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Original article

Characterization, dose dependent assessment of hepatorenal oxidative stress, hematological parameters and histopathological divulging of the hepatic damages induced by Zinc oxide nanoparticles (ZnO-NPs) in adult male Sprague Dawley rats

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ABSTRACT

Nanoparticles are beneficial in many aspects to human life but their excessive use can cause various abnormalities. They dispose in the environment through transport, industrial and agricultural usage and enter in living body through dermal, respiratory route or ingested with the lipsticks and there higher concentration produces toxicity. Therefore, current study characterized ZnO-NPs to evaluate toxic ability by X-rays diffraction (XRD) and Scanning Electron Microscopy (SEM) techniques and showed 29.83 and 35 nm size, respectively with hexagonal crystalline structure. LC50 value of ZnO-NPs was also evaluated as 72.48 ± 10.33 mg/kg BW. Male Sprague Dawley (Post weaning) rats were divided into five groups with five rats in each group. Control (C) group received no treatment, placebo (S) group received normal saline (0.9% sodium chloride) intraperitoneally and three treated groups received different levels of ZnO- NPs intraperitoneally at the dose of either 10 or 20 or 30 mg/kg for 21 days on alternate days and named as 1G1, 1G2 and 1G3, respectively for the assessment of toxicity for better understanding of precautionary measures in future. Oxidative stress enzymes of liver and kidney, hepatorenal function enzymes and hematological parameters along with hepatic histology were measured at the end of the experiment. Results showed highly significant variations in all parameters in a dose dependent manner as compared to control group while groups receiving 10 or 20 mg/kg of ZnO-NPs showed low to moderate pathological changes in both organs. Liver histological analysis showed congestion, necrosis, hemorrhage, RBC's accumulations; inflammatory cells infiltration and severe abnormalities in high dose group while medium, low dose group showed moderate and least effects, respectively. It is concluded that ZnO-NPs are highly toxic at more concentration so their careful usage is needed in daily routine.

1. Introduction

Nanotechnology is the branch of science that deals with the production and development of nanomaterials which are less than 100 nm in size (Hong et al. 2022). Nanoparticles are being studied due to various distinct properties i.e., great surface area towards volume ratio, great surface adsorption capability and more reactiveness (Doolotkeldieva et al. 2022). Among nanoparticles Zinc oxide nanoparticles (ZnO-NP) are the third highest manufactured nano metal globally after Silicon dioxide as well as Titanium dioxide nanoparticles (Swain et al. 2016; Rajput et al. 2018). They are used in food packaging, agriculture and medicinal drugs due to antimicrobial, anticancerous and antifungal abilities (Imade et al. 2022; Kalra et al. 2022) Zinc is also treated as nutritional supplement and its deficiency leads to diverse range of abnormalities like abnormal immune response, growth retardation and delayed sexual maturation (Hussain et al. 2022). They are disposed of in the environment through industrial, transport and agricultural usage

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and enter in living body through dermal, respiratory route or ingested with the lipsticks and there higher concentration produces toxicity (Mendoza-Milla et al. 2022).

In spite its accumulation in the environment, less information is reported about the long term effect of ZnO-NPs. They use to metabolize firstly in hepatic tissues and target other organs like spleen, kidney, lungs, brain, muscles and mammalian tissues, changed heart rate and caused inflammation as well (Al-Ali et al. 2022). ROS changed antioxidant system, damaged lipids, carbohydrates, proteins and deformed DNA (Vimercati et al. 2020). They formed metallic cation (Zn^{2+}) from zinc with free radicals which imbalanced ions, changed ionic homeostasis and blocked iron transport resulting in decrease or increase in blood parameters (Khalid et al. 2022). Free radicals induce aging, cancer as well as atherosclerosis (Selamoglu and Yilmaz, 2014).

The present study was designed to evaluate the toxicity of ZnO-NPs in liver, kidney and blood of male Sprague Dawley rats. They were used in recent research due to their calm behavior, easy handling and very less data availability related to toxicity in selected model. Liver is the vital organ for removal of toxic materials from the body and its high metabolic activity lowers the risk of injury caused by free radicals through biotransformation and detoxification events (Erdemli et al. 2017). Kidney is also important for regulation of minerals, filtrations of toxic particles and homeostasis so they are selected in research to evaluate toxicity (Ozougwu, 2017; Rayner et al., 2017). ZnO-NPs caused lethal changes in all organs of male Sprague Dawley rats, changed biodistribution of minerals and raised Zn concentration in liver, pancreas and kidney of mice (Ko et al. 2015; Salman, 2018). Therefore, this study assessed the in vivo toxicity of ZnO-NPs in male Sprague Dawley rats through hepatorenal oxidative stress, hepatorenal function enzymes along with hematology and histological alterations in the liver.

2. Materials and methods

2.1. Chemicals

The high quality molecular and analytical grade chemicals were used in the study. ZnO-NPs (CAS-number; 67–56-1, <50 nm) were purchased from Sigma-Aldrich company.

2.2. Characterization of ZnO-NPs

The ZnO-NPs were characterized by X-ray diffraction (XRD) and scanning electron microscopy (SEM) from Commission on Science and Technology for Sustainable Development in the South (COMSATS), Lahore and Hi-Tech Lab of Government College University, Faisalabad (GCUF), respectively. XRD graph of ZnO-NPs was studied by using Origin Pro 9.1 software to know 2θ (X) and FWMH (Full width at half maximum). After that size of each peak was recorded by using Scherrer's equation (Bunaciu et al. 2015; Kumar and Rao, 2013).

$$D = K\lambda/\beta Cos\theta$$

K = 0.9, λ = Wavelength of X-ray, β = Full width at half maxima (FWMH) in radian, θ = Diffraction angle.

2.3. Preparation of ZnO-NPs stock solution

Sodium chloride solution (0.9%) was used for the formation of physiological saline solution of ZnO-NPs. Prepared solution was ultrasonicated for one hour in a sonicator (DSA-SK_1-2.8L) to form fine dispersion as much as possible. After ultrasonication, ZnO-NPs solution was vortexed by using vortex machine (BV 1000, Taiwan) for 60 s before injecting to model animal (Shaw et al. 2012).

2.4. Male Sprague Dawley rats' maintenance and treatment plan

25 (post weaning) healthy male (Sprague Dawley) rats (200–220 g) were obtained from Government College University, Faisalabad's animal house. Rats were maintained in cages (ventilated) under normal lighting conditions along with usual day/night cycles with the support of ethical committee (local) on animal experimentations of the Government College University, Faisalabad. The animals were provided with free access to food along with water also adapted for 7 days before the initiation of the trial periods.

2.5. Evaluation of LC_{50} (Sublethal dose) of ZnO-NPs

For the determination of LC_{50}, five doses (30, 60, 80, 120 and 160 mg/kg) of ZnO-NPs were chosen with 5 replicates of each dose. Number of dead rats were counted and removed after twelve hours from the cages. Mortality was noted after 24, 48 and 96 h, rats were not fed during this periods and test was conducted in accordance with the standard methods (APHA, 1998).

2.6. Treatment of 21 days

After acclimatization period of seven days, similar body weight rats were haphazardly distributed into five groups having 5 replicates in each group. A control group (C) was fed with usual water and commercial food while placebo group was injected intraperitoneally (i.p.) with 1 mL normal saline (0.9 % NaCl) solution. Treated groups viz., 1G1, 1G2 and 1G3 were administered intraperitoneally (i.p.) with ZnO-NPs @ 10, 20 and 30 mg/kg BW, respectively on alternate days for 21 days. All treatment was performed in accordance with the established guideline by ethical committee of animal care and maintenance. The doses were nominated on the basis of 1/7th, 1/3.5th and 1/2.4th of LC50 value of ZnO-NPs.

2.7. General observation

Body weight and food consumption were noticed regularly during treatment period. Clinical symptoms like diarrhea, weakness, urination, bumping of skin, tumor growth, stress condition and movement was also noticed on daily basis.

2.8. Sampling

The model animals of all groups were anaesthetized by using chloroform and dissected ventrally at the end of the experimental period. The body weight, health status and external features were also noted carefully. Blood was collected in EDTA tubes for complete blood count (CBC) assessment and in non-EDTA tubes for liver function test (LFT) and renal function test (RFT). Internal organs and color of blood were also observed carefully hepatorenal organs were weighed, stored at −20 °C for the evaluation oxidative stress enzymes analysis. Kidney and Liver tissues were also stored in vials with 20 mL formalin solution for further histological assessment.

2.9. Assessment of oxidative stress enzymes

The homogenates of hepatic and kidney tissues were formed by mixing 0.5 g of each in 5 mL of Tris-HCl (0.1 M) buffer in a bullet blender (BBY5E-CE, USA). Buffer (10 mL) was used to mix blended homogenate and centrifuged in a centrifuge machine (2–16 K, Germany) for 15 min (4 °C, pH 7.4, 9000 rpm, diluting factor 4). The supernatant was kept in a refrigerator at −20 °C for the oxidative stress enzymes analysis. Lipid hydroperoxides (LHP), Glutathione

(GSH), Catalase (CAT) and Melanodialdehyde (MDA) were analyzed. LHP were analyzed by the technique of Noureen et al. (2018). Briefly, 900 μL of reagent was mixed in 100 μL of tissue homogenate (10% w/v) and fox reagent was prepared by mixing 0.0098 g ammonium iron sulphate, 0.0076 g xylenol orange and 0.089 butylated hydroxytoluene with 10 mL of H_2SO_4 in 90 mL of methanol by using a flask (Pyrex, Germany). Afterwards mixture was incubated in an incubator (Compact Shaking Incubator, JSSI-100 T, Korea) at 37 °C for 30 min, pink layer was noticed and read at 560 nm in a spectrophotometer (U-2800 Hitachi). GSH level in the hepatorenal tissues homogenates were assessed at 412 nm by following the technique of Sedalk and Lindsay (1968). 500 μL tissue homogenate was mixed with 50 % trichloroacetic acid (100 L) with distilled water (400 μL) and centrifuged at 1000 rpm for 5 min. 2 mL Tris-EDTA buffer, 0.5 mL supernatant and 0.1 mL 5′ 5′-dithio-bis-2-nitrobenzoic acid were mixed in a cuvette, rested for five minutes and absorbance noted by using spectrophotometer (412 nm). For the CAT estimation, 1.95 mL of phosphate buffer (50 mM) was mixed with 50 μL of prepared tissue homogenate. The absorbance was then noticed after mixing 1 mL of H_2SO_4 at 240 nm at 20 s intervals. The MDA concentration was recorded by following the method of Ohkawa et al. (1979). Briefly, about 0.2 mL of tissue homogenate was mixed in a falcon tube with 20 % acetic acid (1.5 mL, pH 3.5), 8.1 % SDS (0.2 mL) and 0.8 % aqueous thiobarbutaric acid (1.5 mL). The prepared mixture was heated with glass balls in a falcon tube for one hour at 95 °C in a water bath (DIN EN 60529-IP20). After that 5 mL pyridine and n-butanol mixture (1: 15) was mixed in 1 mL distilled water and shaked on vortex (Bv 1000) vigorously. The absorbance of upper organic layer was recorded at 532 nm after centrifugation for 10 min at 4000 rpm.

2.10. Liver function and kidney function

Blood sample's serum was separated using centrifugation for 15 min (3000 rpm) and then stored at −20 °C. Afterwards, enzymatic assays were carried out with the help of enzymatic assay kit and concentrations were presented in IU/L. BIL DRICT, BIL TOTAL, BIL IND, BUN, UREA, URIC ACID and CRAT were also assessed by assay kits and units were expressed in milligram/deciliter (Fazilati, 2013).

2.11. Hematological analysis

The blood samples were subjected to automatic hematology analyzer (M−20GP, MEDONIC Sweden) to evaluate (RBC's, HCT%, HGB, MCV, WBC, MCH, MCHC, GRAN%, LYM%, MID%, and PLT). Hemoglobin were expressed in g/dL, MCV in femtoliters (fL), MCHC and MCH in Pg, RBC' in 10^6/μL while WBC's and PLT were expressed in 10^3/μL, respectively (Noureen et al. 2018).

2.12. Mineral analysis

0.5 g sample and nitric acid (9 mL) were mixed in a flask (Pyrex), retained for overnight and perchloric acid (1 mL) was also added. It was heated on hot plate at 150 °C until minimized to 1 mL afterwards, mixed in distilled water, filtered over filter paper and transferred to 25 mL volumetric flask. Then, Mineral contents were evaluated by inductively coupled plasma-optical emission spectroscopy (Varian Inc., Australia) by using facility of HI-TECH laboratory of Government College University, Faisalabad. Commercial Ca, Zn, $Mg(NO_3)_2$, Na (sodium chloride 99.5 %), P (sodium phosphate 99%) and K (potassium chloride 99.8%) were used as standards (Ramdani et al. 2013).

2.13. Histological analysis

The dehydration of fixed tissues of liver was performed by using ethanol of ascending grades (70–100%). After that the samples were placed in banzol and cedar wood oil. Samples were embedded in paraffin blocks and sections of tissues (4 μm) were cut by using a microtome before staining in a hematoxylin eosin (HE) solution prescribed by Noureen et al. (2018). The stained tissue sections from all groups were observed at 20 magnification power under light microscope (Model: MT4300H-Japan; MEIJI). The histological alterations were expressed by using a four grade technique: normal histological structure (-); mild histological alterations (+), moderate histological alterations (++); and severe histological alterations (+++).

3. Results

3.1. Characterization of ZnO-NPs

XRD study presented that the intensities and 2θ angles of five peaks were 833 (31.827°), 684 (34.47°), 1362 (36.3°), 310 (47.06°) and 450 (57.1°). FWMH of each peak was expressed in degrees by software which was 0.3, 0.25, 0.3, 0.3, and 0.4 converted into radians (Table 1). After that size of each peak was recorded as 29, 36.25, 29, 31.522 and 23.39 nm by Scherrer's equation ($D = k\lambda/\beta\cos\theta$). They showed crystalline, hexagonal structure and average size of 29.83 nm (Fig. 1). SEM analysis confirmed that ZnO-NPs were agglomerates, spherical had tiny grain like appearance with average size ranged from 25 to 65 nm and the mean diameter was 35 nm (Fig. 2). The slight difference in both techniques was due to the ZnO-NPs agglomeration.

3.2. LC50 of ZnO-NPs

In current study LC50 of ZnO-NPs after 96 h exposure was investigated as 72.48 mg/kg BW of rats (Table 2).

3.3. General observation of first phase

Rats treated with ZnO-NPs showed physiological alterations after 21 days. The animals administered with 10 mg/kg ZnO-NPs exhibited normal behavior and health status during three weeks of experimental period and symptoms of less activeness appeared after 18 days but no death was noticed. Rats of 1G2 (20 mg/kg ZnO-NPs) group were less active in second week with goose bumps on yellowish skin and mortality of two rats was also noticed in same week as well while 1G3 (30 mg/kg ZnO-NPs) group exhibited more lethal symptoms like diarrhea, less activeness at the end of the second week with reduced coelomic cavity.

3.4. Analysis of body weight

Table 2 shows body weight of rats on weekly basis and there was a non-significant difference in all groups after first week of experiment while highly significant difference was observed after second week in treated groups as compared to control. After third week there was a highly significant lessening in body weight of treated groups as compared to control in a dose dependent manner while 1G2 and 1G3 group showed non-significant difference among themselves.

3.5. Hepato-renal organ weight

Table 2 shows that there was a highly significant increase in the liver and kidney weight of treated groups as compared to

Table 1
Scherrer's equation calculations for the evaluation of ZnO-NPs average crystalline size.

Peaks No.	K	λ (nm)	FWMH or β in degrees	2θ (X)	FWMH or β in radians	Θ	Cosθ	$\frac{K\lambda}{\beta cos\theta}$(nm)
1	0.94	0.154	0.3 ± 0.006	31.83	0.005	15.91	0.962	29
2	0.94	0.154	0.25 ± 0.0075	34.47	0.0044	17.23	0.96	36.25
3 (Major peak)	**0.94**	**0.154**	**0.3 ± 0.006**	**36.3**	**0.005**	**18.15**	**0.95**	**29**
4	0.94	0.154	0.3 ± 0.01	47.06	0.005	23.53	0.92	31.522
5	0.94	0.154	0.4 ± 0.009	57.1	0.007	28.55	0.88	23.39
Mean size of ZnO-NPs				29.8 nm				

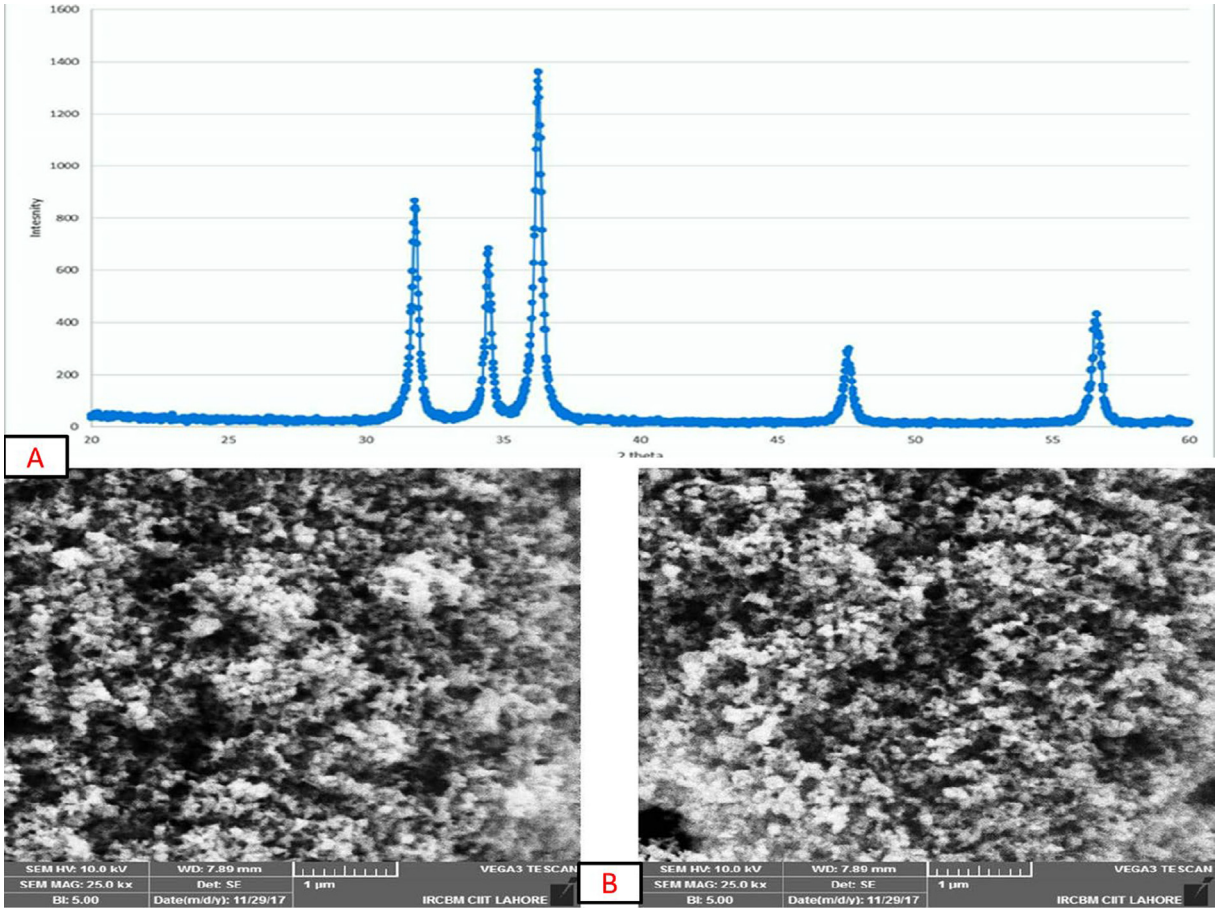

Fig. 1. (A) ZnO-NPs XRD graph along with (B) SEM.

control and placebo groups. Highest significant increment in both organs was observed in 1G3 group while lowest effect was noticed in 1G1 group. Similar trend was noticed in hepatosomatic index (HSI) and renosomatic index (RSI) in a dose dependent manner.

3.6. Oxidative stress enzymes

In present study, there were noticeable alterations in antioxidant enzymes content of model animal exposed with ZnO-NPs. High dose group showed highest reduction in GSH contents as compared to non-treated groups while LHP and MDA contents of both organs were significantly enhanced in treated groups. There was significant enhancement in CAT contents of hepatic tissues of all groups while renal tissues exhibited highly significant increment among medium and high dose group but G1 exhibited non-significant variations (Table 3).

3.7. Liver, renal function and mineral analysis of hepatorenal tissues

Control group mean value of ALT, AST, ALP, BIL TOL, BIL DIC and BIL IND was (38.2 ± 1.85), (32.8 ± 1.82), (299.6 ± 2.27), (0.798 ± 0.02), (0.198 ± 0.01) and (0.502 ± 0.02), respectively which were enhanced with enhancement of dose and highest enhancement was recorded at 30 mg/kg after 21 days. All treated groups exhibited highly significant variation from non-treated groups and similar trend was noticed in renal parameters (Urea, BUN, CRAT and Uric acid) (Table 4). Table also represents highly significant elevation in Zn, Ca, Na and K of hepatorenal tissues as compared to control. There was highest elevation in 1G3 group.

3.8. Hematological analysis

HGB concentration in blood was firstly increased and then lowered in high dose group and there was highly significant variations

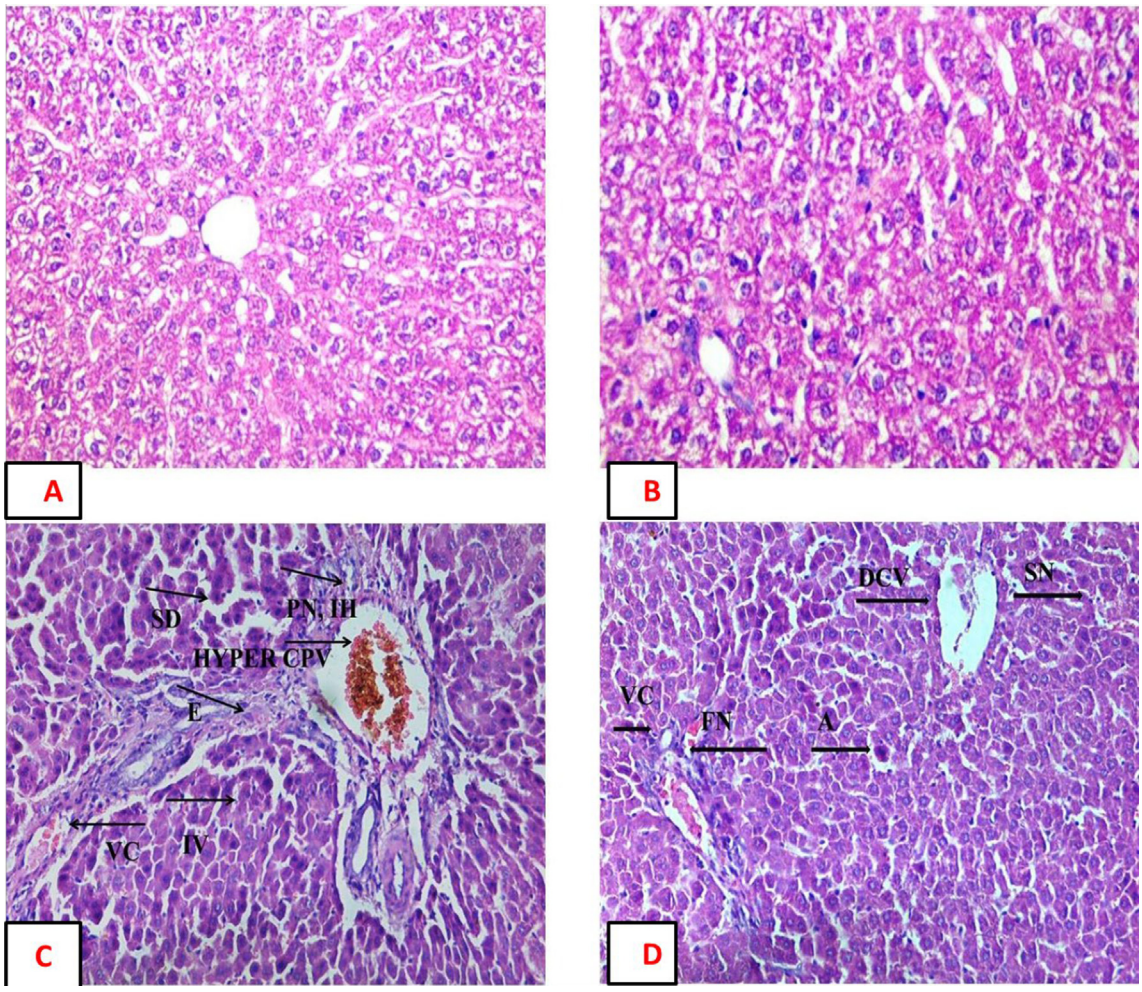

Fig. 2. Photomicrograph (H & E; X200) of hepatic tissues of control and placebo group exhibiting normal hepatocytes (A-B) while 10 mg/kg dose (C-D) showing edema (E), sinusoidal dilation (SD), intracellular vacuolization (IV), vascular congestion (VC), congestion of portal vein (CPV & Hyper.) and hyperemia, piecemeal necrosis (PN) or inflammatory cell infiltration of portal area (interface hepatitis; IH), apoptosis (A), vacuolated cells (VC), focal necrosis (FN), spotty necrosis (SN) and damaged central vein (DSV).

Table 2
Mean ± SE of weekly body weight of male Sprague Dawley rats and analysis of hepatorenal weight, hepatosomatic index (HIS) along with renosomatic index (RSI) after 21 days of exposure.

96 h Confidence Interval				
Metal	LC50 (mg/kg)	Lower		Upper
ZnO-NPs	72.48 ± 10.33	41.12		103.03
Body weight analysis of male Sprague Dawley rats				
Groups	First week (Body weight)	Second week (Body weight)		Third week (Body weight)
C	228.50 ± 0.61^{A}	254.20 ± 0.87^{A}		289.33 ± 3.48^{A}
S	230.20 ± 0.37^{A}	251.73 ± 0.38^{A}		294.33 ± 4.25^{A}
1G1	236.27 ± 0.27^{A}	228.67 ± 2.02^{B}		212.30 ± 1.42^{B}
1G2	234.93 ± 0.93^{A}	217.0 ± 1.73^{C}		198.33 ± 2.73^{C}
1G3	233.73 ± 0.59^{A}	218.0 ± 1.15^{C}		193.67 ± 2.33^{C}
Hepatorenal weight, hepatosomatic index (HIS) along with renosomatic index (RSI)				
Groups	Liver weight	Kidney weight	Hepatosomatic index (HIS)	Renosomatic index (HIS)
C	(g)	(g)		
	6.39 ± 0.25^{D}	0.82 ± 0.02^{C}	2.21 ± 0.01^{D}	0.25 ± 0.06^{D}
S	6.15 ± 0.03^{D}	0.81 ± 0.01^{C}	2.09 ± 0.02^{D}	0.27 ± 0.01^{D}
1G1	9.15 ± 0.03^{C}	0.92 ± 0.01^{C}	4.31 ± 0.03^{C}	0.49 ± 0.02^{C}
1G2	10.22 ± 0.02^{B}	0.97 ± 0.01^{B}	5.27 ± 0.01^{B}	0.55 ± 0.03^{B}
1G3	11.16 ± 0.04^{A}	1.07 ± 0.03^{A}	5.76 ± 0.05^{A}	0.95 ± 0.89^{A}

(Mean values sharing different letter in columns are significantly different ($P < 0.05$).

Table 3
Mean ± SE of oxidative stress enzymes concentrations in hepatorenal tissues of control along with treated rats after 21 days of introduction.

Groups	GSH(μM/mg)	LPH (mM/g) Hepatic Tissues	MDA (nmoles/mg)	CAT (Kunit/mL)
C	14.266 ± 0.280^{A}	0.340 ± 0.015^{D}	0.352 ± 0.013^{D}	122.6 ± 2.83^{D}
S	14.270 ± 0.298^{A}	0.342 ± 0.016^{D}	0.356 ± 0.0121^{D}	122.8 ± 2.89^{D}
1G1	12.79 ± 0.227^{B}	0.554 ± 0.213^{C}	1.057 ± 0.0127^{C}	276.4 ± 1.89^{C}
1G2	9.686 ± 0.206^{C}	0.856 ± 0.019^{B}	2.544 ± 0.015^{B}	325.8 ± 1.93^{B}
1G3	6.450 ± 0.014^{D}	1.132 ± 0.232^{A}	4.544 ± 0.015^{A}	361.2 ± 1.85^{A}
Oxidative stress enzymes of Renal tissues				
C	14.28 ± 0.36^{A}	0.35 ± 0.019^{D}	0.35 ± 0.016^{D}	124.6 ± 3.69^{C}
S	14.29 ± 0.44^{A}	0.40 ± 0.027^{D}	0.36 ± 0.015^{D}	123.2 ± 2.08^{C}
1G1	11.20 ± 0.016^{B}	0.52 ± 0.039^{C}	0.89 ± 0.027^{C}	138.6 ± 14.20^{C}
1G2	10.57 ± 0.023^{C}	0.75 ± 0.016^{B}	1.18 ± 0.035^{B}	226.8 ± 2.15^{B}
1G3	8.28 ± 0.025^{D}	0.96 ± 0.018^{A}	3.40 ± 0.095^{A}	305.2 ± 4.98^{A}

(Mean values sharing different letters in columns are significantly different (P < 0.05; GSH = Reduced glutathione, LHP = Lipid hydroperoxides, MDA = Malondialdehyde, CAT = Catalase).

Table 4
Mean ± SE of liver and kidney profile of control along with treated groups afterwards 21 days of introduction.

Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	BIL TOL (mg/dL)	BIL DIC (mg/dL)	BIL IND (mg/dL)
C	38.2 ± 1.85^{D}	32.8 ± 1.82^{D}	299.6 ± 2.27^{B}	0.798 ± 0.02^{D}	0.198 ± 0.01^{D}	0.502 ± 0.02^{D}
S	38.8 ± 1.28^{D}	37.8 ± 1.52^{D}	298.6 ± 2.29^{B}	0.784 ± 0.03^{D}	0.232 ± 0.02^{D}	0.522 ± 0.03^{D}
1G1	148.8 ± 2.05^{C}	108.6 ± 2.96^{C}	313.6 ± 18.26^{B}	1.276 ± 0.13^{C}	0.41 ± 0.03^{C}	0.632 ± 0.02^{C}
1G2	205.6 ± 2.13^{B}	165.4 ± 1.89^{B}	333.4 ± 18.80^{B}	1.836 ± 0.04^{B}	0.606 ± 0.02^{B}	0.806 ± 0.01^{B}
1G3	246.6 ± 2.13^{A}	187.2 ± 2.06^{A}	464.0 ± 2.25^{A}	2.588 ± 0.18^{A}	0.696 ± 0.02^{A}	0.908 ± 0.02^{A}

Groups	Urea (mg/dL)	BUN (mg/dL)	CRAT (mg/dL)	Uric acid (mg/dL)
C	44.4 ± 1.72^{B}	19.0 ± 1.41^{D}	0.842 ± 0.02^{C}	4.8520 ± 0.018^{D}
S	45.2 ± 1.56^{B}	21.0 ± 1.14^{CD}	0.83 ± 0.02^{C}	4.8280 ± 0.023^{D}
1G1	53.1 ± 0.03^{A}	26.8 ± 1.36^{C}	1.80 ± 0.14^{B}	7.6740 ± 0.017^{C}
1G2	56.3 ± 0.02^{A}	34.6 ± 1.96^{B}	2.38 ± 0.12^{A}	7.7600 ± 0.014^{B}
1G3	57.1 ± 0.01^{A}	42.6 ± 2.01^{A}	2.74 ± 0.23^{A}	7.8580 ± 0.022^{A}

Mean ± SE of Zn, Ca, Na and K in hepatorenal tissues of rats among control and treated groups after 21 days exposure

Groups	Zn (ppm)	Ca (mg/L) Hepatic Tissue	Na (mg/L)	K (mg/L)
C	0.1460 ± 0.02D	0.8840 ± 0.03D	118.8000 ± 4.05D	693.4000 ± 1.99D
S	0.1580 ± 0.01D	0.8860 ± 0.02D	119.2000 ± 4.43D	689.2000 ± 2.65D
1G1	4.2340 ± 0.03C	1.0320 ± 0.03C	274.6000 ± 3.87C	891.8000 ± 3.50C
1G2	5.3180 ± 0.03B	1.4160 ± 0.04B	344.0000 ± 8.60B	1073.4000 ± 3.68B
1G3	6.7380 ± 0.02A	1.8440 ± 0.04A	386.8000 ± 2.97A	1118.4000 ± 3.39A
Mineral analysis in Kidney Tissues				
C	0.2720 ± 0.03^{D}	0.7740 ± 0.01^{D}	106.2000 ± 1.85^{D}	685.8000 ± 2.29^{D}
S	0.3040 ± 0.03^{D}	0.7880 ± 0.02^{D}	105.4000 ± 1.99^{D}	687.6000 ± 2.95^{D}
1G1	5.8520 ± 0.06^{C}	1.0060 ± 0.03^{C}	215.0000 ± 3.74^{C}	881.6000 ± 7.73^{C}
1G2	7.7920 ± 0.05^{B}	1.1980 ± 0.04^{B}	308.2000 ± 4.31^{B}	1066.2000 ± 2.61^{B}
1G3	9.3000 ± 0.02^{A}	1.7380 ± 0.03^{A}	353.8000 ± 17.26^{A}	1096.0000 ± 4.24^{A}

Table 5
Mean ± SE of hematological parameters of rats among control and treated groups rats after 21 days exposure.

Groups	WBC (10^3/ μL)	GRAN (%)	LYMP (%)	MID (%)	HGB (g/dL)	MCH (Pg)	MCHC (Pg)	RBC'S (10^6/μL)	MCV (fL)	HCT (%)	PLT (10^3/ μL)
C	12.6 ± 1.78^{D}	7.46 ± 0.16^{C}	76.6 ± 1.36^{A}	8.08 ± 0.17^{A}	15.32 ± 0.19^{C}	16.94 ± 0.18^{A}	29.04 ± 0.16^{A}	7.18 ± 0.019^{A}	59.22 ± 0.16^{A}	45.93 ± 0.12^{A}	595.2 ± 1.98^{A}
S	13.0 ± 2.0^{D}	7.52 ± 0.19^{C}	76.8 ± 1.93^{A}	8.08 ± 0.18^{A}	15.28 ± 0.18^{C}	16.940 ± 0.17^{A}	29.10 ± 0.20^{A}	7.17 ± 0.020^{A}	59.26 ± 0.18^{A}	45.94 ± 0.02^{A}	595.6 ± 2.20^{A}
1G1	35.4 ± 1.63^{C}	8.90 ± 0.21^{C}	54.0 ± 1.76^{B}	7.08 ± 0.33^{B}	16.80 ± 0.17^{B}	15.67 ± 0.02^{B}	25.33 ± 0.18^{B}	6.14 ± 0.017^{B}	45.8 ± 1.07^{B}	43.62 ± 0.56^{B}	574.2 ± 1.74^{B}
1G2	43.8 ± 2.08^{B}	26.8 ± 1.98^{B}	51.2 ± 2.35^{BC}	6.40 ± 0.16^{BC}	19.84 ± 0.30^{A}	14.59 ± 0.03^{C}	24.63 ± 0.014^{C}	5.68 ± 0.023^{C}	34.8 ± 1.16^{C}	43.42 ± 0.02^{B}	563.8 ± 1.50^{B}
1G3	54.0 ± 1.52^{A}	45.2 ± 1.98^{A}	45.8 ± 1.06^{C}	6.02 ± 0.13^{C}	16.94 ± 0.18^{B}	14.24 ± 0.02^{C}	24.24 ± 0.017^{C}	5.58 ± 0.022^{D}	28.6 ± 1.63^{D}	42.15 ± 0.18^{C}	553.4 ± 1.66^{C}

Values sharing different letters in columns are significantly different (P < 0.05).

were noticed in groups as compare to control and placebo group. RBC's, platelets, monocytes and MID showed highly significant reduction while non-treated rats exhibited normal concentration. WBC's also exhibited increment while MCV, MCH, HCT and MCHC were reduced highly significantly (Table 5).

3.9. Histological analysis of hepatic tissues

In liver tissues, normal hepatic cells were noticed in non-treated groups (control & placebo). The low levels of ZnO-NPs (10 mg/kg) produced edema (E), sinusoidal dilation (SD), intracel-

lular vacuolization (IV) vascular congestion (VC), piecemeal necrosis (PN) or inflammatory cell infiltration of portal area (interface hepatitis; IH), congestion of portal vein (Hyper. and CPV), and hyperemia, focal necrosis (FN), apoptosis (A), damaged central vein (DSV), vacuolated cells (VC), degenerative hepatocytes (DH), spotty necrosis (SN) and sinusoidal congestion (C). The fatty acid accumulation, hemorrhage and pyknotic nuclei were noted at 20 mg/kg dose of ZnO-NPs (medium dose group), while ZnO-NPs accumulation was noticed at 30 mg/kg (high dose group) along with all above observed alterations (Figs. 3–4). Histological modifications in hepatic rat's tissues expressed that ZnO-NPs gained entry in liver cells through blood circulatory system and produced damages. Comparative studies of hepatic tissues of ZnO-NPs exhibited that they are dangerous at higher concentration. Table 6 shows histological changes in hepatic tissues of male Sprague Dawley rats treated with varying concentrations of ZnO-NPs for 21 days.

4. Discussion

4.1. Characterization of ZnO-NPs by XRD and SEM

In current study rats of weight 200–220 g exposed with varying concentrations of ZnO-NPs (10, 20 and 30 mg/kg ZnO-NPs). Moreover, ZnO-NPs characterization, LC50 evaluation, hepatorenal oxidative stress enzymes, hepatorenal function enzymes, hematological assessment along with hepatic tissues histology were analyzed. In present study, XRD evaluation exhibited hexagonal structure and average size range of 29 nm of ZnO-NPs while agglomerized structure with size range of 35 nm was noticed by SEM. XRD results are in accordance with the study by Faisal et al. (2021) who prepared and observed crystalline nature of ZnO-NPs which were because of the formation of peak and they also exhibited hexagonal structure. Khan et al. (2015) also noticed agglomerized ZnO-NPs structure due to association in them by weak forces. Different other scientists also noticed partially same results (Saeed et al. 2021; Imade et al. 2022). Vignesh et al. (2022) synthesized ZnO-NPs by Sol-gel method and noted that SEM images showed greater size than that analyzed from the XRD. This increase in size thus showed that the size of the nanoparticle gained using SEM is not of a single crystal but of all flock of nanoparticles.

4.2. LC50 evaluation of ZnO-NPs

In the start of the study ZnO-NPs acute toxicity was evaluated by 96 h treatment period. Time period was an important factor in evaluating LC50. The tolerance capability of rats was reduced when constant levels of ZnO-NPs were exposed. LC50 value was lowered after 96 h which was due to damaging potential of dose. Present study findings are in partial agreement to the findings of Choi et al. (2015) and recorded 72.48 ± 10.33 mg/L ZnO-NPs LC50.

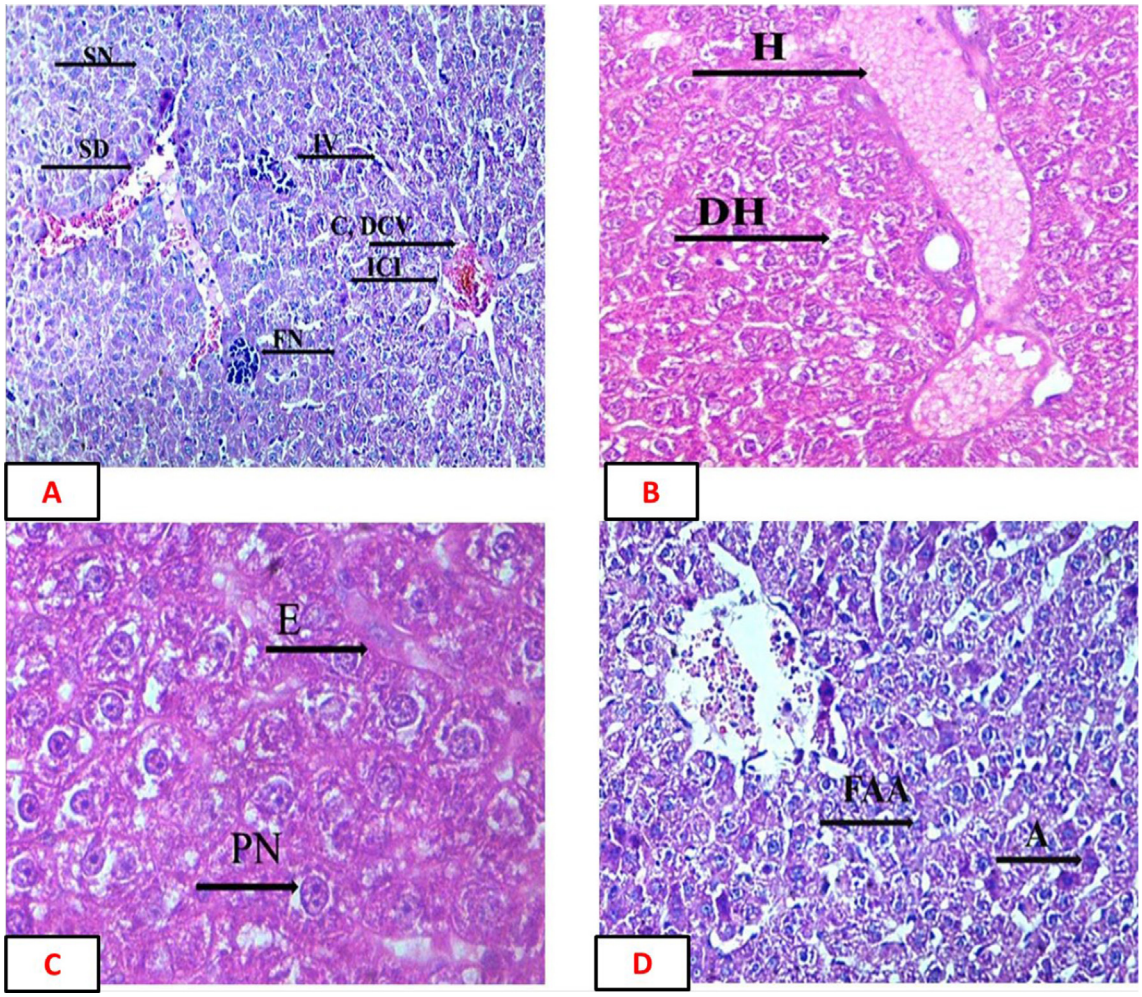

Fig. 3. Photomicrograph (H & E; X 200) of liver tissues of medium dose (20 mg/kg ZnO-NPs) group (A-D) showing spotty necrosis (SN), sinusoidal dilation (SD), damaged central vein (DCV), congestion (C), inflammatory cell infiltration (ICI), degenerative hepatocytes (DH), focal necrosis (FN), hemorrhage (H), intracellular vacuolization (IV), apoptosis (A), fatty acid accumulation (FAA) in circle form, pyknotic nuclei (PN) and edema (E).

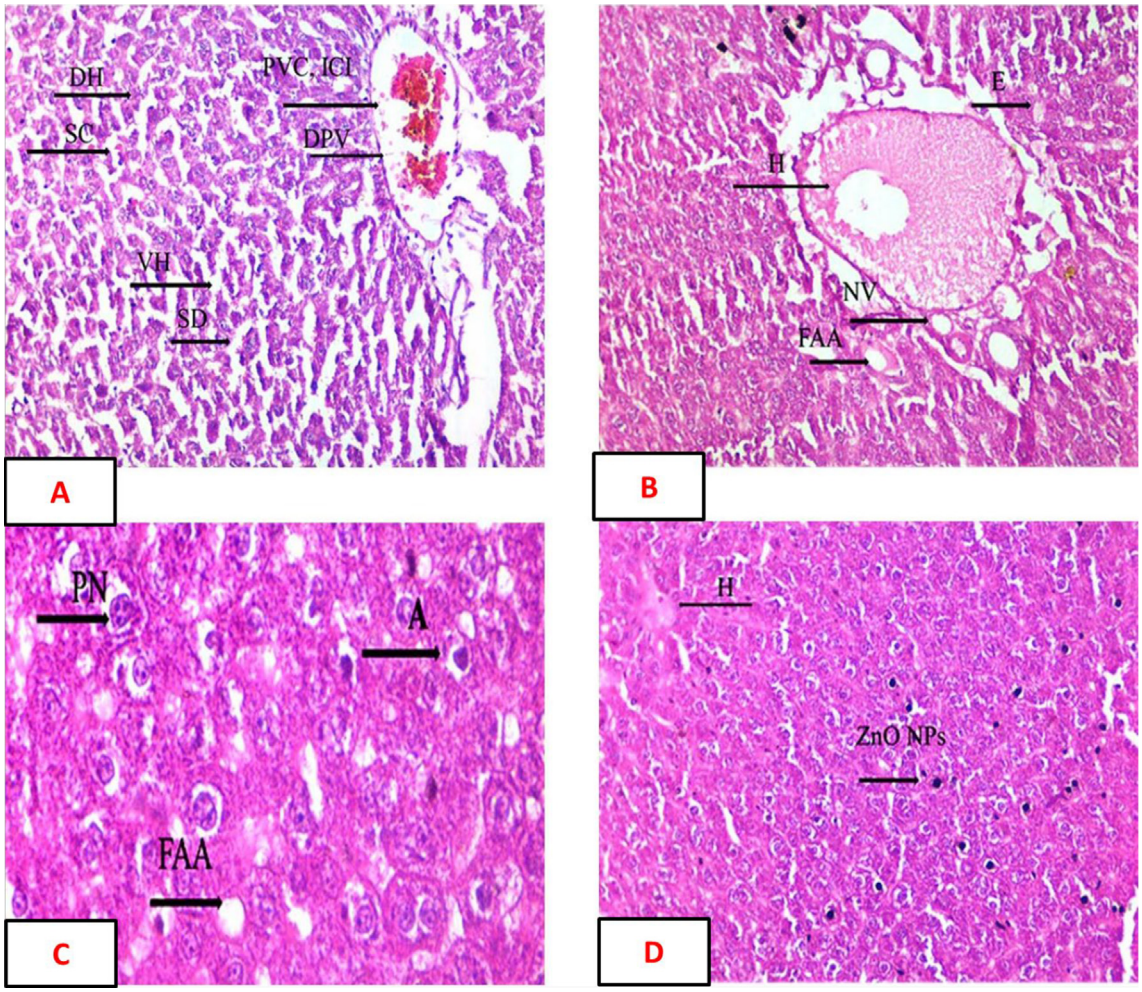

Fig. 4. Photomicrograph (H & E; X 200) of liver tissues of high dose (30 mg/kg ZnO-NPs) group (A-D) exhibiting damaged hepatocytes (DH), vacuolated hepatocytes (VH), sinusoidal dilation (SD), damaged portal vein (DPV), portal vein congestion (PVC), sinusoidal congestion (SC), heamorrhage (H), inflammatory cell infiltration (ICI), fatty acid accumulation (FAA), new vessels formation (NV), pyknotic nuclei (PN), edema, apoptosis (A), fatty acid accumulation (FAA), accumulation of ZnO NPs in hepatocytes and heamorrhage (H).

Table 6
Histological changes in hepatic tissues of male Sprague Dawley rats treated with varying concentrations of ZnO-NPs for 21 days.

Abnormalities	Control	Placebo	1G1 (10 mg/kg ZnO-NPs)	1G2 (20 mg/kg ZnO-NPs)	1G3 (30 mg/kg ZnO-NPs)
Sinusoidal dilation	_	_	+	++	++
Edema	_	_	+	++	++
Vascular congestion	_	_	+	++	++
Intracellular vacuolization	_	_	+	++	+++
Hyperemia	_	_	+	_	_
Congestion of portal vein	_	_	+	++	+++
Piecemeal necrosis	_	_	+	_	_
Apoptosis	_	_	+	_	+++
Focal necrosis	_	_	+	++	_
Vacuolated cells	_	_	+	_	+++
Damaged central vein	_	_	+	++	_
Spotty necrosis	_	_	+	++	_
Degenerated hepatocytes	_	_	_	++	_
Hemorrhage	_	_	_	++	+++
Fatty acid accumulation	_	_	_	++	+++
Pyknotic nuclei	_	_	_	++	+++
Damaged hepatocytes	_	_	_	_	+++
Damaged portal vein	_	_	_	_	+++
Sinusoidal congestion	_	_	_	++	+++
New vessel Formation	_	_	_	_	+++
Accumulation of hepatocytes	_	_	_	_	+++
Inflammatory cell infiltration of portal area	_	_	+	++	+++

ZnO-NPs LD50 was 22.25 ± 0.48 μg/mL by Shaker et al. (2017) who worked on Hepatoma cells (HEPG2) of human and variation in LD50 might be due to variations in organs and route of exposure.

4.3. Animal body and organ weight

In current study reduction in body weights was recorded in dose dependent manner in rats exposed to ZnO-NPs (10, 20 and 30 mg/kg) on weekly basis for 21 as compared to control. The present study findings are in line with the findings of Xu et al. (2020) and Rahman et al. (2022) who reported toxic effect of ZnO-NPs on rats. Ramadan et al. (2022) assessed genotoxicity exposed by ZnO-NPs in rats and showed reduce body weight due to less feed intake. Hosseini et al. (2020) described that ZnO-NPs caused decrement in body weight of rats while working on the hepatic and pancreatic impairment in female rats. He also concluded that heavy metals used to loss body weight. Some scientists reported increment in body weight of rats which is dissimilar to our results (Yan et al. 2022; Kociova et al. 2020).

Liver weight was enhanced in present study in dose dependent manner and results are similar with the reported interpretations of Hosseini et al. (2020). Liver is the main toxicity organ and affected by heavy metals which increase liver weight. It was due to white blood cells infiltration, red blood cells congestion, ZnO-NPs accumulation, vacuolization or fats deposition in liver, damaging of lobular structure and necrosis which was confirmed by Mansouri et al. (2015). Senapati et al. (2015) confirmed that ZnO-NPs communicate with enzymes and proteins in the interstitial hepatic tissues, interfere with defense system (antioxidant) and generate ROS which induces inflammatory response. Abbasalipourkabir et al. (2015) found apoptosis in liver cells by oxidative stress and noticed ballooning of hepatocytes. Hegazy et al. (2018) reported decrement in hepatic weight after exposure of ZnO-NPs which is in disagreement with recent results. Sakr and Steenkamp, (2021) also found same results of hepatic weight decrement.

Kidney weight was also enlarged in all treated groups according to given dose in recent research. ZnO-NPs caused increase in kidney weight due to major target organ for toxins and filtering ability of renal tissues (Zayed and Luaibi, 2018). Noori et al. (2014) evaluated damages in distal and proximal tubules and deposition of inflammatory cells in capillaries of glomerulus of rats. Wang et al. (2016) also confirmed toxicity in kidney by increasing their size at high dose which is similar to our results. Salman (2018) showed pathological alterations including enlargement of collecting tubules, cortex region and inflammatory cells deposition along with mononuclear cells deposition among renal tubules and blood vessels.

4.4. Oxidative stress enzymes

In present study, alterations in kidney and liver oxidative stress enzymes were more at high dose (30 mg/kg) of ZnO-NPs while others concentrations also exhibited oxidative stress. ZnO-NPs produce toxicity by generating ROS e.g., singlet oxygen (O_2), superoxide anion (O^{2-}) and hydroxyl radical (OH^-) which change macromolecule's structure. Zn^{2+} ions are also produced to create toxic condition in living things (Khalid et al. 2022; Vimercati et al. 2020). MDA is being used as indicator of stress which reacts with omega-3 and omega-6 fatty acid and causes lipid peroxidation and oxidative damage. MDA production is usually linked with many pathological conditions (Alvarez-Mon et al. 2022). There was enhancement in MDA concentration in dose dependent manner. The current study is in line with the findings of Selamoglue et al. (2015) who noted increment in MDA levels in L-NAME exposed rats. Nagar et al. (2022) also noted rise in MDA levels of renal tissues. Consequences were according to the results of Aboulhoda et al. (2020), who noticed enhancement of MDA in liver tissues after ZnO-NPs exposure. ZnO-NPs caused more cytoxicity that non-metals nanoparticles because of their ion releasing capacity (Vimercati et al. 2020) and enhances MDA concentration along with depletion of glutathione levels (Bashandy et al. 2021). Another study of El Shemy et al. (2017) also noticed high concentration of MDA of liver tissues in dose dependent manner.

There was reduction in GSH concentration and enhancement in CAT level in renal and hepatic tissues according to administered dose. The findings of Yang et al. (2015) and Mansouri et al. (2015) also noticed reduced GSH concentration in liver after exposure of nano-ZnO. The recordings of Khorsandi et al. (2018) are in line with the findings of present study and increased lower GSH values in renal tissues. Alarifi et al. (2013) also noticed reduced CAT activity and demonstrated apoptosis and oxidative stress in cancerous skin cell lines. Ansar et al. (2018) also noticed lower CAT contents in liver tissues which disagree with recent study due to change in duration, time and exposure routes. LHP act as a disease mediator and cause death of cells (Gaschler and Stockwell, 2017). LPO concentration was enhanced as compared to non-treated groups of liver and kidney tissues and results are similar to the findings of Lee et al. (2012) in human epidermal keratinocyte HaCaT cells.

4.5. Liver function and kidney functions parameters

The present study results approve a significant enhancement in hepatic enzymes e.g., AST, ALT along with ALP at (10, 20 & 30 mg/kg) of ZnO nanoparticles and are harmonious with the results of Tamimi et al. (2020). They clarified the effect of ZnO nanoparticles on hepatic function in mice when were they nourished with ZnO nanoparticles at 100 as well as 300 mg/kg, for 25 days where detected the enhancement in hepatic enzymes. Bilirubin was also increased in this study which is similar to the findings of Hosseini et al. (2020) who studied hepatic impairment in female rats. They also confirmed that increment in liver enzymes is the sensitive indicator of hepatic damage. Aboulhoda et al. (2020) had recorded enhancement in hepatic enzymes (AST, ALT & ALP) after administering various doses of ZnO nanoparticles to rats. The level of AST along with ALT enzymes in the serum is a sign of injured hepatic tissues. AST for the most part is a mitochondrial enzyme that is majorly exploited to evaluate hepatotoxicity in association with other enzymes. ALT is mainly present in hepatocytes cytosol and is an early necrosis indicator. There was also increase in these enzymes after five days exposure of ZnO-NPs at 10 mg/kg concentration (Sakr and Steenkamp, 2021).

The findings of current study demonstrated that ZnO-NPs administered rats at a level of 10, 20 and 30 mg/kg caused significantly increase in serum urea, creatinine, BUN and uric acid levels which is consistent with the results of Tamimi et al. (2020). They found nephrotoxicity in male mice at different doses of ZnO-NPs and noted increment in BUN, urea and creatinine levels. The concentration of the kidney plasma markers were altered under the impact of the renal disorders which are present in the nephrons proximal cells. They excreted out in the blood during renal damage; therefore the enhanced concentration of them represents cell damage. The furthermost delicate markers for assessing renal function are CAT along with Urea because they chiefly released from the renal tissues (Asmaa et al. 2018).

4.6. Hematology

Hemoglobin levels were first enhanced and then lowered at high concentration while normal range was observed in control

group. High metabolism altered hemoglobin levels which caused anemia (Ko et al. 2015). Platelets and RBC's were lowered in treated rats as compared to control. Abass et al. (2017) also noticed similar findings and confirmed that decrement in platelets and RBC's was due to stressing environment. Shirsekar et al. (2015) reported that RBC's levels were dependent on ZnO-NPs dose. White blood cells alterations were noticed in present study to fight for stress situation. These alterations indicated presence of xenobiotics, different diseases and abnormalities in blood cells. Leucocytes were enhanced in recent study due to increment in granulocytes while monocytes and lymphocytes were lowered similar to the findings of Tang et al. (2016). MCH is the average mass of hemoglobin which was also decreased according to dose in recent study similar to the findings by Tang et al. (2016). MCHC, MCV and HCT were also lowered and decreased levels of RBC's were due to deformation, damage, hemolysis and bleeding or decreased RBC's formation (Aula et al. 2014). Lower concentration of MCV, MCHC and HCT is due to copper deficiency, iron and hemorrhage produced by ZnO-NPs (Kim et al. 2014). Blood parameters usually altered to fight against toxins by generating erythrocytes (Bresnick et al. 2018).

4.7. Mineral analysis

In present study, Zn, Na, K and Ca levels of hepatic and renal organs were altered in dose dependent manner. Zn, Ca, Na were enhanced along with K with increasing dose (30 mg/Kg ZnO-NPs). More Zn was accumulated in liver than kidney which is similar to the results of Du et al. (2018) along with Chen et al. (2016), they represented that liver is the major target organ for nanoparticles. Same results were observed in studies of many scientist (Wang et al. 2016; Ben-slama et al. 2015). Homeostasis of minerals in liver was disturbed by a protein (metallothionien).

4.8. Histology of liver

ZnO-NPs showed various histological changes in hepatic tissues in recent research similar to the alterations noticed by Ben-Slama et al. (2015), who exhibited inflammation and deposition of RBC's in rats. Further, their results also exhibited kupffer cells hyperplasia, apoptosis, portal triads and lobular inflammatory cells infiltration, karyolysis, hemosidrosis, depletion of glycogen contents, anisokaryosis and nuclear membrane irregularity (Almansour et al. 2017). Pkynotic nuclei were noticed by Subashkumar and Selvanayagam (2014) in liver of *Cyprinus carpio* exposed with ZnO-NPs which is similar to recent study at medium and high dose. Badr et al. (2019) exposed rats with CCl_4 and noted necrosis, congestion, fatty changes and sinusoidal dilation in hepatocytes which is also in line with our study. Alferah, 2018 detected hepatocytes karyomegaly, central vein disruption and infiltration of hepatocytes in various groups of rats receiving ZnO-NPs.

Going with above outcomes, ZnO-NPs were recorded to encourage an imbalance among oxidant as well as antioxidant systems in which there was an increament in oxidants that produced damages. The ROS can interrupt mitochondrial role and causes changes in genes expression that are entangled in apoptosis and inflammation and it is necessary in pathogenesis of various diseases (Mohammed et al. 2022).

There were no cytotoxic effects by ZnO-NPs in liver of mice due to lower dose concentration (Yan et al. 2022). Tang et al. (2016) noted swelling, intracellular spaces and minor hemorrhages in hepatocytes treated with ZnO-NPs. ZnO-NPPs accumulation was noticed at 30 mg/kg dose similar to the results recorded by Liu et al. (2017) in rats liver.

5. Conclusion

It is concluded that ZnO-NPs are toxic to the health of rats as these caused various lethal effects, including alterations in body and organ weight (kidney & liver), oxidative stress enzymes, blood parameters, liver function along with kidney function enzymes and histology of liver. We investigated dose dependent increment in hemato-, hepato- and renotoxicity in rats exposed to ZnO-NPs.

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CRediT authorship contribution statement

Sana Kausar: Writing – original draft. **Farhat Jabeen:** Conceptualization. **Muhammad Asif Latif:** Writing – review & editing. **Muhammad Asad:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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