSO_4$ for 16 days had significantly reduced PCV, TRBC, TWBC and MCV than $ZnSO_4$ untreated fish. All other studied parameters remained unaffected for *C. idella* exposed to the toxicant for 8 and 32 days (Table II).

Serum biochemical parameters

Data analysis indicated that during short term experimental phase, *C. idella* exposed to $ZnSO_4$ had reduced serum cholesterol, LDL while elevated ALT as compared to their untreated control group. In 4 days treatment group, total protein, ALT, cholesterol and LDL were significantly reduced in $ZnSO_4$ treated fish as compared to their untreated control group. While total protein, cholesterol and LDL levels were significantly reduced in the serum of *C. idella* treated with $ZnSO_4$ for 6 days as compared to their untreated control group (Table I).

During long term experimental phase, serum TG and LDL (P= 0.007) were significantly elevated while HDL levels were significantly reduced in *C. idella* exposed to $ZnSO_4$ for 8 days than their untreated control group. In 16 days experimental group, HDL was reduced while ALT was elevated in serum than untreated *C. idella*. Cholesterol (P < 0.001), HDL and LDL were reduced while ALT and AST levels in serum were significantly elevated in *C. idella* exposed to $ZnSO_4$ for 32 days than their untreated control group (Table II).

DISCUSSION

The elevated presence of zinc in freshwater ecosystems has been associated with varying degrees of toxicity on aquatic organisms, including fish. Exposure of fish to zinc can induce detrimental effects on organ functionality and lead to behavioral, physiological, and biochemical alterations (Heath, 1995). In freshwater fish, the uptake of zinc from water occurs mainly through gills by calcium mediated pathway, while intestinal zinc uptake take place mainly by carrier mediated pathway (Fiaz *et al*., 2015). Once these toxic substances enter body, they damage and alter the fish physiology (Khan and Badroo, 2021). Many previous reports have confirmed the toxic effect of zinc on the blood profile of several fish species (Joshi, 2011; Velisek *et al*., 2006). In the present study fresh fish water fish grass carp was exposed $ZnSO_4$ to observe the effect of toxicant on hematology and serum biochemistry under short and long term experimental conditions.

Chronic Zinc exposure to fish is known to cause a variety of histopathological, behavioral, biochemical and physiological changes including loss of appetite, reduced growth, decreased aerobic scope and mortality as it is known for its strong action on biological tissues (Khan and Badroo, 2021). Our results indicated that only few of the studied hematological parameters were affected upon exposure to sub lethal concentrations of $ZnSO_4$ under short and long term experimental conditions. TRBC, TWBC and MCV values were disturbed when compared between *C. idella* exposed to 38 mg L^{-1} of $ZnSO_4$ for 2 (Table I) or 16 days (Table II) with their untreated control groups. Control group had higher values for all these parameters than $ZnSO_4$ treatments indicating severe effect of $ZnSO_4$ on hematological profile of *C. Idella.* Similar results with significant reduction of RBCs in fishes exposed to different heavy metals have been reported previously by Goel *et al*. (1985) and Goel and Sharma (1987). The PCV was the other hematological parameter that was significantly reduced in *C .idella* exposed to $ZnSO_4$ for 4 (Table I) and 16 (Table II) days as compared to their respective control groups indicating immune depression due to Zinc intoxication. The observed reduction in hematocrit values in the fish could also be attributed to the lyses of erythrocytes. Similar results were also reported by Samprath *et al*. (1993) and Musa and Omoregie (1999) when they exposed fish to Ekalus Malachite green laboratory conditions. In *C. idella* exposed to $ZnSO_4$ for two days, a significant elevation in blood glucose levels was observed compared to their control group (Table I). Goss and Wood (1988) had documented that the hyperglycemic condition in metal treated fish is probably an effort to provide additional energy required during times of high metabolic activities such as fight or flight response in order to counter the metal toxicity.

The exposure to heavy metals significantly impacted the serum biochemical profile of *C. idella*, with pronounced effects observed on the liver as evidenced by the elevated levels of ALT and AST enzymes associated with liver function (Tables I and II). Our findings are in line with Srivastava and Prakash (2018) as they had reported significant increase in glucose, lipids (TG and cholesterol), serum phosphatases (acid and alkaline phosphatases) and serum transaminases (SGOT and SGPT) where as a decrease in bilirubin and protein level in *Clarias Batrachus* exposed to various concentrations of $ZnSO_4$. Humtosoe *et al*. (2007) had also reported that change in ALT and AST activities were indicator of disturbance in the structure and integrity of organelles, like endoplasmic reticulum and transport system and Marie (1994) had reported that the disturbed level of ALT and AST in fish could be due to high accumulation of metal in fish, disturbing the normal liver physiology.

The cholesterol concentration decreased significantly

in all short-term experimental treatments (Table I) and 32 day $ZnSO_4$ treated group during long term experimental phase (Table II). Lowering of cholesterol was probably due to an increase in lipid utilization to cope with additional energy requirements under stress conditions (Sirvastava *et al*., 2002). Sindhe *et al*. (2002) had reported that alterations in cholesterol content might be due to disturbed steroidogenesis due to heavy metal exposure. LDL concentrations decreased significantly in $ZnSO_4$ treatments in short term experiments (Table I) while it significantly increased in 8 days and decreased in 32 days treated groups in long term experimental phase (Table II). These findings align with the observations made by Dhanpakian and Ramasamy (2001) and Desi *et al*. (2002), who reported that heavy metal exposure could lead to alterations in hepatic protein content due to lysosomal enzyme activity disruption, thereby affecting protein metabolism in fish.

It has been an established fact that serum proteins are highly sensitive to heavy metals and their levels are commonly used as indicator of heavy metal poisoning (Srivastava and Prakash, 2018). In the present study, we have observed a $ZnSO_4$ induced significant decline in the serum proteins of *C. idella* under short term experimental conditions (Table I). This hypoproteinemia can be attributed to the enhanced proteolysis that is offering a physiological mechanism to provide energy to cope up with the stressful situation caused by the metal toxicity. This reduction in serum protein level in metal exposed fish is probably due to enhanced use of proteins to build up new cells or enzymes to reduce the stress. Thus, it can be proposed that declined serum protein content are due to the increased cost of homeostasis, tissue repair and detoxification during stress (Shalaby *et al*., 2006).

In summary, our findings illustrate that ZnSO4 has a pronounced impact on the hematological and serum biochemical characteristics of *C. idella*, both in the short and long term experimental scenarios. These results underscore the direct deleterious effects of heavy metals on the physiological processes of *C. idella*. Importantly, this influence extends indirectly to humans as this fish species is a common component of the human diet. As $ZnSO_4$ is commonly introduced into aquatic environments through sewage discharge and due to its presence in the pesticides, it is imperative that immediate measures must be taken to mitigate the various forms of water pollution to safeguard the aquatic ecosystems.

DECLARATIONS

Funding

No specific funding was involved in this project.

Availability of data and material

All the data associated with this project is presented in this manuscript.

Consent to participate

Informed consent was obtained from livestock owners before including their animals in this study.

Statement of conflict of interest

The authors have declared no conflict of interest.

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