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Subacute toxicity study of calcium zincate nanoparticle exposed to common carp *Cyprinus carpio*

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Abstract
The current investigation aim was to study the hematological and serum biochemical changes under sublethal calcium zincate nanoparticle ($CaZnO_2$ NP) concentration in the fresh water fish *Cyprinus carpio*. Fish were exposed to two sublethal concentrations 3.53 and 7.05ppm of $CaZnO_2$ NP for 7, 14 and 21 days. Simultaneously control was maintained without nanoparticle. Fish were sampled at the end of each interval to measure the hematology and biochemical variables such as red blood cells (RBC), white blood cells (WBC), hemoglobin (Hb), glucose, total protein (TP), cholesterol, creatinine, alanine aminotransferase (ALT/GPT) and aspartate aminotransferase (AST/GOT). From the results it is inferred that $CaZnO_2$ NPs affect the hematology and biochemical parameters of fish *Cyprinus carpio*.

Keywords: toxicity, calcium zincate, nanoparticles, biochemical alteration, *Cyprinus carpio*

1. Introduction
Nano sized materials are increasingly used in the fields of industry, science, pharmacy, medicine, electronics, communication and consumer products. The "nano" is derived from the Greek word "nanos" meaning "dwarf" [1]. A nanomaterial (NM) defined as a substance with at least one dimension <100 nm in length. There are numerous nano-sized materials in our life. They can take different forms such as tubes, rods, wires or spheres. Depending on their origin, they can be categorized as either engineered or incidental NMs. Engineered nanoparticles (NPs) are particles generated to use the size-related properties inherent in the nanoscale (e.g. conductivity, spectral properties, biodistribution). Incidental NPs are defined as particles either from unintended anthropogenic sources (e.g. combustion derived) or of natural origin (e.g., particles generated in forest fires) [2].
Nanotoxicology is the study of the toxicity of NMs. It has emerged only recently, years after the beginning of nanotechnology that is considered one of the key technologies of the 21st century, when numerous NMs had already been introduced into some industrial processes and consumer products. Donaldson *et al.* quoted "discipline of nanotoxicology would make an important contribution to the development of sustainable and safe nanotechnology" [3]. Growing concerns about the nanotoxicology were derived from prior experiences with air pollution and asbestos [4, 5].
Among the various types of nanoparticles, Zinc Oxide nanoparticles (ZONPs) are widely produced and applied in many products including sunscreen, waste water treatment, and environmental remediation [6]. Increased production and aplication of NPs have increased the exposure chances of humans and ecosystems to NPs. The toxic action of metal and metal oxide NPs can potentially involve at least three distinct mechanisms [7]. First, particles may release toxic substances into exposure media, e.g. free Zn+ ions from zinc particles. Second, surface interactions with media may produce toxic substances, e.g. chemical radicals or reactive oxygen species (ROS). Third, particle or their surfaces may interact directly with, and disrupt biological targets, e.g. carbon nanotube interaction with membranes or intercalation with DNA [8].
Ferry *et al.* (2009) first reported that NPs could pass from the water column to the aquatic food web and accumulated by the organisms through internal or external exposure routes and it is necessary to understand the biological behavior of nZnO in the aquatic environment [9]. Because ZnO NPs partially dissolve in water exposures in aquatic systems are expected to involve both soluble and particulate species, suggesting that these three mechanisms of toxic action were tenable for ZnO. Solubilized Zn+ from ZnO NPs has proven to contribute substantially to the cytotoxicity of these NPs [10].
Another unique feature of NP toxicity is the formation of a biomolecular layer on their surface, referred to as "Protein Corona" (PC). Via Vander Waals, electrostatic, hydrogen bonding and hydrophilic/hydrophobic interactions, biomolecules such as lipids, sugars and especially Proteins, will bind to the surface of NPs once in contact with biological fluids [11, 12]. The composition of this PC is important because research has shown it can affect agglomeration, toxicokinetics, signaling, and ultimately, toxicity [13]. For instance, negatively charged NPs will attract positively charged biomolecules, which in turn will increase interactions with the negatively charged cell membrane [14]. This also has implications for interpreting toxicity results across different animal models and exposure scenarios [15].
Hematological and serum biochemical studies furnishes an index of physiological changes in fish [16] and the fish blood acts an impressive tool for detection of alterations in the tested organism [17, 18]. The most common hematological variables

measured during stress included Red and White blood cells count, hemoglobin content and red blood cells indices [19]. Hematological parameters are an indicator of water balance, nutritional status and overall health condition of fish [20]. For this reason hematological variables have been used as indicators of fish health status in a number of fish species to detect physiological changes as a result of stress condition such as exposure to pollutants, hypoxia, transportation, anesthetic and acclimation [21]. Measurement of serum biochemical parameters can be especially useful to help identify target organs of toxicity as well as the general health status of animals and has been advocated to provide early warning of potentially damaging changes in stressed organisms [22, 23].

Fish *Cyprinus carpio* is a freshwater teleost, one of the major edible fish and very sensitive to pollutants. Therefore it is necessary to evaluate the toxicity of nanoparticles on fish species. Hence the present investigation is aimed to study the subacute of $CaZnO_2$ NP on certain hematological and biochemical parameters in a freshwater fish *C. carpio*. Moreover, the selected parameters can be used as biomarkers in environmental monitoring of nanoparticle contamination in aquatic ecosystem.

2. Materials and methods

2.1 Experimental fish

The fresh water fish *Cyprinus carpio* (18.5 ± 2.0g) were procured from the State Fisheries Department, Bhadra Reservoir Project, Bhadravati and acclimatized to laboratory conditions for about twelve days before the commencement of the experiment. During acclimatization, fish were fed with CP 9932 herbivorous fish feed once a day. $CaZnO_2$ NP was dispersed in distilled water and used in this study. A preliminary study was then conducted to determine the 96-h $LC^{[50]}$ of $CaZnO_2$ NP for common carp according to Finney's Probit Analysis and it was 35.251 mg/l [24, 25]. The sublethal test was performed by semi static bioassay method. The acclimatized fishes were divided into 3 groups. The first group of fishes served as control (0.0ppm), Group II were exposed to one tenth of $LC^{[50]}$ (3.53ppm) concentration and Group III were exposed to one fifth of LC_{50} (7.05ppm) concentration of NP. The experiment for each concentration of NP was performed for a period of twenty one days. Blood sample was collected from control and exposure groups at 7, 14 and 21 days. No mortality was observed during the experiment.

2.2 Blood sample collection

Blood was collected by direct heart puncturing using sterile disposable plastic syringe. The syringe was flushed with EDTA. The whole blood was used for the estimation of hematological parameters. For biochemical estimations, blood was collected from each fish using disposable syringe without EDTA and pooled into the dry tube which was allowed to coagulate and later centrifuged and serum was collected.

2.3 Hematology and Serum biochemical studies

Whole blood was used for the estimation of RBC, WBC and Hb. RBC and WBC counts were calculated using haemocytometer [26]. Hb content was estimated by cyanmethaemoglobin method [27]. Serum glucose was quantified by GOD-POD method [28]. The serum total protein was measured by Biuret method [29]. Total cholesterol was estimated by COD-PAP enzymatic test [30]. The creatinine level was determined according to Jaffe's method [31]. The serum activities of alanine aminotransferase (ALT/GPT) and aspartate aminotransferase (AST/GOT) were determined according to IFCC method [32]. All the biochemical estimation was measured using spectrophotometric method (prietest-Touch Semi – Automated Chemistry Analyzer (Indian) with reagents provided in standard analyses kits (Robonik India Pvt. Ltd.).

2.4 Statistical analysis

Statistical analyses were performed using the computer program SPSS ver. 16.0. Differences between the control and treated sub lethal exposure groups were tested using one way analysis of variance (ANOVA). A P value <0.05 was considered to be significant, while a P value <0.01 was considered to be highly significant.

3. Results & discussion

Effect of nanoparticle exposure to hematology and serum biochemical studies are summarized in Table 1 and Figs. 1 to 9. Blood is a pathophysiological reflector of the whole body, and blood parameters are important in the diagnosis of the structural and functional statuses of animals exposed to toxicants [33]. The value of total RBC and Hb count decreased significantly in 3.53 mg/L, 7.05mg/L NPs at 14 and 21 days in relation to the control groups (Fig 1&2). In both the treatments (Group II and III), a maximum percent decrease of RBC (-38.80%) was noted at the end of 21day and Hb (-24.10%) at 14day Fish exposed to 7.03mg/l. Panigrahi and Misra evidenced reduced hemoglobin and red blood cell count in fish *Anabas scandens* treated with mercury [34]. Reduced RBC and Hb were reported in *Tinca tinca* exposed to lead and mercuric chloride [35]. The decreased hemoglobin concentration represents the reduced supply of adequate oxygen to the tissues and resulted in decline of physical activities [36]. James and Sampath found that the oxygen carrying capacity of blood of Heteropneustes fossilis declined due to the reduction of RBC count and Hb content which reflected on tissue respiration [37]. Oxygen carrying capacity of blood was declined in metal-exposed *O. mossambicus* due to the reduction of RBC count and Hb content [33]. The pollutants were entering into fish via gills which were continuously exposed to ambient waters. This causes hindrance to O_2 absorption through gill surface which was reflected in the reduced O_2 carrying capacity of blood [38]. Similar results were observed in our work as decreased RBC & Hb concentration and as a result there was a reduced O_2 carrying capacity in ZnO nanoparticle exposed fishes. Reduced erythrocyte count and Hb levels were observed due to severe anemia. Decreased Hb provides an indication of the status or size of the erythrocytes and reflects an abnormal or normal cell division during erythropoiesis indicates that the erythrocytes have shrunk, either due to hypoxia or a microcytic anemia [39].

The WBC count was significantly increased ($P < 0.05$) in day 7 and significantly ($p<0.05$) decreased in 7.05mg/L at 14 day (-25.77%) and 21day (-34.83%) compared to control group. Our studies showed reduced leucocyte count in treated groups

of fish at 14 and 21 days (Fig 3). Some researchers reported the decrease in WBCs count in Clarias and Heteroclarias species in response to Zn [40, 41]. Decrease in WBC may attributed to the movement of white blood cells The white blood cells to the infected tissue leaving the circulating blood, to protect the body, resulting in reduced cell number in blood [42]. The decreased in number of white blood cells (leucopenia) may also be the result of bioaccumulation of Zn in different tissues that cause toxicity and effect on cell production from spleen due to an increased level of corticosteroid hormones because these hormones are important for prevention and healing of inflammation [43,44].

Blood glucose has been employed as an indicator to different environmental stresses [45]. In the present study, the Glucose level significantly increased in the highest exposure group (Fig 4). At 14day, the Glucose level of the both the exposure groups significantly increased by 50.18% and 34.16% compared to the control group ($p>0.05$). Thus, Glucose levels in fish are affected by $CaZnO_2$ NPs. However. In the case of other heavy metals, an increase in serum glucose levels was linked to a decrease in glycogen reserves in the liver [46]. The observed results are in agreement with F±rat and Karg±n who reported an increase in serum glucose levels of O. niloticus that were exposed to different concentrations of Zn, Cd, and Zn+ Cd compared with control fish [47]. The increase in the glucose level and vice versa decrement of tissue glycogen in stressed fish make it clear that the glycogen reserves are being used to meet the stress caused. Depleted glycogen levels under heavy metal stress were observed by Bedii and Kenan [48]. This can be attributed to several factors and one of them is the decrease in the specific activity of some enzymes like phosphofructokinase, lactate dehydrogenase and citrate kinase that decrease the capacity of glycolysis that initially enhance glucose breakdown and glycogen formation [49].

An important function of serum proteins is the maintenance of osmotic balance between blood and tissue spaces as well as these proteins are highly sensitive to metal poisoning [50]. The serum protein significantly decreased throughout the experimental period for both the sublethal exposures except for an insignificant increase after 7days at 3.53mg/L of nanoparticle concentration. Highest %decrease (-32.59) was recorded after 14 days of exposure at 7.05mg/L of nanoparticle concentration (Fig 5). The lowering of protein concentration was perhaps accompanied by the glucose increase, to meet the high energy demand necessary to struggle with the stress. Kori-Siakpere and Ubogu reported a decrease in plasma proteins with Zn exposure [42]. Kumar and Banerjee also noticed depletion of glycogen and proteins from the hepatic and muscular tissue of C. batrachus following arsenic exposure [51]. They attributed this decrease to renal excretion, impaired protein synthesis or due to liver disorder. On the other hand, this decrease could result from the breakdown of protein into amino acids then into nitrogen and other elementary molecules [52]. Zutshi *et al.* observed a reduction in serum protein levels in Labeo rohita under stress of pollution from lakes of Bangalore and this might be due to breakdown of proteins and other macromolecules (e.g. to meet the higher energy demand during the prevailing stress), liver cirrhosis, nephrosis or due to alteration in enzymatic activity involved in protein biosynthesis as reported by several studies [53, 54, 55]. Tripathi *et al.* reported a decrease in the protein content in Colisa fasciatus exposed to sub-lethal concentration of zinc sulfate for 30 days [56]. Depletion in total protein after NP exposure may be due to overproduction of ROS within the tissue, which can damage macromolecules as DNA, proteins, lipids, and carbohydrates [57, 58]. Nel *et al.* stated that, in a biological environment, NPs are coated with proteins resulting in a nanoparticle–protein corona that causes a diverse change in the level of proteins [59]. Some researchers reported that the capacity of ZnO NPs to induce DNA damage could affect protein synthesis [60, 61].

Total cholesterol in serum shows an insignificant decrease at an initial exposure periods. Increase was significant ($p<0.05$) after 14 and 21 days at 7.05mg/L nanoparticle concentration. For 3.53mg/L of nanoparticle concentration, significant increase ($p<0.05$) was recorded at 14 days of exposure (Fig 6). Similar trend was observed by Desai *et al.* when freshwater fish *Channa punctatus* exposed by nickel [62]. In an another similar study Jain observed a decrease in the soluble protein, RNA and glycogen contents in the liver and body weight but increased the cholesterol contents when teleost fish H. fossilis exposed to sublethal concentrations of lead nitrate in water solution for short (35 days) and long (120 days) period [63]. Cholesterol concentrations in the serum of nanoparticle exposed fish generally increased when compared to the control value. The concentrations of cholesterol is an essential structural components of membranes and the precursor of all steroid hormones, may increase due to the liver failure causing the release of cholesterol into the blood. Heavy metals are known to have hazardous effects on cell structure, especially on the membranes. Therefore, increase in cholesterol may be the indications of environmental stress [64, 65, 66].

Creatinine level in serum was increased significantly ($p<0.05$) for 3.53mg/L of nanoparticle concentration and highly significant ($p<0.01$) for 7.05mg/L of nanoparticle concentration at 14 and 21 days and highest increase (45.31%) was recorded at 21days for 7.05mg/L of nanoparticle concentration (Fig 7). Kidney functions indicated by creatinine can be used as a rough index of the glomerular filtration rate and kidney dysfunction. Abdel-Tawwab *et al.* found that Zn toxicity in Nile tilapia increased with increasing concentration and time of exposure [67]. This is in accordance with Al-Zahaby *et al.* who found that the exposure of fish to high concentrations of heavy metals led to disintegration of the renal epithelium, displacement of nuclei, shrinkage of glomeruli, breakdown of Bowman's capsule and heavy infiltration by inflammatory cells [68].

Liver function tests (AST and ALT enzymes) are widely used to demonstrate liver function or toxicant induced hepatotoxicity [69, 70]. An increased trend in the SGOT level was observed throughout the experimental period for both the treated groups compared to control. Highest %changes in 14 day were recorded at 7.05mg/L (Fig 8). However SGPT levels in serum also showed increase in both the exposures and highly significant ($p<0.01$) increase was recorded in 7.05mg/L exposure at 14 and 21days (Fig 9). This is in agreement with Nemcsok and Hughes who observed an increase in liver enzyme activities of fish *Oncorhynchus mykiss* exposed to Cu and Vaglio and Landriscina in the case of Sparus aurata exposed to Cd metal [71, 72]. Wu *et al.* recorded an increase of

liver enzyme activities in stressed *Epinephelus areolatus* fish due to hepatic cells injury or increased synthesis of these enzymes by the liver [73]. Kim and Kang observed an increase in serum AST and ALT concentrations in the rock fish, *Sebastes schlegeli* after sub-chronic dietary Cu exposure for 40 days with increasing time and dose. They suggested that, liver damage can result in the liberation of large quantities of enzymes into the blood. Therefore, increases in liver enzyme activities in the serum of heavy metal treated fish are assumed to be a result of liver damage by heavy metals [74]. F± rat and Karg ± n reported an increase in the serum ALT and AST activities in O. niloticus exposed to concentrations of Zn, Cd, and Zn+ Cd compared with controls at 7 and 14 days [47]. Younis *et al.* observed a significant increase in AST and ALT levels in zinc treated *O. niloticus* fish with short and long term sub-lethal exposure which indicates hepatic damage due to zinc accumulation which in turn releases these enzymes into the blood stream [75]. The elevation in the AST and ALT enzymes could be due to a variety of conditions, including hepatopancreatic injury that reflect potential damage to parenchymal cells, muscle, intestinal and hepatic injury [76,77].

4. Tables and figures

Table 1: Hematology and biochemical parameters of *Cyprinus carpio* exposed to sub lethal concentrations of $CaZnO_2$ NP

$CaZnO_2$ (Acetamide)	Exposure Day	Group-I	Group-II		Group-III	
		Control	3.53 mg/L	%Change	7.05 mg/L	%Change
RBC ($Cellx10^6.mm^{-3}$)	7day	2.65±0.37	3.14±0.25	18.49	3.15±0.43	18.68
	14day	3.05±0.42	2.80±0.22	-8.29	2.31±0.31	-24.12
	21day	2.93±0.36	1.97±0.16	-32.74	1.79±0.26	-38.80
WBC ($Cellx10^3.mm^{-3}$)	7day	21.75±1.71	27.25±3.77	25.29	30.75±1.89	41.38
	14day	24.25±2.99	25.00±2.94	3.09	18±2.45	-25.77
	21day	22.25±3.77	16.25±1.89	-26.97	14.5±1.73	-34.83
Hb (g/dl)	7day	9.45±0.67	10.98±1.58	15.61	10.50±1.29	11.11
	14day	10.38±1.25	8.38±0.85	-19.28	7.88±0.63	-24.10
	21day	9.95±0.71	8.05±0.42	-19.10	7.65±1.03	-23.12
Glucose (mg/dl)	7day	66.30±7.12	69.75±4.11	5.20	85.25±10.81	28.58
	14day	70.25±6.85	105.50±15.52	50.18	94.25±10.87	34.16
	21day	69.38±5.12	86.25±13	24.32	92±11.63	32.61
Protein (g/dl)	7day	1.92±0.17	1.98±0.220	3.12	1.57±0.11	-18.10
	14day	2.03±0.13	1.89±0.217	-6.79	1.37±0.20	-32.59
	21day	2.00±0.14	1.61±0.162	-19.38	1.40±0.21	-30.13
Cholesterol (mg/dl)	7day	101.50±15.18	101.75±13.12	0.25	97±10.30	-4.43
	14day	103±12.99	101.25±9.25	-1.70	129.75±13.18	25.97
	21day	92.50±12.58	138.50±24.47	49.73	130.25±20.16	47.30
Creatinine (mg/dl)	7day	1.21±0.19	1.00±0.16	-17.60	0.95±0.12	-21.53
	14day	1.24±0.11	1.6±0.11	28.89	1.65±0.17	33.33
	21day	1.09±0.15	1.57±0.18	43.25	1.59±0.20	45.31
SGOT (U/L)	7day	23.00±2.45	23.25±2.87	1.09	29.25±3.77	27.17
	14day	21.25±2.5	29±2.94	36.47	32.25±4.19	51.76
	21day	22.50±2.94	30.00±3.37	36.36	29.25±3.59	32.95
SGPT (U/L)	7day	11.63±1.25	11.50±1.73	-1.08	12.48±2.09	7.31
	14day	10.75±1.50	14.50±1.29	34.88	19.25±2.87	79.07
	21day	12.50±1.29	18.50±2.08	48.00	20.75±3.59	66.00

Data represent mean ± SD (n = 4)

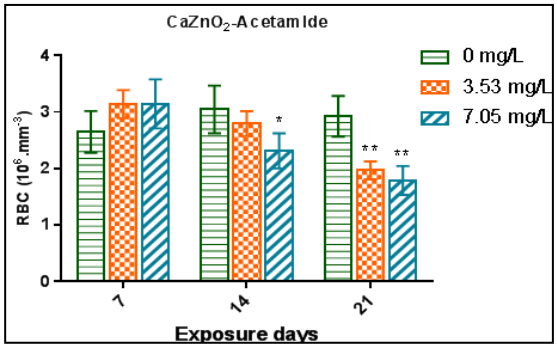

Fig 1: Changes in the RBC count of *C. carpio* exposed to sub lethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP forz 21days. Data represent mean ± SD (n = 4). (*=p<0.05,**p<0.01).

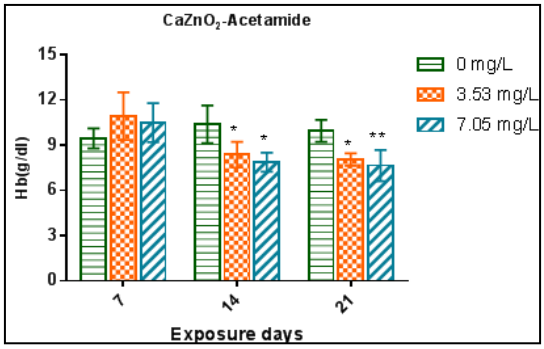

Fig 2: Changes in Hb content of *C. carpio* exposed to sub lethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

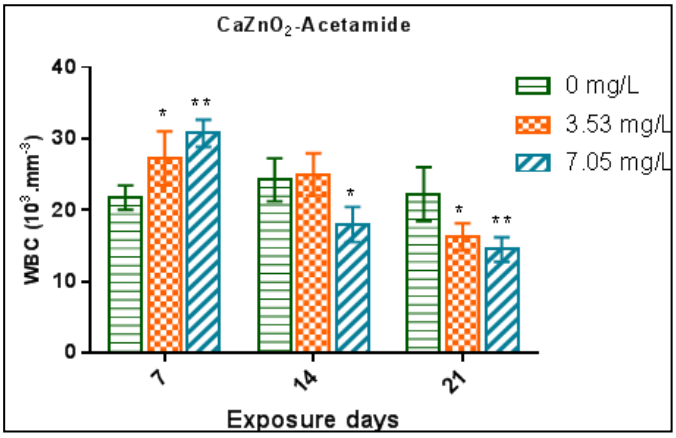

Fig 3: Changes in the WBC count of *C. carpio* exposed to sublethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

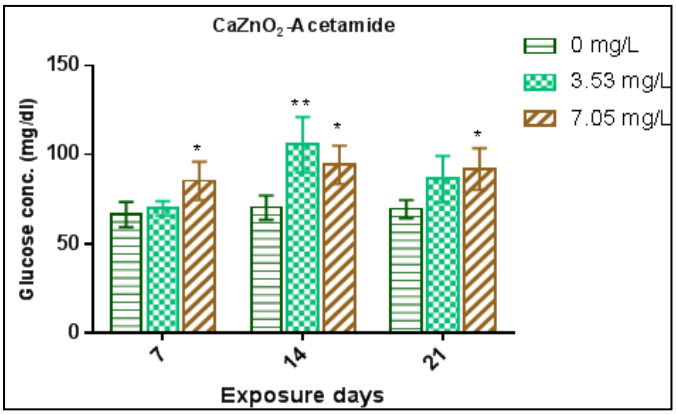

Fig 4: Alteration in the serum glucose level of *C. carpio* exposed to sublethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

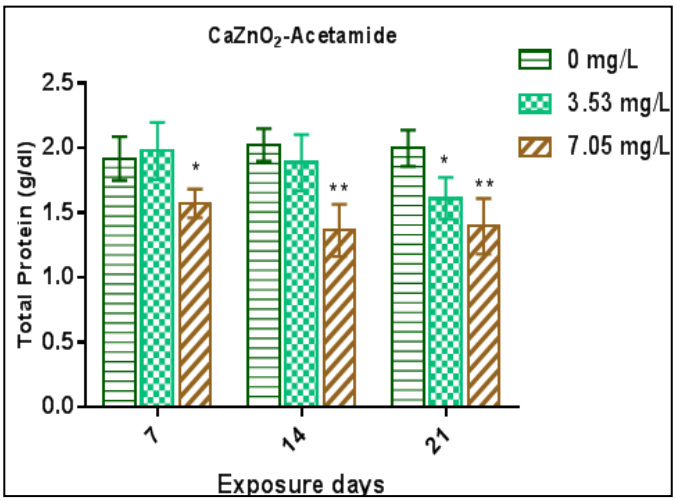

Fig 5: Alteration in the serum total protein level of *C. carpio* exposed to sublethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

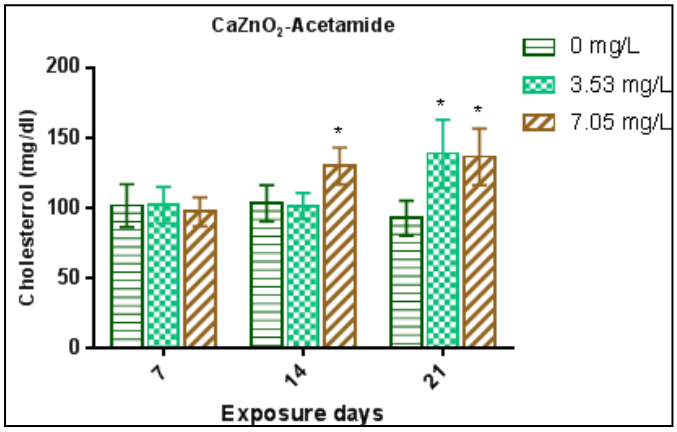

Fig 6: Alteration in the serum cholesterol level of *C. carpio* exposed to sublethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

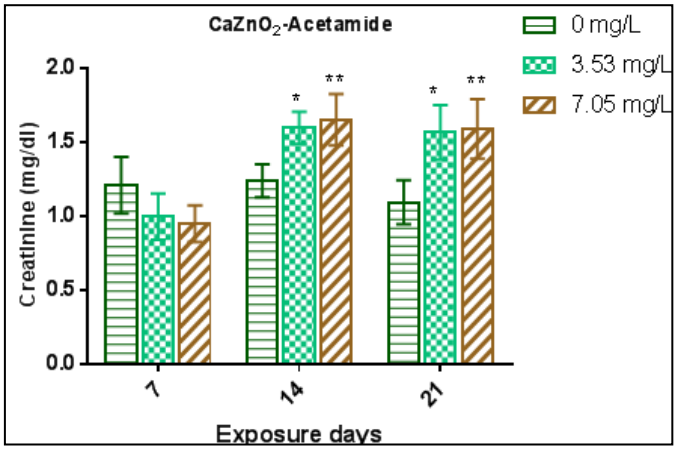

Fig 7: Alteration in the serum creatinine level of *C. carpio* exposed to sublethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

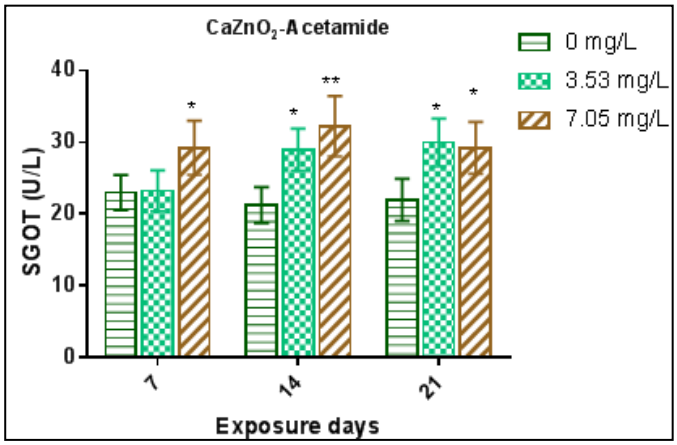

Fig 8: Changes in the SGOT activity of *C. carpio* exposed to sublethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

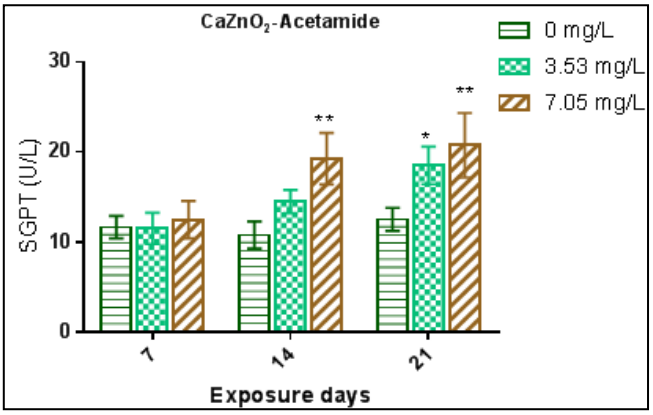

Fig 9: Changes in the SGPT activity of *C. carpio* exposed to sublethal concentrations (7.05 mg L^{-1} and 3.53 mg L^{-1}) of $CaZnO_2$ NP for 21days. Data represent mean ± SD (n = 4). (*=p<0.05, **p<0.01).

5. Conclusions

Results of the present investigation indicate that administration of sublethal concentration of $CaZnO_2$ NP is toxic to fish *Cyprinus carpio* and caused alterations in the hematological and biochemical parameters of fish. The alterations of these parameters may provide better understanding of toxic level of nanoparticles and their effects in aquatic environment.

6. References

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