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Sub-Acute Oral Toxicity of Zinc Oxide Nanoparticles in Male Rats

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Abstract

ZnO-NPs suspended in distilled water were administered to Wistar rats at dose of 10 mg/kg body weight through oral gavage for 5 consecutive days. The mean body weight gain in rats given ZnO-NPs was similar to this of control group, so no significant differences in the relative organs weight were observed between the ZnO-NPs treated-rats and control. Moreover, ZnO-NPs-exposed rats showed normal values for the complete blood count test. However, biochemical assays showed that sub-acute exposure to ZnO-NPs induced a marked increase of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Therefore, uric acid, creatinine and glucose levels are not modulated by ZnO-NPs administration. These biochemical findings were supported by histopathology examination, which showed minor morphological changes in rat tissues. Sub-acute exposure to ZnO-NPs does not affect the exploratory behaviors and the anxious index of rats.

Keywords: ZnO-NPs; Oral administration; Rat; Toxicity

Introduction

In the past few years, the rapid development of nanotechnology has contributed to the production and control of engineered nanoparticles, which are generally defined as particles in the size range of 1-100 nm in one [1]. The small particle size of NPs creates a large surface area per unit mass and makes them more reactive in a cell [2]. Among the varieties of engineered nanoparticles being used today, ZnO NPs are one of the most widely used in consumer products. They are extensively used in cosmetics and sunscreens because of their efficient UV absorption properties. ZnO NPs are being used in the food industry as additives and in packaging due to their antimicrobial properties. They are also being explored for their potential use as fungicides in agriculture and as anticancer drugs and imaging in biomedical applications [3]. With increased use of ZnO NPs, exposure to these nanoparticles has been rising steadily, resulting in more attention being paid to their potential toxicity, including cytotoxic, genotoxic, and proinflammatory effects [4,5]. Nanoscale particles can enter the human body through different routes such as inhalation, ingestion and injection [6]. They may then translocate to blood causing adverse biological reactions in several organs [7]. Some researchers consider that ZnO-NPs as a material of low toxicity , because zinc is an essential trace element in the human body and is commonly present in foods or added as a nutritional supplement, so zinc attracts little attention during assessment of toxicity of nanoparticles [8]. On the other hand, it is known that a high concentration of zinc is responsible for toxic effects [9]. Several studies have reported that ZnO nanoparticles at a high dose of 1-5 g/kg can cause apoptosis in murine liver cells and induce severe oxidative stress [1,10]. Morover, recent studies have demonstrated that ZnO-NPs are toxic to microorganisms and rodents, the release of metallic cations Zn^{2+} are the main causes of toxicity [11]. Preliminary results indicated that affected organ systems may show inflammation, altered heart rate and functions, and oxidative stress [12,13]. Ingested nanoparticles may be absorbed through the intestinal lining and translocate into the blood stream where they undergo first pass metabolism in the liver [14]. Again, the effects of this translocation are largely unknown. Biodistribution experiments have revealed liver, kidney and spleen as the target organs for engineered nanoparticles after uptake by the gastrointestinal tract [15]. Recently, Yongling et al., [16] reported that ZnO NPs could ameliorate the behavioral and cognitive impairment in mice with depressive-like behaviors, probably through up-regulating neuronal synaptic plasticity and functions. Moreover, ZnO NPs may regulate ionic homeostasis and the physiological functions of neurons and have potential influence in central nervous system which shed light on the possible application and treatment in neurotransmitter system disorders [17].

In this study, the oral administration was selected as the route of exposure for rats to nanoparticles because the ZnO-NPs are used in food packaging and may penetrate the body directly. Even when used in other consumer products like coating and dermatological applications, there is a risk of ingestion during use [10]. Jani et al. [18] have shown that nanoparticles, when administered orally, can be absorbed across the gastrointestinal tract, and pass through the mesentery lymph supply and lymph node to liver and spleen. The present study was undertaken to investigate the sub-acute oral toxicity of ZnO nanoparticles. For this the effects of nanoparticles on the emotional behavior, hematological and biochemical parameters were analysed and organ damage was examined by histopathology. Additionally, the distribution of trace element in different tissues was investigated.

The potential use of ZnO and other metal oxide nanoparticles in biomedical and cancer applications is gaining interest in the scientific and medical communities, largely due to the physical and chemical properties of these nanomaterials, and is the focus of this article.

Zinc oxide nanoparticles (ZnO) applications

Electronic industry, instrumental industry, manufacture, electrical device, radio, wireless fluorescence lamp, image recorder, rheostat,

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phosphor; Sunscreening agent used in cosmetics, antibacterial and health protection antiager; UV protection; Piezoelectrics; High-temperature lubricant in gas turbine engines; Flame retardant; Adsorption; Dental cements; Environmental remediation; Gas sensors; Photocatalytic decontamination; Attenuation of ultraviolet light; Demilitarization of chemical and biological warfare agents; Cosmetics and cosmeceuticals; Electrodes for solar cells; Varistors; Pigments for paints.

Materials and Methods

Nanoparticles preparation and characterization techniques

ZnO-NPs were provided by the Laboratory of Physics of Materials and Nanomaterials applied to environment, College of Sciences in Gabes, Tunisia. The preparation of nanocrystalline ZnO aerogels were prepared by dissolving 2 g of zinc acetate dehydrate ($ZnCH_3COO)_2.2H_2O$) in 14 ml of methanol under magnetic stirring for 2 h. The water for hydrolysis was slowly released by esterification of acetate with methanol. Nanoparticle aerogel as obtained by supercritical drying in ethyl alcohol (EtOH) [19]. The crystalline data were obtained by X-ray diffractometry (XRD; Bruker D8 Advance; 40 kV, 30 mA). To determine the lattice parameters of the different phases the diffraction X-rays were collected by scanning between 2θ=5 and 70° in 0.02° steps (Figure 1). To examine the size and morphology of oxide nanocrystals transmission electron microscopy [(TEM) JEM-200CX] was used (Figure 2). The sample preparation for TEM observation was as follows: the powder was firstly put in EtOH, and the ultrasonic dispersed solution was dropped on a Cu net.

Animals and treatment

Zinc oxide is an inorganic compound with the formula ZnO. It usually appears as a white powder. We prepared ZnO suspension using physiological saline solution (9‰ sodium chloride). The powdered ZnO nanoparticles were dispersed in the fresh sterilized physiological saline solution, and the suspension was ultrasonicated for 20 min to disperse completely as well as possible (Ultrasonic Liquid Processor : Sonicator 4000). ZnO suspension was vortexed for 1min before injections. Male Wistar rats, weighing 135-140 g at the beginning of the experiment were housed in groups of 6 in cages at 25°C, under a 12:12 light/dark cycle (lights on at 07:00), with free access to food and water. Animals were then randomly divided into two groups

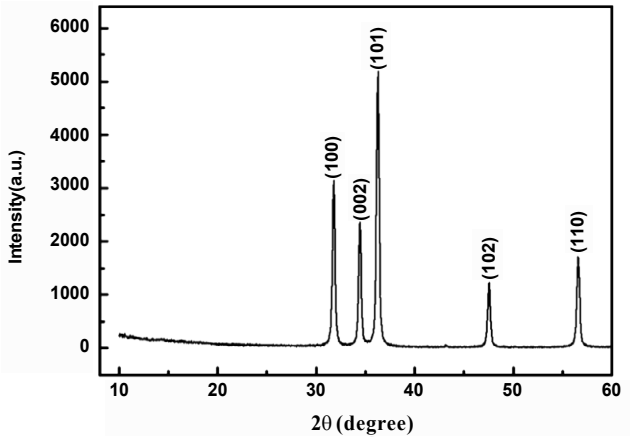

Figure 1: X-ray diffraction patterns for the crystal quality of ZnO nanoparticles.

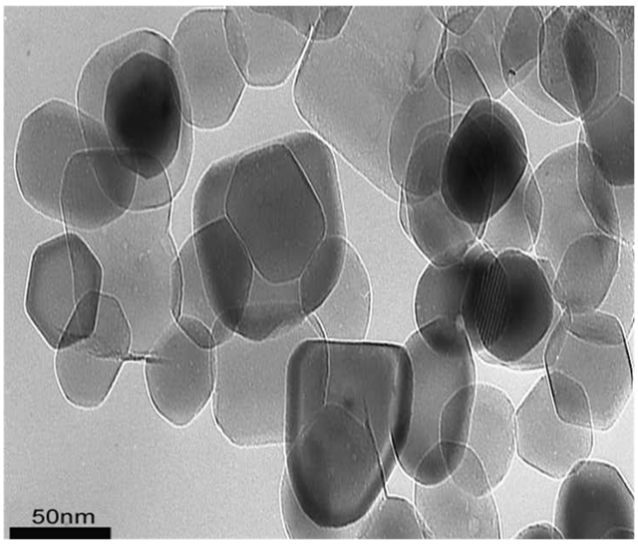

Figure 2: Transmission electron microscopy (TEM) images of synthesized ZnO nanoparticles.

(n=6 in each group). Group 1 (control) received physiological saline solution (9‰ sodium chloride), group 2 received (ZnO-NPs) at dose of 10 mg/kg body weight through oral gavage for 5 consecutive days. The experimental protocols were approved by the Faculty Ethics Committee in accordance with the Medical Ethical Committee for the Care and Use of Laboratory Animals of Pasteur Institute of Tunis (approval number: LNFP/Pro 152012).

Emotional behavior testing

The elevated plus maze test was used accordingly to previously published methodologies [20-22]. The maze was made of clear painted wood. The arms were 50 cm long and 10 cm width and the apparatus was elevated at a height of 60 cm. The closed arms were surrounded by a 50 cm wall while open arms had 0.5 cm edges in order to maximize open arms entries [23]. The test was 5 min long and began with the placement of a rat in the centre of the maze, with its head facing an open arm. The time spent in the different parts of the maze (i.e. open arms, closed arms and central part) was recorded along with the numbers of entries into closed and open arms. In addition, total activity into the maze was evaluated via the total number of arm and central part entries. Since total activity was found to be different among groups, a ratio for open arm entries and open arm time was also calculated. The maze was cleaned with a 10% alcohol solution between each animal.

Open field locomotor activity

The exploratory and anxiety-related behavior was assessed in the open field test. Control and ZnO-NPs-treated rats were tested in the open field for one daily session of 5 min during three consecutive days, with an intertrial interval of 24 h. The device is a metallic gray circular enclosure (100 cm in diameter and 60 cm high). It is divided into a central circle and a peripheral part which is also divided into 6 parts of equal area (0.135 m^2). Three identical objects (length 15 cm; width 10cm; height 5cm) were placed in the same positions of the peripheral part during al trials [24]. The compartmentalization of the device is maintained for all presented data. At the beginning of the test, the rat was placed in a peripheral part of the open field. The following behavioral components were measured: locomotion in the peripheral and central part, total immobility time, number and duration of

contact with an object. The open-field and objects were wiped clean between trials using a 10% alcohol solution before the next animal was introduced, as to prevent the possible cuing effects of odors left by previous subjects.

Biochemistry panel analysis

All animals were sacrificed at the same time. Blood samples were taken from all rats in heparinized tubes and then centrifuged. In the present study, we choose plasmatic biochemichal parameters related to liver and kidney function. We determined the glucose content, uric acid, creatinine and levels of enzymes such as aspartate aminotransferase (AST) and alanine aminotrasferase (ALT). These enzymes were examined by routine colorimetric methods using commercial kits. Organes of the control and treated groups were harvested immediatly. Organs were rinsed with ice-cold deionized water and dried with filter paper. After weighing the body and tissues, the coefficients of liver and kidneys to body weight were calculated as the ratio of tissues (wet weight, mg) to body weight (g).

Determination of trace element content

The concentrations of trace element (Zn, Fe, Ca, Na, K and Cu) in rat liver, kidney and brain were determined with an atomic absorption spectrometer (Avanta, GBC, Australia). The standard solution of zinc used in this assay resulted by the dissolution of $ZnCl_2$ in deionized water. Liver and kidney tissues were lyophilized, weighed and digested in 2 ml of concentrated HNO_3 in pressurized Teflon containers at 160°C for 3 h. After cooling at room temperature, samples were diluted with 10 ml of deionized water [25]. Trace elements analyses were performed using acetylene gas as fuel and air as an oxidizer. Operational conditions were adjusted to yield optimal determination. The calibration curves were prepared separately for all the trace elements by running suitable concentrations of the standard solutions. Digested samples were aspirated into the fuel rich air-acetylene flame and the concentrations of the trace element were determined from the calibration curves. Average values of three replicates were taken for each determination. Suitable blanks were also prepared and analysed in the same manner. The detection limits for iron (Fe), zinc (Zn), calcium (Ca), sodium (Na), potassium (K) and copper (Cu) were 0.05, 0.008, 0.025, 0.04, 0.05, 0.05 ppm respectively. Trace elments concentration was calculated in µg/g of the dry mass of tissues.

Histology

A small piece of liver, kidney and brain were collected 24 h after the last administration. The portion of organs were fixed in 10% neutral buffered formalin solution, dehydrated in a graded series of ethanol and xylene solutions, and embedded in paraffin. Sections were cut with a microtome, deparaffinized, rehydrated in a graded series of ethanols, and stained with hematoxylin and eosin.

Statistical analysis

Results were expressed as the mean ± S.E.M. and data were analyzed by means of one way analysis of variance (ANOVA) with the post hoc test to determine significance relative to the unexposed control using Statistica 5.0. In all cases, $p<0.05$ was considered significant.

Results

Animal observation and effect on body weight

No toxic signs or mortality was observed related to ZnO-NPs administration. Also, there were no significant changes in the body weight and the coefficient of brain, liver and kidney (as the ratio of tissues wet weight (mg) to body weight (g)) between the exposed rats and the control group (Table 1 and Figure 3).

Effects of ZnO-NPs exposure on the emotional behavior of rats

The spontaneous activity, exploratory behavior, and habituation to novelty were examined in the open field test. All the parameters did not differ between control and ZnO-NPs exposed-rats (Table 2). Moreover, the behavioral performances measured in the pulse mize test indicated that the ZnO-NPs exposed group seemed to show no significant difference in the anxiety index (Figure 4).

Effects of ZnO-NPs exposure on homeostasis of essential trace elements

Sub-acute oral exposure to ZnO-NPs decreased the concentration of copper (Cu^{2+}), calcium (Ca^{2+}) and sodium (Na^{2+}) in the brain. However, the content of trace elements remained unchanged in the liver and kidney (Table 4). Interestingly, the determination of zinc distribution in the liver, kidney and brain shows no significant variations in the concentration of this element in the ZnO-NPs treated group compared to the control (Table 3).

Effects of ZnO-NPs on hematological and biochemical parameters

In the present study, we choose plasmatic biochemical parameters related to liver and kidney function. Experimental group treated by oral administration of ZnO-NPs (10 mg/kg) showed a significant increase of ALT and AST. However, uric acid and creatinine levels remained unchanged (Table 3). On the other hand, sub-acute exposure to ZnO-NPs modulates slightly the RBC, WBC, hematocrit, hemoglobin and platelet count (Table 4).

	Liver	Kidney	Brain
Control	3.82 ± 0.10	0.76 ± 0.09	0.154 ± 0.007
ZnO-NPs	3.80 ± 0.11	1.01 ± 0.07	0.161 ± 0.009

Data represent the means ± SEM of 6 animals *per* group

Table 1: Effect of ZnO-NPs treatment on the coefficients of organs.

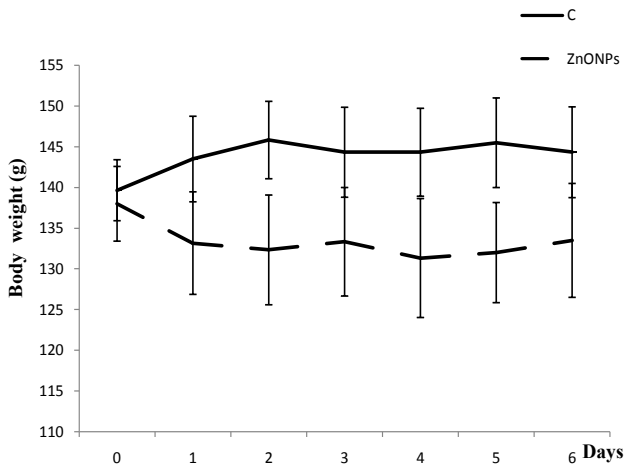

Figure 3: Body weight changes for rats treated with ZnO-NPs at doses of 10 mg/kg. Data represent the means ± SEM of 6 animals *per* group.

Variables	Session 1		Session 2		Session 3	
	C	ZnO_{NPs}	C	ZnO_{NPs}	C	ZnO_{NPs}
Locomotion in the peripheral part (s)	172.16 ± 13.3	194.5 ± 16.4	104 ± 8.6	112.7 ± 13.4	71.16 ± 7.14	113.5 ± 22.1
Locomotion in the central part (s)	2.83 ± 1.7	5.7 ± 2.4	0.83 ± 0.54	2.66 ± 1.7	0.5 ± 0.5	2.83 ± 1.8
Immobility time (s)	100.3 ± 14	81.5 ± 18.56	171.5 ± 8.7	172 ± 12.54	213.16 ± 6.7	165 ± 26.8
Time of contact with objects (s)	19 ± 3.6	18.33 ± 2.66	18.16 ± 2	12.83 ± 2.18	15.16 ± 3.12	8.66 ± 4.62
Number of contact with objects	10.83 ± 1.53	11.83 ± 2.4	9.16 ± 1.16	7.83 ± 0.94	7.66 ± 1.7	9 ± 1.77

Data represent the means ± SEM of 6 animals *per* group

Table 2: Effects of ZnO-NPs treatment on the open field parameters.

	Liver		Kidney		Brain	
	C	ZnO-NPs	C	ZnO-NPs	C	ZnO-NPs
Zn	399.74 ± 43.54	507.04 ± 75.51	97.77 ± 33.95	165.74 ± 21.4	263.07 ± 57.14	230.85 ± 32.3
Fe	2.62 ± 0.33	2.08 ± 0.22	2.58 ± 0.95	1.62 ± 0.32	1.33 ± 0.29	0.78 ± 0.11
Cu	0.12 ± 0.01	0.12 ± 0.01	0.36 ± 0.03	0.32 ± 0.03	0.29 ± 0.01	0.22 ± 0.01*
K	11.28 ± 1.13	13.78 ± 0.50	14.08 ± 0.69	11.66 ± 0.36*	17.45 ± 1.11	18.80 ± 0.03
Ca	0.82 ± 0.09	0.95 ± 0.26	1.81 ± 0.27	1.68 ± 0.07	5.99 ± 1.35	1.50 ± 0.15*
Na	4.66 ± 0.48	4.87 ± 0.33	15.35 ± 0.53	13.38 ± 0.41	11.46 ± 0.04	10.55 ± 0.20*

Data represent the means ± SEM of 6 animals *per* group. *p<0.05, compared to control

Table 3: Effects of ZnO-NPs treatment on the trace element content.

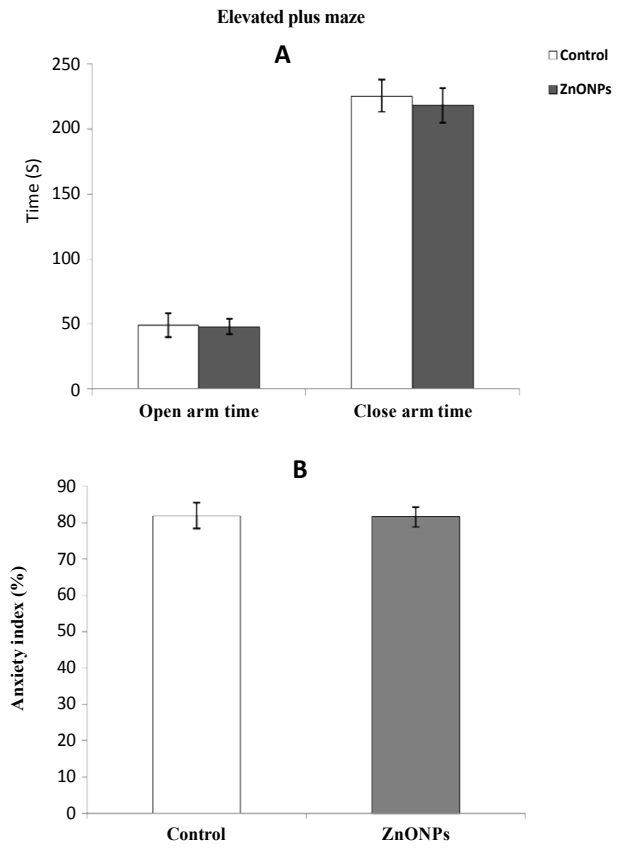

Figure 4: Effect of ZnO-NPs treatment on the time spent in the open and closed arms (A). Graphical representation of anxious index (AI) from pulse mice assay of control and ZnO-NPs treated rats (B). Data represent the means ± SEM of 6 animals *per* group.

Parameters	Experimental group	
	Control	ZnO-NPs
WBC 10^9/l	14.44 ± 1.82	13.35 ± 0.82
RBC 10^{12}/l	6.77 ± 0.11	6.812 ± 0.24
Hematocrit %	34.06 ± 0.66	34.42 ± 1.33
Hemoglobin g/dl	12.44 ± 0.43	12.22 ± 0.29
Platlets 10^9/l	603.8 ± 87.90	584.2 ± 88.92
AST U/l	38.85 ± 5.19	116.9 ± 24.83*
ALT U/l	12.6 ± 1.92	23.27 ± 2.48*
LDH U/l	586.88 ± 183.86	270.37 ± 63.21
Creatinine mg/dl	0.67 ± 0.06	0.67 ± 0.057
Uric acid mg/dl	6.34 ± 0.38	7.01 ± 0.40
Glucose mg/dl	159.27 ± 10.30	139.56 ± 16.23

Data represent the means ± SEM of 6 animals *per* group. *p<0.05, compared to control

Table 4: Effects of ZnO-NPs treatment on the hematological and biochemical parameters in rats.

Histopathological examination

Our findings indicate that a pathological damage in the liver was manifested by sinusoidal congestion (SC), RBC deposition in the vein and inflammatory response after oral administration of ZnO-NPs for 5 consecutive days (Figure 5). However, the histopathology of kidneys tissues showed intratubular protein deposition and vascular congestion (Figure 6). Histological analyses in the brain tissues showed vascular congestion and edema in rats treated with ZnO-NPs (Figure 7).

Discussion

Nanotechnology and nanoparticles could be risk factors for neuropathological and toxicological processes. Along with extensive application of ZnO nanoparticles in the industrial field, it is conceivable that the human body may be intentionally or unintentionally exposed to nanoparticles via several possible routes, including oral ingestion, inhalation, intravenous injection, and dermal penetration. Among

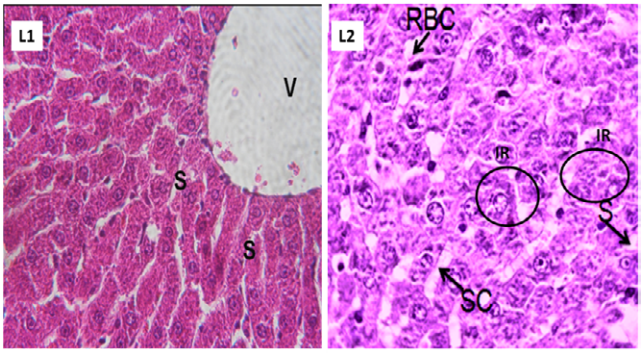

Figure 5: Histopathology of liver tissues in rats treated with ZnO-NPs for 5 consecutive days. (L1) Control group showing normal liver, (L2) pathological alterations in the liver of ZnO-NPs (10 mg/kg) treated rats manifested by sinusoidal congestion (SC) and RBC (Red Blood Cells) deposit and inflammatory response (IR),Sinusoide (S), Vein (V). L1 and L2 magnification x 400.

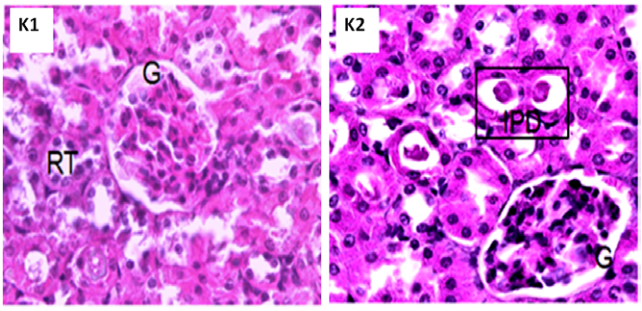

Figure 6: Histopathology of kidney tissues in rats treated with ZnO-NPs (10mg/kg) for 5 consecutive days. K1 control group showing normal architecture of renal corpuscles with their glomeruli (G) and renal tubules (RT); K2 treated group with 10 mg/kg body weight/day ZnO-NPs for 5 days, showing intratubular protein deposition (IPD) but no significant glomerular changes (G). K1 and K2 magnification x 400.

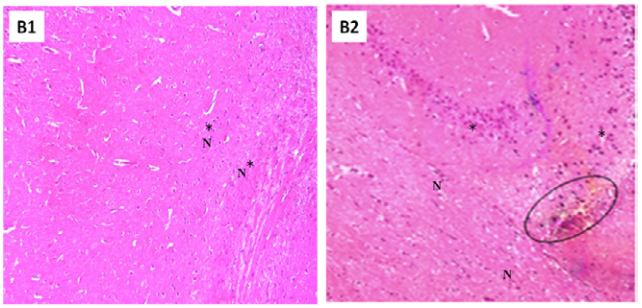

Figure 7: Histological analyses in the brain tissues in rats treated with ZnO-NPs (10mg/kg) for 5 consecutive days. B1control group, the histological analyses in the brain tissuesdid not show significant changes in morphology and nuclei of neurons were observed (N). B2 treated group showing congestion of vascular (asterisk) and edema (round). (B1, B2 magnification x400).

these, uptake of nanoparticles by the gastrointestinal tract is one of the most important routes [26]. Retention of metal oxide nanoparticles in the environment and food chain is high and continuous exposure to them may affect human health [27]. Very small particles have the ability to enter, translocate within, and damage living organisms. This ability, results primarily from their small size, which allows them to penetrate physiological barriers, and travel within the circulatory systems [28]. Earlier studies have shown that nano-forms of different particles are more toxic than their micro-counterparts after acute exposure via the oral route [29]. Biodistribution experiments have revealed liver, kidney and spleen as the target organs for engineered nanoparticles after uptake by the gastrointestinal tract [29]. This study investigates the effects of ZnO-NPs on the blood cell count and biochemical parameters in adult rat. Additionally, the histopathological examination may be helpful in detecting organs abnormalities after ZnO-NPs uptake by the gastrointestinal tract. The results of this experimental study indicated that ZnO-NPs in mentioned concentration (10mg/kg) don't show significant effect on the body weight gain and the relative organs weight. This is in accordance with our previous finding indicating the absence of toxic signs and mortality in adult rats exposed to ZnO-NPs [30,31]. Our results obtained in the treated rats are consistent with a work done by Sharma et al., [10] suggesting that oral exposure to ZnO-NPs did not exert any major visible impairment in the health status of animals. Since the biological responses to toxic substances and distribution pathways may be different depending on the doses accumulated in the body. The nanoparticles when ingested into the body can be distributed to different regions because of their small size. They can cross the intestine and further distribute into the blood, brain, lung, heart, kidney, spleen, liver, intestine and stomach [32]. Ragarding the distribution of zinc in the treated rats, no appreciable amount of this metal was accumulated in rat tissues. Our results showed that the orally administrated ZnO-NPs induced a no significant increase of zinc concentration in the liver and kidney. Although the number of published oral exposure studies has increased during the recent years, only very few well performed studies on intestinal absorption are available. Sharma et al., [10] showed that nanoparticles were mainly found to be retained in the liver after 14 day of sub-acute oral exposure to ZnO-NPs at a higher dose (300 mg/kg). However, Matsumoto et al., [33] suggested that repeated oral gavage of nanoparticles reached the gastro-intestinal tract as agglomerates and were mostly excreted via faeces but no investigations and results to support this suggestion were presented. Further characterization of nanoparticles in the gastrointestinal tract, including both absorption and excretion in response to ZnO-NPs intake are needed. Hematological profile and biochemical parameters such as blood cells count and enzymes activity are used to provide useful information for diagnosis in routine clinical evaluation of the state of health of a patient and animals. There were no abnormal findings at hematological parameters, glucose content, creatinine and uric acid levels in ZnO-NPs treated-rats. The unchanged levels of creatinine and uric acid in the present study indicated normal renal functions in the ZnO-NPs exposed group. However, the oral administration of a moderate dose of ZnO-NPs (10 mg/kg) induced a marked increase of plasma AST and ALT enzymes. From our results we can deduce that the elevated levels of transaminases, which are located primarily in the cytosol of hepatocytes, is a sign of damage which leads to liver dysfunction in treated rats. These biochemical findings were supported by histopathology examination, which showed signs of cytotoxicity (inflammatory response, vascular congestion and edema formation) in rat liver. This is in agreement with previous report indicating that sub-acute oral exposure to ZnO nanoparticles (300 mg/kg) for 14 consecutive days induced hepatocellular necrosis [10,26]. However, the histopathological analysis of the kidney showed intratubular protein deposition (IPD) but no significant glomerular changes. Nonetheless, the experimental rats developed minor morphological changes and the disturbance of trace element homeostasis (calcium, sodium and copper) in the brain. The basis for the modulation of trace element in rat brain

under ZnO-NPs is not clear, but may involve probably the impairment of the active transport processes or ions exchange mechanisms in the brain. Our previous finding have clarified that intraperitoneal injection of ZnO NPs (25 mg/kg) disrupt trace elements homeostasis in rat brain, but this effect is insufficient to promote emotional behavior impairments [30]. In the current study, oral exposure to ZnO-NPs did not cause significant changes in the activity of rats measured in the plus maze and open-field tests such as exploratory, locomotion and anxiety-like behaviors. This result is in accordance with our previous study indicating the absence of correlation between zinc accumulation in brain following ZnO-NPs treatment and behavioral performances of rodents [31].

The results of the present study together with those of previous investigations showed that effects of oral nanoparticles exposure are less remarkable than those observed for parenteral administration. The oral route is probably one of realistic way of modeling nanoparticles exposure in humans. In the present investigation oral exposure to moderate dose of ZnO-NPs has no significant main effect on the behavior of the rodents and causes subtle signs of toxicity.

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