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Evaluation of the Toxicological Effects of Zinc Oxide Nanoparticles in Albino Male Mice

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Abstract

The acute and sub chronic toxicity effects of 25.16 nm intraperitoneally- injected zinc oxide nanoparticles (ZnO NPs) were evaluated. Albino male mice were exposed to three different doses (25, 50 ,and 100 mg/kg), depending on the value of calculated LD50, for 2 and 4 weeks . Considerable changes in organ indexes were shown with a good relevance to the illustrated histopathological effects which ranged from multiple hemorrhagic foci in liver, mild swelling and dilatation in kidney tubules, thickening of intestinal villi, moderate interstitial pneumonia, especially with the high dose , and sever necrosis of seminiferous tubules in testes of all studied groups. Significant changes in both hematological and biochemical parameters as well as thyroid hormones were observed with a considerable increase in the levels of antioxidant enzymes, in dose and exposure time dependent manner. The highest accumulated Zn mean values were recorded in the small intestine, kidney, liver, and spleen, respectively, followed by testes , heart , lung , and brain. These values followed the same order of the dose dependent manner, which explains the adverse effects that were recorded. This study proved the ability of using organ indexes as good tools side by side with the biochemical indicators to explain the histopathological changes. This study also revealed some histopathological effects that were not previously recorded as a toxicological effect of ZnO NPs in animal models.

Keywords: ZnO NPs, bioaccumulation , Organ index , Biochemical parameters , Histopathology

تقييم التأثيرات السمية لدقائق أوكسيد الزنك النانوي في ذكور الفئران البيض

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الخلاصة

تم تقييم التاثيرات السمية الحادة وتحت المزمنة ادقائق الزنك النانوي (25,16 نانومتر) وذلك بتعريض ذكور الفئران البيض لثلاث تراكيز مختلفة (25، 50 ، و100 ملغم/ كغم) بالحقن البريتوني لمدة 2 و 4 أسابيع ، اختيرت التراكيز اعتماداً على القيمة المحسوبة للتركيز نصف القاتل (LD50). أظهرت النتائج تغيرات معنوية في معاملات اوزان الأعضاء وبارتباط وثيق مع التأثيرات النسيجية الملاحظة والتي تراوحت بين

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النزف متعدد المراكز في الكبد ، تورم خفيف وتمدد في النبيبات الكلوية ، تثخن في جدار الزغيبات المعوية ، الألتهاب الرئوي الخلالي المعتدل خاصة مع الجرع العليا ، و التنخر الشديد في الحويصلات المنوية في الخصيتين ولجميع المجاميع المعاملة . كما اظهرت النتائج تغيرات معنوية في خصائص الدم ، العوامل البايوكيميائية وهرمونات الغدة الدرقية مع الزيادة المعنوية في معدلات تراكيز الأنزيمات المضادة للأكسدة وبنمط معتمد على الجرعة وفترة التعرض . سجلت اعلى قيم معدلات تراكيز الزنك في الأمعاء الدقيقة ، الكليتين، الكبد ، الطحال على التوالي متبوعة بقيم التراكيز في الخصيتين ، القلب ، الرئتين ، والدماغ وبترتيب مرتبط مع الجرعة والذي يفسر التاثيرات المسجلة ، اثبتت الدراسة امكانية استخدام معامل وزن الأعضاء كوسيلة جيدة جنباً الى جنب مع المؤشرات البايوكيميائية للكشف عن التغايرات النسيجية التي لم تسجل سابقاً على مستوى العالم كنوع من التاثيرات السامة لدقائق اوكسيد الزنك النانوي ZnO NPs في الحيوانات كموديل .

Introduction

With the vast developments in nanotechnology fields, a numerous number of various scales of nanomaterials have been utilized in different aspects of our life. Nanoparticles are defined as objects with one or more dimensions ranging from 1 to 100 nm [1]. Among metals containing nanoparticles, ZnO NPs were the third highest produced NPs over the world with 550 tons, being widely used in cosmetic products for UV light scattering, especially in sun screens, beauty products, and toothpastes [2]. ZnO NPs are mostly used in the production of solar cells, LCDs pigments, electronics, rubber, textiles and chemical fiber [3- 4]**.** Due to their high surface-to-volume ratio, greater oxidant capacity, and biopersistence, nanoparticles became the primary source for toxicity research, as they can penetrate easily through the epithelium and reach to the interstitial pulmonary area. Recently, ZnO NPs play important roles in nanomedicine, due to their high biocompatibility, easily surface functionalization, cancer targeting, and drug delivery capacity. They also demonstrated the potential to overcome the side effects of chemotherapy, radiotherapy, and surgery [5- 6] due to their large surface area, versatile surface chemistry, phototoxic effect, among other properties. In comparison with other NP types such as titanium oxide (TiO2) and others, ZnO NPs are considered as one of the most toxic types of NPs [7- 8], which is attributed to their ion shedding potency, high solubility in acidic environments, and restricted tendency in neutral one with considerable effect of particle size [9- 10]. The toxicological evaluation of zinc oxide was reported by the Scientific Committee on Cosmetic Products and Non-food Products (SCCNFP), which indicated that the LD50 of ZnO NPs for rats was more than 5-g/kg body weight, belonging to the non-toxic chemicals as illustrated by a single oral ingestion [11]. Despite the growing literature on nanomaterial applications, the information related to biological effects of nano-ZnO is still scarce and often controversial [12]. There are real needs to protect our public and environmental health and safety, especially when the standards or guidelines that can directly control the effects of the nanomaterials do not exist in the present time [13]. The objective of the current study was to illustrate the lethal as well as the sub chronic hematological, biochemical, and histopathological effects of intraperitoneally-injected ZnO NPs using three different doses that were determined depending on the LD50 values. This study is characterized by the employment of organ indexes as good tools, side by side with the biochemical indicators, to explain the histopathological changes, especially those which were not previously recorded worldwide as related to the toxicological effects of ZnO NPs in animal models.

2. Materials and methods

2.1. Nanoparticles

ZnO NPs (10-30 nm as in the specification sheet) were purchased as white to light yellow nano powder from Skyspring Nanomaterials incorporation , USA , with 99.8% purity, 5.606 g/cm^3 density and spherical morphology. Size average and surface morphology were characterized using scanning probe microscope (SPM) and atomic force microscope (AFM).

2.2. Animal Housing

Healthy adult Swiss albino male mice with an age range of 8-110 weeks and an average weight of 25±2 gm were purchased from the National Center for Drug Control and Research – Ministry of Health. All mice were housed under controlled conditions of temperature, humidity, and feeding. All

animals were treated in accordance with the guidelines of the Care and Use of Laboratory Animals-National Research Council and the international guidelines for animal experimentation.

2.3. ZnO NPs suspension preparation

The concentrations of ZnO NPs suspension were prepared with deionized distilled water and homogenized for 20 sec., then exposed to a probe ultrasound sonication in the pulsed mode in an ice bath for 60 min, as previously described [14].

2.4. Acute toxicity assay of ZnONPs and mortality percentage

The calculation of LD50 for ZnO NPs was carried out by the Probit analysis method from LD0 to LD100, using nine experimental groups and one as a control (6 animals per group). Nine series of concentrations were intraperitoneally administrated as a single dose and two replicates per concentration. Live/dead animals were counted after 48 of administration. The probit analysis was carried out as in the previously published method [15].

2.5. Sub chronic toxicity test for ZnO NPs

One control and three treated groups of mice males (10 animals per group) were used in this experiment. Depending on the calculated LD50 value, treated groups were intraperitoneally-injected with 25 mg/kg , 50 mg/kg , and 100 mg/kg of ZnO NPs, three times a week, for four weeks. The control group received 0.1 ml of distilled water. After 24 of the last dosing, blood samples were collected for hematological and biochemical tests. Organs (liver, spleen, kidney, brain , small intestine , testes, lung , and heart) were collected for the bioaccumulation study, organ index, and histopathological examination.

2.5.1 Body weight and organ index

Initial and weekly body weights of animals were recorded for all groups and the organ index was calculated for organs (liver , spleen , kidney , small intestine , brain , testis , lung , and heart) using the equation described below:

$$\text{Organ Index} = \frac{\text{Weight of organ (mg)}}{\text{Weight of the body (gm)}} \times 100$$

2.5.2. Hematological parameters

Hematology analyzer was used to measure the hematological parameters in the collected blood samples according to the manufacturer instructions and using Quality Control Reagent to assess the validity of the assays.

2.5.3. Biochemical parameters

Separated sera were used to determine biochemical parameters and hormone levels which were assayed using specific kits and Quality Control Reagents for validation.

2.5.4. Bioaccumulation study

Sub samples about of 0.25-0.5 gm of organ tissues (liver , spleen , kidney , small intestine , brain , testis , lung , heart) were obtained from the control and the three experimental groups and digested individually using a microwave digestion system [16- 17]. The concentrations of silver and zinc were detected in each sample using a flame atomic absorption spectrophotometer (AAS), with the concentrations expressed in µg of Zn/gm of the sample .

2.5.6. Histopathological study

At the end of the experiments of the toxicological study, liver, kidneys, spleen, and small intestine were collected from three animals for each of the experimental and control groups. and the organs were kept in 10% formalin, then routine histological preparations were carried out. The tissues were further examined using an optical compound microscope to detect any abnormal changes in the organ tissues.

2.6. statistical analysis

The results are presented as mean ± standard error of means (SE). Analysis of variance (ANOVA) and least significant difference (LSD) were used to explain the differences between means of parameters in treated and control groups ($p \leq 0.05$) [18].

3. Results and discussion

3.1. Characterization of ZnO NPs

According to the granularity distribution chart, the average diameter for ZnO NPs in the sample was 25.16 nm with a spherical shape as determined using AFM (Figure-1).

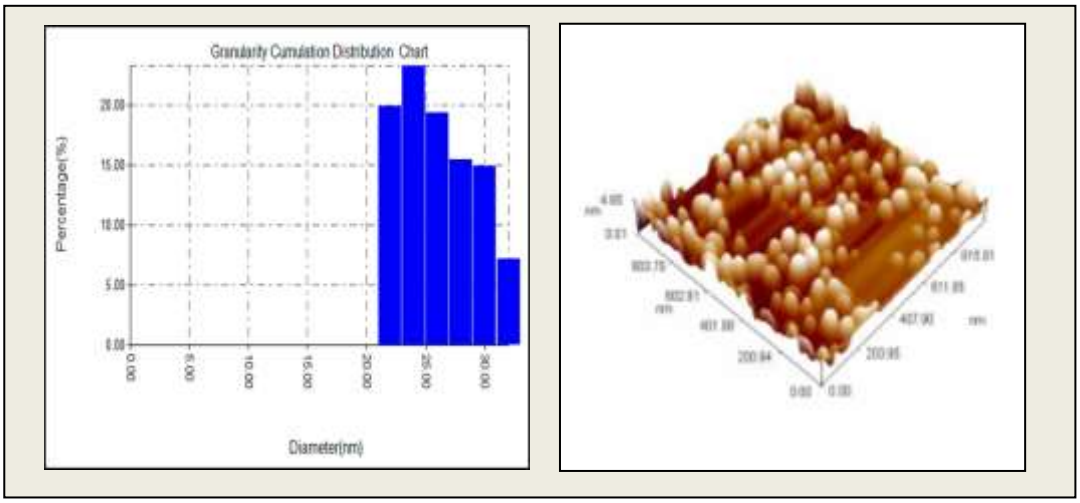


Figure 1-Characteristics of ZnO NPs, granularity distribution chart (left), and spherical shape using AFM (right) .

3.2 Acute toxicity assay of ZnO NPs

Acute toxicity study is better described as LD50, which is defined as the dose which kills 50% of animals in a group [19]. The LD50 value for the intraperitoneally-injected ZnO NPs was estimated to be 506.67mg/kg (Table-1; Figure -2). The calculated value of LD50 in the current study was lower than that recorded by a previous study [20], which was 2225 mg/kg for intraperitoneally-injected ZnO NPs in mice. The animals used in the sub-chronic study received 1/5th, 1/10th,and 1/20th of the calculated LD50, standing for 25, 50 , and 100 mg/kg

Table 1- Median lethal dose (LD50) after 48 h of intraperitoneal injection with ZnO NPs using probit analysis method

Con. (mg/kg)	log of Con.	Mortality (%)	Probits	LD50 (mg/kg)
100	2.00	0	0.00	506.67
200	2.30	16	4.01	
300	2.48	16	4.01	
400	2.60	33	4.56	
500	2.70	33	4.56	
600	2.78	50	5.00	
700	2.85	50	5.00	
800	2.90	83	5.95	
900	2.95	100	7.33	

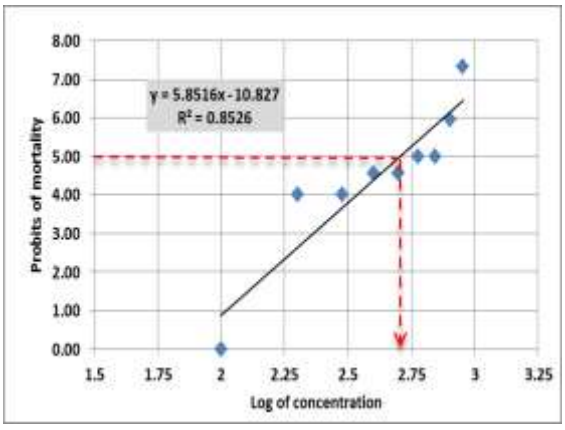


Figure 2- Toxicity curve of ZnONPs after 48 h of intraperitoneal injection.

3.3 Sub chronic toxicity

3.3.1 Body weight and organ index

Body weight in all exposed groups was decreased after 2 and 4 weeks of injection as compared with the control group. Statistically, the decrease in body weight was significant ($p \leq 0.05$) in all ZnO NPs-treated groups over the time of injection**.** Although diminished eating and drinking may contribute to decreased body weight in mice, it does not needs effect in death. Thus, injury caused by ZnO NPs in mice may be associated closely with the weight leakage [21]. Chen and his group proposed that the crossing of the brain-blood barrier (BBB) by metal NPs has a direct injurious effect on the central nervous system which probably results in anorexia and an eventual weight loos [22].

Another study [23] reported a slight decrease in body weight after daily intraperitoneal injection with ZnO NPs with concnetrations of 1, 10 , and 100 mg/kg for 14 days. On the other hand, a slight increase was recorded after oral administration of the same doses. In toxicological evaluation studies, the organ indexes were vastly used to provide a general concept of toxicity [24]. In this study, clear increases in the organ index for kidney , heart , lung , and brain were recorded for all three groups over 2 and 4 weeks of injection compared with control group. Some increase in the small intestine's organ index was also observed, especially after 4 weeks in G2 and G3. These increased values were well correlated with the ZnO NPs accumulation in the organs, as well as with some histopathological effects such as the swelling in renal tubules and some thickness in the small intestine villi that lead to increased value of the organ index. Focal necrosis associated with infiltration of mononuclear leukocytes in lungs was also associated with the mentioned increase in lung weight index. Results of this study demonstrate a considerable decrease in the mean values of the testes organ index, which refers to a severe damage in the testicular parenchyma and disorganization and severe necrosis of the seminiferous tubules in all groups after 4 weeks of injection with different ZnO NPs doses. The decreased spleen index, except after 2 weeks in G2, and liver index values in comparison with the control group were histologically manifested as multiple hemorrhagic foci and sever hepatitis (Table-2 and 3). From all the results above, this study concluded a considerable association between weight loss and the toxic effects of ZnO NPs, which appeared to be dose dependent .

Table 2- Weekly change in body weight (gm) after 4 weeks of intraperitoneal injection with different doses of ZnONPs.

Body weight (gm)	Experimental groups			
	Control	weight (gm) (G1) 25 mg/kg ZnONPs	weight (gm) (G2) 50 mg/kg ZnONPs	weight (gm) (G3) 100 mg/kg ZnONPs
Time zero	26.7 ± 0.36 a	27.7 ± 0.66 ab	28.6 ± 0.55 c	28.4 ± 0.48 c
1st. week	27.8 ± 0.67 a	27.2 ± 0.97 ab	27.6 ± 1.08 c	27.5 ± 0.95 c
2nd. week	28.4 ± 1.16 a	26.7 ± 1.25 ab	24.0 ± 1.14 b	25.9 ± 1.06 c
3 rd. week	29.8 ± 1.37 a	24.5 ± 1.33 a	22.5 ± 0.70 ab	22.0 ± 1.43 b
4 th week	30.7 ± 1.16 b	24.3 ± 1.19 a	20.2 ± 0.81 a	19.3 ± 1.21 a
LSD	4.00	3.05	2.50	2.70

All data are expressed with mean ±standard error of means (SEM). Similar small letters indicate no significant difference between mean values on $p<0.05$

Table 3- Organ index values after 2 and 4 weeks of intraperitoneal injection with different doses of ZnONPs.

Organ index

Organs	Control	G1 (25 mg/kg ZnO NPs)		G2 (50 mg/kg ZnO NPs)		G3 (100 mg/kg ZnO NPs)		LSD
		2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	
Liver	6.40 ± 0.10 c	5.78 ± 0.12 a	5.83 ± 0.29 a b	6.32 ± 0.23 bc	6.73 ± 0.12 c	5.77 ± 0.07 a	6.05 ± 0.08 ab	0.53
Spleen	0.64 ± 0.05 ab	0.73 ± 0.02 c	0.60 ± 0.04 ab	0.64 ± 0.03 ab	0.67 ± 0.04 bc	0.57 ± 0.01 a	0.61 ± 0.01 ab	0.09
Kidney	1.35 ± 0.03 a	1.53 ± 0.08 ab	1.55 ± 0.03 ab	1.46 ± 0.02 ab	1.58 ± 0.03 ab	1.65 ± 0.03 b	2.04 ± 0.14 c	0.24
Small intestine	12.50 ± 0.03 b	9.95 ± 0.25 a	11.37 ± 0.17 ab	11.25 ± 0.66 ab	14.37 ± 0.38 ab	11.61 ± 0.41 ab	19.18 ± 0.74 c	1.82
Testes	1.20 ± 0.01 c	0.88 ± 0.05 b	0.67 ± 0.10 a	0.88 ± 0.03 b	0.68 ± 0.05 a	0.76 ± 0.04 ab	0.65 ± 0.01 a	0.15
Brain	1.43 ± 0.00 a	1.61 ± 0.02 b	1.76 ± 0.01 c	1.76 ± 0.03 c	2.07 ± 0.01 d	1.61 ± 0.04 b	2.16 ± 0.04 d	0.09
Lung	0.83 ± 0.02 a	0.86 ± 0.04 ab	0.92 ± 0.04 ab	0.94 ± 0.03 bc	1.10 ± 0.04 d	0.85 ± 0.00 ab	0.98 ± 0.03 c	0.11
Heart	0.50 ± 0.00 a	0.66 ± 0.01 bc	0.65 ± 0.02 b	0.73 ± 0.02 de	0.75 ± 0.00 e	0.67 ± 0.03 bc	0.70 ± 0.01 cd	0.05

All data expressed with mean ± standard error of means (SEM), simillar small letters means no significant difference between means values on $p<0.05$

3.3.2. Hematological parameters

All ZnO NPs-treated groups exhibited clear decreases in red blood cells (RBC) count and hemoglobin (Hb) values ($p \leq 0.05$), along with an increase in the concentration and prolonged injection period in comparison with the control group (Table-4**).** The lowest mean values of RBC counts and Hb concentrations were recorded with clear differences among the groups which were related to anemia due to the increase in immunogenic responses [25], or among subjects with disorders in signaling pathways and disturbance in cell maturation that affects RBC's division and other cell development events [26]. According to another report [27], growth inhibition and anemia were induced by copper and iron deficiency as excessive zinc was present in animal dietary, which was also confirmed later by other studies [28-29]. White blood cells (WBCs) are considered the functionally active cells of specific and nonspecific immune responses, with their count reflecting a whole picture of the immune system function [30]. WBCs mean counts were decreased in all groups, reaching their lowest values after 4 weeks of injection. This result indicates the suppression of the immune system that renders the animal susceptible to any external dangerous agent [31], which could explain the clear histopathological effects that are described below. These results are in agreement with those obtained by other authors [32] after intravenous and intraperitoneal injection with 96 nm Cu NPs and ZnO NPs in male Wistar rats for 14 and 28 days. Ben-Slama and her group stated an increased WBC count with no significant difference compared with the control group after five days of 10 mg/kg ZnO NPs oral administration [33]. The decrease in lymphocyte and monocyte counts follows the same manner described for the WBC in ZnO NPs-injected groups, which marks an immune system dysfunction [34].

3.3.3. Biochemical parameters

A significant increase ($P \leq 0.05$) was found in CHO, TG, and LDL concentrations in the experimental groups, in a manner that depended on both the injected dose and time of exposure. The highest mean values recorded were 130.00± 00.82 mg/dL , 104.00±2.08 mg/dL , and 83.10±0.41 mg/dL for CHO , TG , LDL, respectively after 4 weeks of treatment in G3 (Table-4). This elevation in CHO levels was related to the increase in the level of LDL-associated cholesterol, as indicated by the apparently unchanged HDL concentration. According to a previous investigation [35], these deteriorations in lipid profile could fundamentally lead to the induction of inflammation and oxidative stress along with increased production of free radicals resulting, as a result of treatment with ZnO NPs. These are the major mechanisms that can trigger lipid metabolism deterioration by the interaction of generated reactive oxygen species with unsaturated chains of fatty acid in the membrane lipids, leading to lipid peroxidation that promotes the lipid profile's disturbance [36].

Many previous studies [27, 37, 23] demonstrated that excess zinc oxide adminstration can result in liver damage and dysfunction, leading to elevated serum levels of liver enzymes such as AST , ALT , and ALP. These elevations in levels of liver function markers in response to the adminstration of ZnO NPs were correlated to the elevation in the intracellular Zn concntration that eventually led to hepatic injury [27]. Another report [19] also elucidated that the elevation of intracellular zinc concentration levels can drive hepatic injury or failuer. The current study showed significantly increased concentrations of AST , ALT , and ALP ($p \leq 0.05$) in mice injected for 4 weeks with 25 , 50 , and 100 mg/kg ZnO NPs in comparison with the control group. These results still suggested a liver damage or dysfunction which is confirmed by the histopathological finding described later. These results come in agreement with those of a previous investigation [23] which showed liver dysfunction in ICR mice after intraperitoneal and oral adminstration of 1, 10 , and 100 mg/kg ZnO NPs . Also, another study [38] showed a clear a dose-dependent increase in the levels of liver enzymes after adminstration of ZnO NPs , as also described later after intravenous and intraperitoneal administration of three different doses of ZnO NPs [32]. The results demonstrated significant increase ($p \leq 0.05$) in urea concentration in the experimental groups compared with the control one, with a clear concentration-dependent manner which marked the severity of toxicological dysfunction effects in the kidney. These effects are confirmed by the high creatinine mean concentrations that provides similar implications as those derived from levels of urea, especially under the consideration of using creatinine as a good index of renal function [39- 40]. These increased levels in urea and creatinine were used as markers of kidney damage which was also proved by histological microscopic examination. A statically significant and dose-dependent increase ($p \leq 0.05$) in serum mean levels of SOD and GPx enzymes was also observed in the treated animals. This increase could be related to the increase in oxidative stress,

as stated by previous study [41]. In addition, the results showed of reductions in meanvalues of T3 and T4 hormones, with significant difference between the treated and control groups ($p \leq 0.05$). These reductions in hormone concentrations might be related to the increased oxidative stress [42], as a result of the high levels of ROS generated by nanoparticles in addition to the extensive ability of these particles of tissue penetration that lead to inflammation or structural and functional disruption [43-44]. On the other hand, the results also recorded TSH mean values that were significantly increased in the ZnO NPs-injected groups ($p \leq 0.05$) compared with the control one. Such an elevation was obvious in the three experimental groups G1, G2 , and G3. The highest mean value was recorded in G3 with 0.26 ±0.01 µU/ml. Previous data suggested that the high intake of zinc can change the processes of thyroid hormones production and secretion, in addition to the reduction of thyroid effects which may be due to the elimination of the regulatory action of thepituitary gland, and the attenuation in circulated thyroid hormones that may mark the hypothyroidism as Zn toxicity [45]. Also, zinc-induced thyroid hormone reduction could be related to its effects on the activity of deiodinase through its antioxidant properties [46]. Results of the current study agree with those of another group [47] which demonstrated the deleterious effects resulting from the intraperitoneal injection of 5, 10. 20 and 40 mg/kg ZnO NPs in rats, as represented by the dose-dependent increased TSH levels, which was significant with the high dose .

Table 4- Hematological and biochemical parameters means values in mice after 2 and 4 weeks in control and intraperitoneal injected groups with different doses of ZnO NPs 25 mg/kg (G1) , 50 mg/kg (G2) , and 100 mg/kg (G3)

Parameters	Experimental groups								LSD
	control		G1 (25 mg/kg) ZnO NPs		G2 (50 mg/kg) ZnO NPs		G3 (100 mg/kg) ZnO NPs		
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	
RBC ($\times 10^6$/ul)	8.33 ± 0.09 ^{de}	8.37 ± 0.12 ^{c}	8.45 ± 0.04 ^{e}	7.61 ± 0.13 ^{c}	8.22 ± 0.01 ^{d}	6.71 ± 0.02 ^{b}	8.16 ± 0.00 ^{d}	6.33 ± 0.08 ^{a}	0.19
Hb (g/ml)	12.53 ± 0.15 ^{d}	12.57 ± 0.31 ^{d}	12.30 ± 0.00 ^{d}	11.05 ± 0.04 ^{c}	11.70 ± 0.00 ^{cd}	9.75 ± 0.04 ^{b}	10.80 ± 0.00 ^{c}	8.14 ± 1.04 ^{a}	1.03
WBC ($\times 10^3$/ul)	9.00 ± 0.44 ^{c}	9.00 ± 0.38 ^{bc}	17.67 ± 0.34 ^{e}	7.05 ± 0.04 ^{b}	11.93 ± 0.03 ^{d}	7.05 ± 0.12 ^{b}	9.20 ± 0.00 ^{c}	2.65 ± 0.04 ^{a}	0.67
lymphocyte ($\times 10^3$/ul)	65.50 ± 0.10 ^{e}	60.83 ± 2.74 ^{d}	62.55 ± 0.35 ^{d}	47.25 ± 0.25 ^{b}	51.35 ± 1.15 ^{c}	44.50 ± 0.00 ^{b}	50.55 ± 3.85 ^{c}	22.05 ± 0.75 ^{a}	2.84
Monocyte ($\times 10^3$/ul)	2.80 ± 0.37 ^{e}	2.90 ± 0.37 ^{d}	2.20 ± 0.08 ^{d}	1.10 ± 0.00 ^{b}	1.45 ± 0.04 ^{c}	0.90 ± 0.00 ^{ab}	0.95 ± 0.12 ^{b}	0.70 ± 0.08 ^{a}	0.25

All data expressed with mean ± standard error of means (SEM), similar small letters means no significant difference between means values on $p<0.05$

Parameters	Experimental groups								LSD
	control		G1 (25 mg/kg) ZnO NPs		G2 (50 mg/kg) ZnO NPs		G3 (100 mg/kg) ZnO NPs		
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	
CHO (mg/dL)	123.00 ± 1.15 ^{ab}	124.00 ± 1.53 ^{ab}	122.50 ± 2.04 ^{ab}	125.50 ± 0.41 ^{bc}	123.0 ± 2.31 ^{ab}	118.7 ± 1.20 ^{a}	128.7 ± 1.86 ^{c}	130.0 ± 0.82 ^{c}	5.59
TG (mg/dL)	92.00 ± 2.00 ^{a}	92.67 ± 1.76 ^{a}	94.67 ± 2.40 ^{ab}	97.00 ± 1.53 ^{ab}	100.3 ± 2.60 ^{bc}	94.67 ± 3.48 ^{ab}	101.3 ± 2.40 ^{bc}	104.0 ± 2.08 ^{c}	7.35
HDL (mg/dL)	27.67 ± 0.67 ^{a}	28.33 ± 0.67 ^{a}	27.67 ± 0.88 ^{a}	26.67 ± 0.88 ^{a}	27.00 ± 0.82 ^{a}	26.33 ± 1.20 ^{a}	27.00 ± 0.82 ^{a}	26.00 ± 0.58 ^{a}	2.42
LDL (mg/dL)	75.00 ± 1.15 ^{a}	74.00 ± 2.08 ^{a}	75.20 ± 0.96 ^{a}	76.20 ± 0.82 ^{ab}	77.90 ± 3.18 ^{ab}	79.00 ± 1.63 ^{b}	77.90 ± 1.06 ^{ab}	83.10 ± 0.41 ^{c}	3.48
TP (gm/dL)	5.80 ± 0.06 ^{c}	5.83 ± 0.03 ^{c}	5.70 ± 0.06 ^{bc}	5.63 ± 0.03 ^{ab}	5.63 ± 0.03 ^{ab}	5.53 ± 0.03 ^{a}	5.63 ± 0.03 ^{ab}	5.53 ± 0.03 ^{a}	0.13
AST (U/L)	71.67 ± 1.20 ^{a}	72.00 ± 1.15 ^{a}	78.00 ± 1.53 ^{b}	92.00 ± 1.73 ^{c}	92.00 ± 2.31 ^{c}	94.00 ± 2.00 ^{c}	96.67 ± 1.45 ^{cd}	101.7 ± 1.67 ^{d}	5.25
ALT (U/L)	13.00 ± 1.00 ^{a}	12.33 ± 1.33 ^{a}	11.67 ± 0.88 ^{a}	14.00 ± 1.15 ^{a}	14.33 ± 0.88 ^{a}	18.00 ± 1.53 ^{b}	18.00 ± 0.58 ^{b}	20.33 ± 1.20 ^{b}	3.24
ALP (U/L)	93.00 ± 1.00 ^{c}	92.33 ± 0.33 ^{c}	85.00 ± 3.27 ^{a}	90.67 ± 1.45 ^{b}	94.00 ± 2.89 ^{c}	88.00 ± 1.63 ^{ab}	92.50 ± 0.41 ^{bc}	99.00 ± 0.58 ^{d}	4.94
ALB (gm/dL)	3.27 ± 0.03 ^{a}	3.30 ± 0.00 ^{a}	3.30 ± 0.06 ^{a}	3.23 ± 0.03 ^{a}	3.37 ± 0.03 ^{a}	3.30 ± 0.03 ^{a}	3.33 ± 0.03 ^{a}	3.22 ± 0.03 ^{a}	0.17
BIL (mg/dL)	0.34 ± 0.02 ^{a}	0.35 ± 0.01 ^{a}	0.35 ± 0.00 ^{a}	0.41 ± 0.00 ^{b}	0.47 ± 0.01 ^{d}	0.42 ± 0.01 ^{b}	0.44 ± 0.00 ^{c}	0.48 ± 0.01 ^{d}	0.03
UR (mg/dL)	29.67 ± 0.33 ^{a}	29.33 ± 0.67 ^{a}	28.00 ± 0.82 ^{a}	35.50 ± 0.41 ^{bc}	33.50 ± 0.41 ^{b}	36.50 ± 2.04 ^{c}	39.50 ± 0.41 ^{d}	42.50 ± 1.22 ^{e}	2.15
CR (mg/dL)	0.35 ± 0.03 ^{a}	0.34 ± 0.03 ^{a}	0.44 ± 0.01 ^{b}	0.48 ± 0.03 ^{bc}	0.52 ± 0.01 ^{cd}	0.57 ± 0.04 ^{ef}	0.53 ± 0.02 ^{de}	0.65 ± 0.00 ^{g}	0.05
SOD (U/L)	10.63 ± 0.38 ^{a}	10.00 ± 1.01 ^{a}	11.47 ± 0.49 ^{ab}	11.93 ± 0.13 ^{ab}	12.83 ± 0.18 ^{bc}	12.97 ± 0.09 ^{bc}	13.30 ± 1.10 ^{c}	13.60 ± 0.40 ^{c}	1.54
GPx (U/L)	6.93 ± 0.38 ^{a}	6.87 ± 0.07 ^{a}	8.65 ± 0.12 ^{bc}	8.20 ± 0.08 ^{b}	9.00 ± 0.40 ^{c}	9.00 ± 0.08 ^{c}	9.30 ± 0.24 ^{c}	11.20 ± 0.64 ^{d}	0.90
T3 (ng/ml)	0.88 ± 0.01 ^{b}	0.88 ± 0.01 ^{b}	0.84 ± 0.02 ^{ab}	0.83 ± 0.02 ^{ab}	0.82 ± 0.04 ^{ab}	0.78 ± 0.03 ^{a}	0.79 ± 0.02 ^{a}	0.78 ± 0.02 ^{a}	0.07
T4 (ng/ml)	4.83 ± 0.01 ^{c}	4.83 ± 0.01 ^{c}	4.80 ± 0.03 ^{bc}	4.79 ± 0.08 ^{bc}	4.75 ± 0.11 ^{ab}	4.72 ± 0.01 ^{ab}	4.71 ± 0.00 ^{ab}	4.65 ± 0.04 ^{a}	0.12
TSH (ng/ml)	0.21 ± 0.00 ^{a}	0.21 ± 0.01 ^{a}	0.21 ± 0.02 ^{a}	0.21 ± 0.00 ^{a}	0.22 ± 0.01 ^{ab}	0.24 ± 0.02 ^{bc}	0.23 ± 0.00 ^{b}	0.26 ± 0.01 ^{c}	0.03

Total cholesterol (CHO) ,triglyceride (TG) , high density lipoprotein (HDL) , light density lipoprotein (LDL), total protein (TP) , aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP) , albumin (ALB), bilirubin (BIL), blood urea (UR) , creatinine (CR) , Super oxide dismutase (SOD), Glutathion peroxidase (GPx) , thyroid hormones (T3) , (T4), and thyroid stimulating hormone (TSH) . All data expressed with mean ± standard error of means (SEM), similar small letters means no significant difference between means values on $p<0.05$

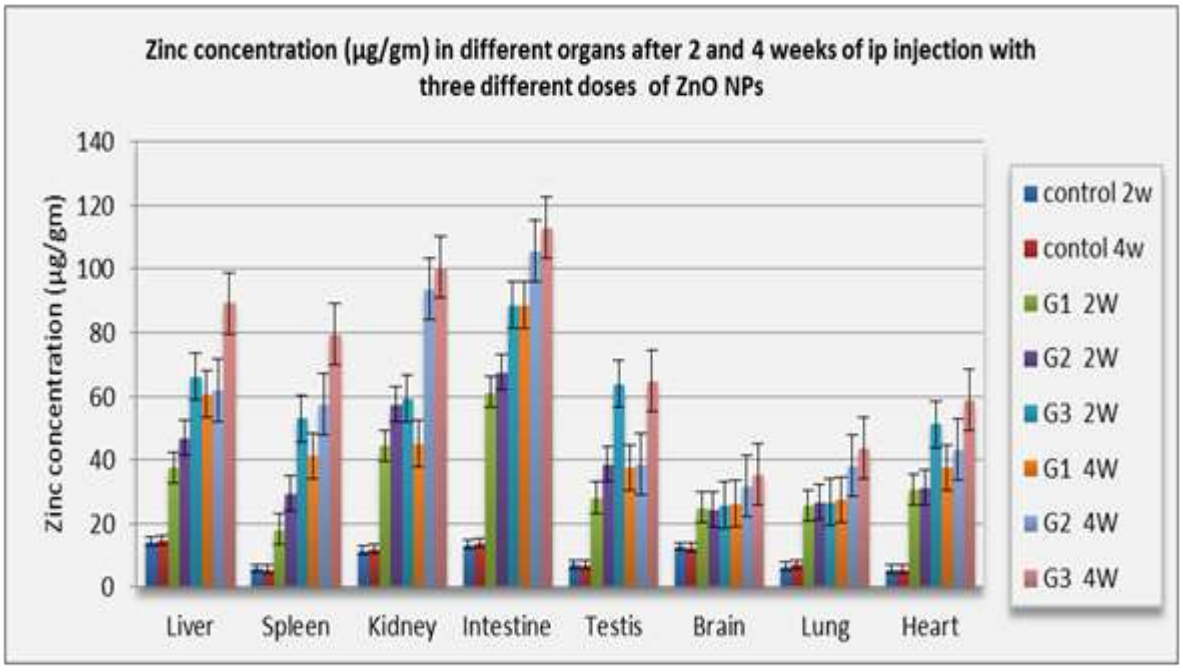
3.3.4 Bioaccumulation study

Among their unique properties, nanoparticles have a tendency to aggregate. Furthermore, some metal-containing nanoparticles have the ability of forming and releasing ions that easily enter the blood stream and reach the vital organs. The overload of metal ions may lead to a serious tissue damage [48-49]. In the present study, the accumulation of ZnO NPs (G1 25, G2 50 , and G3 100 mg/kg) in different organs was determined after 2 and 4 weeks of repeated intraperitoneal injection. Statistically, highly significant differences ($p \leq 0.05$) were obvious among concentration means in different organs, with a tendency to increase as the dose concentration and treatment duration increase. Liver, the major reticuloendothelial phagocytic system, recorded significant levels of Zn, especially after 4 weeks of injection in the G3 experimental group (89.38±1.53 µg/gm; Figure-3; table 5). This is attributed to the highly capturing and retaining capacity [50 - 51] of Kupffer cells mainly, followed by hepatocytes and endothelial cells [52- 53]. Important concentrations of Zn were observed in spleen after 2 and 4 weeks of injection in all groups (with a range of 5.94±0.18 µg/gm -79.38 ±0.51 µg/gm). These values can be explained by the massive filtering activity by non specific scavenging properties of the red pulp macrophages and marginal zone to nanoparticle uptake [54]. Roughly all nanoparticle types, especially those with small dimensions of size, are known to have a high probability of clearance through kidneys. Thus, they have a high chance of accumulation and causing some adverse effects [55]. Important mean values of Zn were observed in kidney (in a range of 11.56±0.18 µg/gm – 100.63±0.51 µg/gm), again with dose, concentration. and duration-dependent manner . These considerable levels may indicate the deposition of an uncleared portion of Zn in the basement membrane of renal glomerulus units [56]. The highest mean levels of accumulated Zn were found in the intestine in all experimental groups and over the two time points of 2 and 4 weeks (with a range of 13.44±0.18 µg/gm -113.13±0.51 µg/gm), iindicating the high excretion ability of the intraperitoneally-injected ZnO NPs by the intestine into the feces . In the testes, considerable concentrations were observed in ZnO NPs treated groups compared with the control group. The highest mean value(56.00±2.04 µg/gm) were observed in G3 after 4 weeks of repeated injection, with non significant difference compared to those recorded in the same group after 2 weeks of injection. These results identified the ability of ZnO NPs to cross the blood- testes barrier with an obvious deposition ability in testes, which explains the potential adverse effects [57]. Gradual accumulation of Zn in the brain in a dose and duration-dependent manner was also demonstrated (with a range of 12.81±0.18 µg/gm -35.63±0.51 µg/gm), but still with lower levels than those recorded in other organs [28]. Other researches demonstrated the ability of ZnO NPs to penetrate the BBB and considered brain as one of the target organs. In the lung, the bioaccumulation profile followed a trend that depended on the administrated dose, recording their highest mean levels of Zn after 4 weeks in the G3 experimental groups (43.75±1.02 µg/gm). This trend was explained by a numbers of researchers to be related to an increased deposition percentage in the sub epithelial zone of basement membrane. The predominant accumulation of ZnO NPs in lungs of Sprague-Dawley rats was illustrated [58] after two weeks of repeated exposure at levels relevant to occupational exposure levels (<5 mg/m3). In the heart, the highest mean levels of 56.75± 3.06 µg/gm were observed in G3 after 4 weeks of repeated injection. These results agree with a number of previous studies. One investigation [59] evaluated the level of ZnO NPs deposition in the heart, following the oral administration of 100 and 300 mg/kg ZnO NPs for 28 days. The authors noted a significant increase in Zn concentration in the heart compared with the control group.

Table 5 - Zinc concentrations means in different organs after 2 and 4 weeks in control and intraperitoneal injected groups with different doses of ZnO NPs , G1:25 mg/kg , G2: 50 mg/kg , and G3: 100 mg/kg

Organs	Experimental groups								LSD
	control		G1 (25 mg/kg) ZnO NPs		G2 (50 mg/kg) ZnO NPs		G3 (100 mg/kg) ZnO NPs		
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	
Liver	14.39 ± 0.18 ^{a}	14.75 ± 0.00 ^{a}	37.50 ± 0.00 ^{b}	60.63 ± 0.51 ^{d}	46.88 ± 0.51 ^{c}	61.88 ± 0.51 ^{d}	68.25 ± 0.51 ^{e}	89.38 ± 1.53 ^{f}	1.84
Spleen	5.94 ± 0.18 ^{a}	5.63 ± 0.36 ^{a}	18.13 ± 0.28 ^{b}	41.25 ± 1.02 ^{d}	29.38 ± 1.53 ^{c}	57.50 ± 1.02 ^{f}	53.13 ± 0.28 ^{e}	79.38 ± 0.51 g	1.88
Kidney	11.56 ± 0.18 ^{a}	12.19 ± 0.18 ^{a}	44.38 ± 0.28 ^{b}	45.00 ± 0.00 ^{b}	57.50 ± 1.02 ^{c}	93.75 ± 0.00 ^{d}	59.38 ± 0.77 ^{c}	100.6 ± 0.51 ^{e}	1.77
Small intestine	13.44 ± 0.18 ^{a}	13.75 ± 0.00 ^{a}	61.25 ± 0.51 ^{b}	88.75 ± 1.02 ^{d}	67.50 ± 1.02 ^{c}	105.6 ± 0.51 ^{e}	88.75 ± 0.51 ^{d}	113.1 ± 0.51 ^{f}	1.77
Testes	7.19 ± 0.18 ^{a}	6.88 ± 0.00 ^{a}	28.13 ± 0.28 ^{b}	37.50 ± 0.00 ^{c}	38.75 ± 1.02 ^{c}	38.75 ± 1.02 ^{c}	63.75 ± 0.51 ^{d}	85.00 ± 2.04 ^{d}	2.24
Brain	12.81 ± 0.18 ^{a}	12.50 ± 0.00 ^{a}	25.00 ± 0.00 ^{bc}	26.25 ± 1.02 ^{d}	24.38 ± 0.51 ^{b}	31.88 ± 0.51 ^{e}	25.63 ± 0.28 ^{cd}	35.63 ± 0.51 ^{f}	1.19
Lung	6.56 ± 0.18 ^{a}	7.19 ± 0.18 ^{a}	25.63 ± 0.28 ^{b}	27.50 ± 0.00 ^{d}	26.88 ± 0.51 ^{cd}	38.13 ± 0.51 ^{e}	26.88 ± 0.28 ^{cd}	43.75 ± 1.02 ^{f}	1.19
Heart	5.63 ± 0.00 ^{a}	5.66 ± 0.00 ^{a}	30.63 ± 0.28 ^{b}	37.50 ± 0.00 ^{c}	31.25 ± 0.00 ^{b}	43.13 ± 0.51 ^{d}	51.25 ± 0.51 ^{e}	58.75 ± 3.08 ^{f}	2.68

Figure 3- Zinc concentrations means in different organs after 2 and 4 weeks of of intraperitoneal injection with different doses of ZnO NPs , G1:25 mg/kg , G2: 50 mg/kg , and G3: 100 mg/kg



3.3.5. Histopathological study

Normal cytoarchitecture of liver lobules and hepatocytes were observed in most liver sections of G1 after 4 weeks of injection with 25 mg/kg ZnO NPs (Figure 4-B). The zone of hepatocytes showed mild cellular swelling with hyper cellulartiy of kupffer cells at the lobules periphery in G2 (50 mg/kg ZnO NPs) (Figure 4-C). In G3, these effects were developed to show disorganized hepatic lobules and hepatic cords with severe cloudy swelling of hepatocytes in the level of the sub capsular area. The

whole liver parenchyma showed multiple hemorrhagic foci, which was first detected as a histopathological effect of ZnO NPs, along with severe hepatitis associated with infiltration of mononuclear cells after injection with 100 mg/kg ZnO NPs (Figure 4-D). Thus was correlated with the above mentiioned increase in the levels of ALT and AST in the blood, which can be used as good indicators for the hepatic alternations on the cellular level. Therefore, the considerable increase in the activity of these enzymes in mice treated with ZnO NPs could explain the ability of this type of nanoparticles to cause hepatic injury. Normal renal cortex and medulla appeared in kidney sections after 4 weeks of injection in G1 (Figure 5-A), while the sections showed severe cloudy swelling in the tubules of the renal cortex and medulla in G2 (Figure5-C). In addition to these marked signs, other tubules showed mild cloudy swellings with mild dilatation of the collecting tubules in G3 (Figure4-D). These results were indicated by previous biochemical tests of renal function, with elevation in urea and creatinine mean levels and increase in the organ index of kidney, showing possible renal damages that were approved the by histopathological examination. The damage in membrane sodium-potassium pumping process may have led to the observed swelling in the renal tubules in some groups treated with ZnO NPs, as a first appearance of almost all sorts of cellular injury that led to the increase in kidney organ index as mentioned before. The results of ZnO NPs histopathological examination in the current study agree with previous studies in regard to the demonstration of glomeruli epithelial cell degeneration and swelling in the renal tubules after 5 days of oral administration of 333.33 mg/kg ZnO NPs (20-30 nm) in Wister female rats [1], and after 75 days of oral administration of 100 mg/kg of ZnO NPs (100 nm) in Wister male rats [60]. No effects were detected in the spleens of all three ZnO NPs treated groups (Figure 6- B, C and D). Sections of the small intestine in G1group were similar to those in the control (Figure 7-B), while the sections illustrated moderate enteritis in G2, characterized by thickening of the villi with focal thickening of the lamina propria associated with disappearance of the crypts duet infiltration of mononuclear leukocytes (Figure 7-C). This thickening in the villi was marked with an increase in the population of goblet cells associated with mucus in G3 (Figure 7-D). The results of the current study clearly indicated the disrupting effects of the intraperitoneally-administrated ZnO NPs on the normal structure of microvilli that led to infiltration of inflammatory cells and mucoid degeneration as a response to the attempts of the nanoparticles to cross the protective small intestinal barrier and disturb the function of microvilli. From these histological results, we can explain the observed reduction in body weight in the experimental groups as resulting from the impairing effects on the microvilli, leading to the reduction of the absorption function. The infiltration of inflammatory cells, mainly lymphocytes. along with the thickening in the villi and the increased mucoid degeneration clearly explained the increase in the intestinal weight index. Normal appearance of lung parenchyma was shown after 4 weeks of injection in G1 and G2 (Figures 8-B and C). A first worldwide record of multiple focal necroses as an adverse effect of intraperitoneally-injected ZnO NPs is indicated here and associated with infiltration of mononuclear leukocytes (lymphocytes) and moderate interstitial pneumonia, as illustrated in G3 (Figure 8-D). These effects were also correlated with increased serum levels of antioxidant enzymes (SOD and GPx) and increased lung organ index compared with the control group, as mentioned before. It was previously shown [61] that rapid ZnO NPs dissolution inside the phagosomes was the main reason of the severe lung injury. Moderate to severe inflammation and alveolar wall devastation with moderate interstitial inflammation were also illustrated by another investigation [23] after 14 days of intraperitoneal injection with 1, 10 and 100 mg/kg ZnO NPs. An obvious alveolar inflammation was also reported [62] after eight weeks of the last dose of intratracheal instillation of ZnO NPs. In comparison with the control, the sections of testes in G1 , G2 , and G3 (Figure9-B,C, and D) showed severe damages of the testicular parenchyma, disorganized and severely necrotic seminiferous tubules, and macrovacuolation of germ cells, which may be related to the inflammatory response and explains the above mentioned significant decrease in testes weight index . This is the first record of this type of ZnO NPs histopathological effects in the testes following the intraperitoneal injection of all studied doses. A previous investigation [63] demonstrated a degeneration in seminiferous tubules with interstitial edema in rats after 10 week intraperitoneal injection with 5 mg/kg ZnO NPs. However, no obvious histopathological effects on the testes were reported by another study [64] after 6 weeks of 500 mg/kg oral administration in rats .

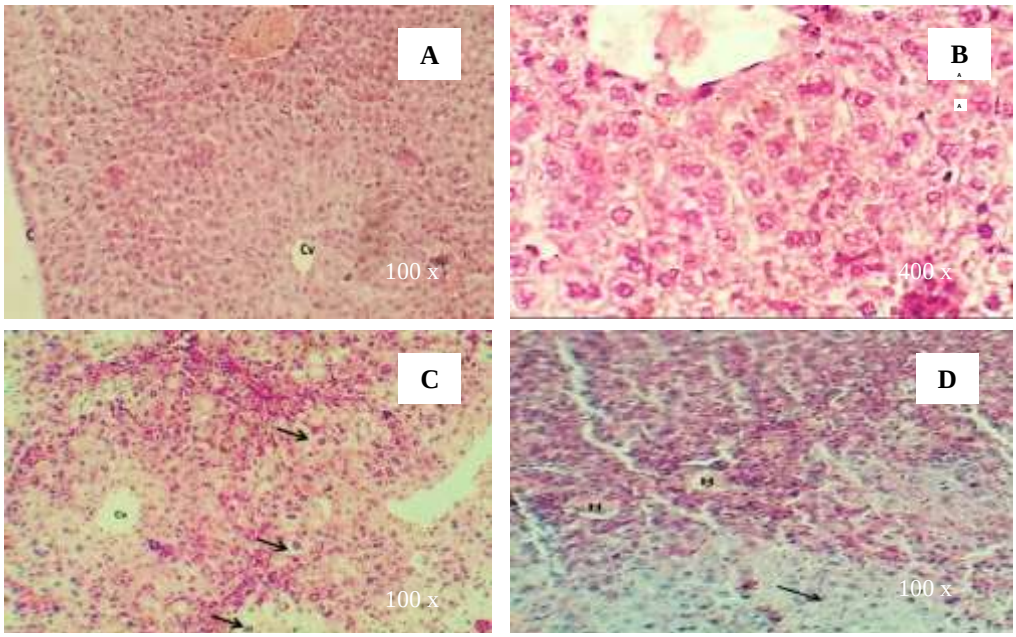


Figure 4 – Liver sections showing histopathological effects in control and G1, G2, and G3 groups after 4 weeks of intraperitoneal injection with 25 , 50 , and 100 mg/kg ZnO NPs, respectively. Control group (A) shows normal structure of the liver including the capsule, central vein (Cv), Hepatocyte cords, and sinusoids. (B) normal appearance of liver G2, (C) swelling of hepatocyte at the periphery of lobules (Arrows), (D) area of cellular swelling (arrows) & multi hemorrhagic foci (H) in G3. H&E stain.

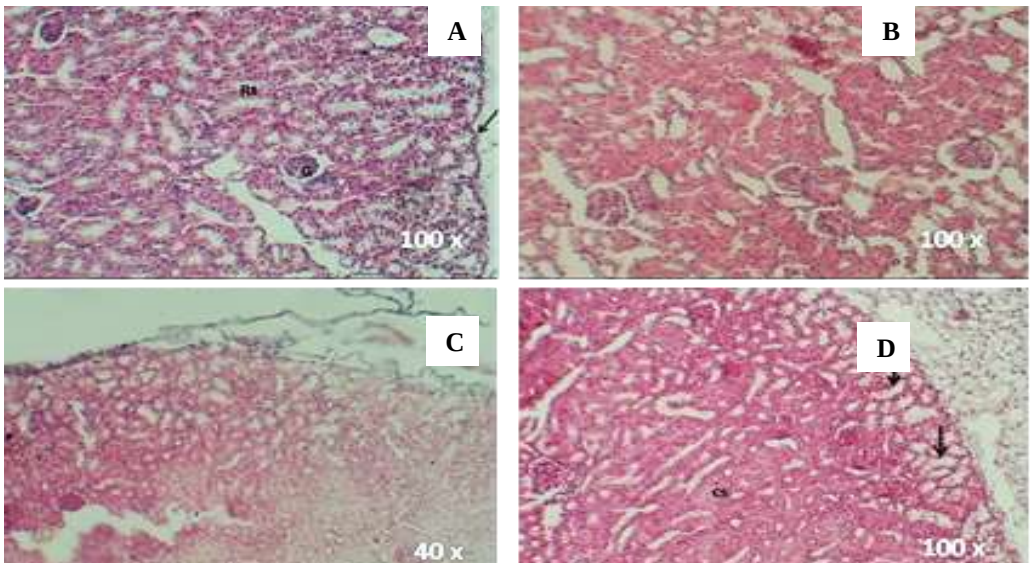


Figure 5- Sections in kidney. Control group (A) section in renal cortex shows capsule (arrow), glomerulus and normal renal tubules (Rt), normal appearance of renal parenchyma G1(B), cortical cloudy swelling of renal tubules in G2 group (C), sub capsular vacular degeneration (arrows) & cloudy swelling in G3(D). H&E stain.

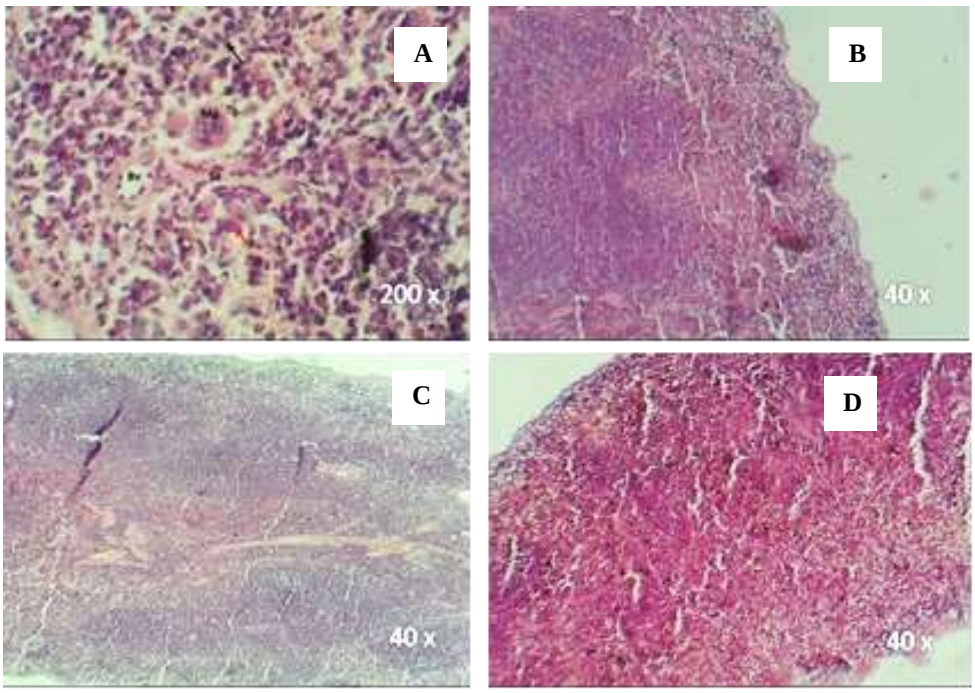


Figure 6- Sections in the spleen. Control group (A) a magnified section showing megakaryocytes (Mg), lymphocytes (black arrow), macrophages (red arrow), blood vessel (Bv) & septum (se), normal appearance of splenic parenchyma in G1(B) , G2(C) , G3(D)**.** H&E stain.

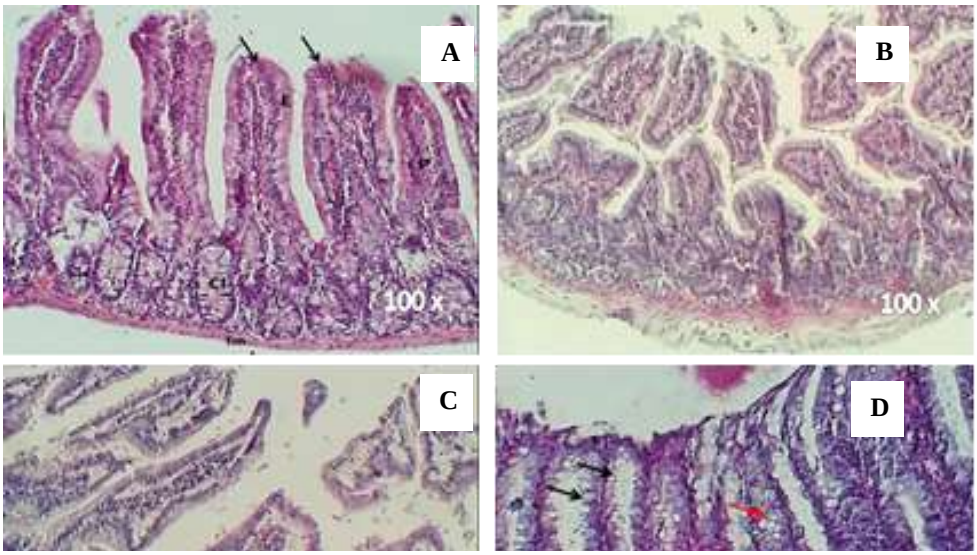


Figure 7- Sections in the small intestine. Control group (A) showing villi (arrows) , lamina propria (P), epithelium (E), crypt of Lieberkühn (Cl) and tunica muscularis (Tm) in G1(B) and G2(C), normal appearance of intestinal villi and epithelial crypts , increased goblet cells (Black arrows) & muocoid degeneration (Red arrow) in G3 (D). H&E stain.

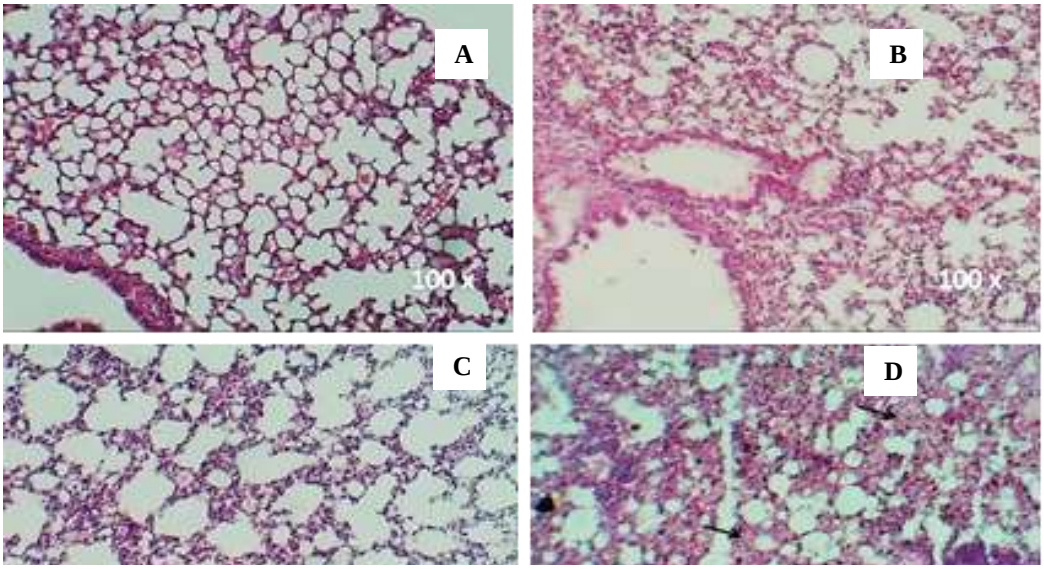


Figure 8- Sections in the lung. Control group (A) showing bronchioles (B), alveolar sac (As), alveolar duct (Ad), alveolus (A) & blood vessel (Bv) in G1 (B) and G2 (C), normal appearance of lung parenchyma in G3 (I **A** /ith multiple focal necrosis (N) **B** ıoderate interstitial pneumonia (Arrows). H&E stain.

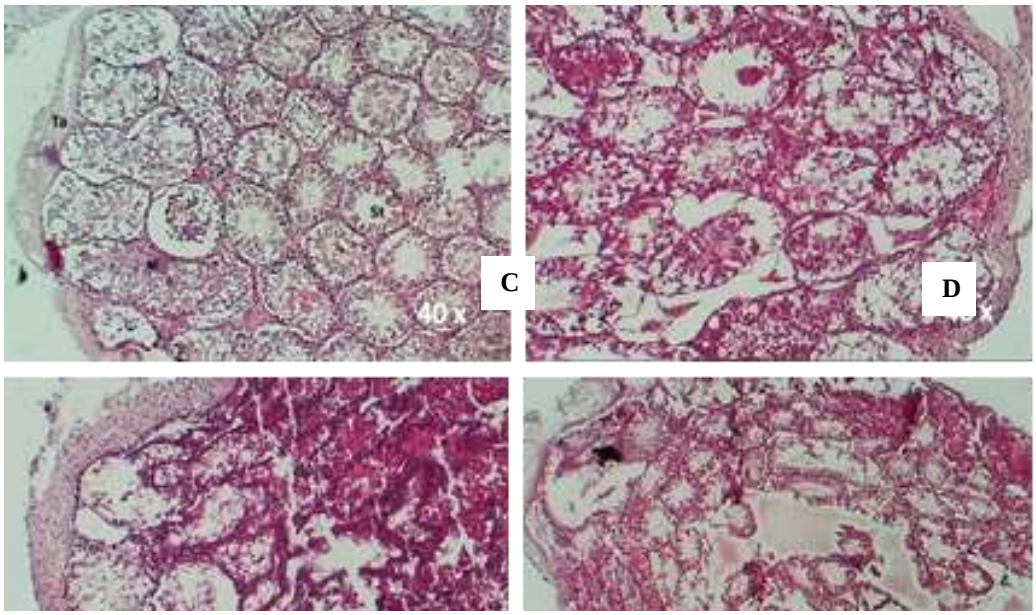


Figure 9- Sections in the testes. (A) Control group showing tunica albuginea (Ta) & seminiferous tubules (St). , severe necrosis of seminiferous tubules and macrovacuolation of germ cells in G1 (B) and G2 (C) , disorganized testicular lobules with necrosis and depletion of seminiferous tubules in G3 (D). H&E stain.

Conclusions

Marked effects on body weight and organ index along with hematological and biochemical parameters were noticed, combined with severe histopathological effects as adverse effects of intraperitoneally-injected ZnO NPs. The small intestine , kidney , liver , and spleen were the main target organs of the accumulated ZnO NPs. This study proved the ability of using the organ index as a good tool side by side with the biochemical indicators to explain the histopathological changes. Also, this study indicated some adverse histopathological effects that were not recorded before as toxicological impacts of ZnO NPs in animal models.

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