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Toxicity assessment of zinc oxide nanoparticles in mice: Correlation between different doses and particle sizes

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ZnO NPs are previously reported to have diverse toxic effects. However, this study aims to attain a safe dose correlated with a small particle size of these NPs to be used in biomedical applications in further research. Investigation of toxicity-induced disorders using different sizes and doses of ZnO NPs and selection of the most convenient dose and size to be utilized in medical applications. Two doses of ZnO NPs (50 and 150 mg/kg. B.wt) for each of three different particle sizes (14, 30, 50 nm) were administrated to mice for 14 consecutive days. Biochemical and histopathological investigations were performed to confirm the toxicity of ZnO NPs. Comet and qRT-PCR assays were performed on liver tissues. Data were analyzed using SPSS coupled with Co-Stat (version 8). A significant increase in both liver and kidney coefficients as well as in serum liver function enzymes was obtained using the higher dose of ZnO NPs. Also, the hepatic defense mechanisms showed significant disorders in the different antioxidants measured. Comet assay revealed a significant hepatic DNA damage. On the molecular level, an up regulation in Bax and caspase-3 gene expressions were observed while, Bcl-2 was down-regulated. ZnO-NPs also showed a significant depletion in brain neurotransmitters (dopamine and serotonin) and an elevation in noradernaline level. The higher dose of ZnO NPs using particle size 30 nm induced the most toxic effect on liver and brain tissues. While the lower dose using 14nm was considerably safe for therapeutic applications.

Keywords: ZnO NPs, antioxidant, toxicity, Bax, Bcl2, neurotransmitters.

INTRODUCTION

NPs are characterized by their small size (1 to 100 nm), high number per given mass and large specific surface area. These properties have aroused global concern regarding their metabolic fate in biological systems (Lynch et al., 2006). Previous studies have reported the toxicity of NPs since they can easily penetrate cell nuclei owing to their small size. Although the liver is highly prone to toxicity as it is the major organ involved in metabolism and detoxification of xenobiotics, these NPs can also cause serious effects to other organs, namely, kidney, spleen, lung, heart, and brain using 20- and 120-nm[3]. This is represented in increased production of reactive oxygen species (ROS), induction of apoptosis; genotoxicity and DNA damage (Wang et al., 2007). Additionally, NPs cause various inflammatory responses and leading to dysfunction of these organs (Borm et al., 2002).

Nano-sized particles have wide applications in many medical and industrial products (Mcintyre, 2012). Metallic (NPs) are used in daily life in cosmetics, sunscreens, food products, implanted medical devices as cardiovascular stents, dental implants, spinal fixation devices and drug carriers (Beckett et al., 2005). The success of engineered nano-materials is related to their physical properties and high reactivity. Also, the novel and useful properties possessed by these engineered nano-materials can lead to unpredictable outcomes in terms of their interactions with biological systems.

Exposure to some nanoparticles is associated to the occurrence of autoimmune diseases such as systemic lupus erythematous, scleroderma, and rheumatoid arthritis (Buzea et al., 2007). Published data indicated that numerous metallic nanoparticles are selective nephrotoxins that preferentially accumulate and produce cellular injury in the kidney which permits unrestricted use, distribution and reproduction in any medium (Pujalté et al., 2011).

Earlier studies have shown that nano forms of different particles are more toxic than their micro counterparts after acute exposure via the oral route (Wang et al., 2007).

ZnO NPs are one of the most widely used in consumer products andthis makes them transparent and more aesthetically acceptable as compared to their bulk counterpart (Schilling et.al, 2010). ZnO NPs are being as food additives, in packaging, as fungicides (He et al., 2010)and as anticancer drugs and imaging in biomedical applications (Rasmussen et al., 2010). However, ZnO NPs were selectively toxic towards potential disease causing cells, and have been regarded as a possible treatment for cancer and/ or autoimmune diseases (Hanley et al., 2008).Some studies have demonstrated the toxicity of ZnO NPs in bacteria (Sinha et al., 2010)and mammalian cells (Wang et al., 2010) causing membrane injury, inflammatory response, DNA damage and apoptosis (Sharma et al., 2011).

Workers involved in the synthesis of ZnONPs can be exposed by unintentional hand-to-mouth transfer of nano-materials. When discharged accidentally into the environment, these nanoparticles may enter the human body through the food chain. Some of the nanoparticles can be swallowed into and reach the gastrointestinal tract when they are expelled from the mucociliary system of the lungs after inhalation (Oberdorster et al., 2005). NPs could be then translocated from the lumen of the intestinal tract and blood into different organs like the liver.

Also, exposure to low concentrations of nano-ZnO indicates a genotoxic potential mediated by lipid peroxidation and oxidative stress in epidermal cells (Sharma et al., 2009).

More recently, ZnO NPs are also being developed as bio imaging probes (Khan et al., 2010) and drug delivery vehicles (Khan et al., 2010). However, there are many reports in the literature documenting the cytotoxic effects of ZnO NPs in vitro (Heng et al., 2010). Concerns are also rising with respect to the possible genotoxic effects of these particles (Yin et al., 2010), due to their potential devastating long term damage caused to humans. The present study aimed to evaluate toxicity of different sizes and doses of ZnO NPs in healthy mice aiming to therapeutic application on cancer cells in further study.

MATERIALS AND METHODS

This research was performed in the laboratory of Biomaterials and in the Department of Therapeutic Chemistry, National Research Centre, Egypt. The study lasted for a period of two years.

Chemicals:

All chemicals were of high analytical grade products of Sigma-Aldrich Co (St. Louis, MO, USA). Kits utilized for liver function and oxidative stress biomarkers determination were purchased from Randox Company (UK).

Zinc oxide nanoparticles were synthesized using aqueous solution of zinc acetate and a solution of sodium hydroxide. The different solutions were prepared using deionized water while stirring and heating, respectively. The hydroxide solution was slowly added into the zinc salt solution at room temperature under vigorous stirring, which resulted in the formation of a white precipitate. The precipitate was centrifuged at 6500 r.p.m while cooling to -10°C for 20 min. The resulted product was washed with distilled water several times followed by absolute alcohol. The obtained product was calcined at 500°C in air atmosphere for 3hr. The resulted NPs were tested by TEM in order to evaluate the particle morphology; shape and size. ZnO NPs were then redisposed in deionized water to give the requested concentrations (Hingoranim et al., 1993).

Experimental animals:

Male albino mice, weighing 20–25 g were obtained from the animal house of National Research Centre. Animals were housed in cages

kept at standardized conditions (22 ± 5◦C, 55 ± 5% humidity, and 12 h light/dark cycle). They were allowed free access to water and pelleted standard chow diet.

Ethical procedure:

All procedures related to animal care and treatments strictly adhered to the ethical procedures according to the Guide for Care and Use of Laboratory published by the US National Institute of Health policies and approved by Animal Care and Use of the Committee of National Research Centre (approval no:13-088).

Experimental design:

After careful selection on the basis of health and inclusion criteria, animals were left for 1 week for acclimatization, and were randomly divided into two main groups according to the following schedule:

G1: Control group (normal healthy animals) (n=15).

G2: Animals orally administered different doses and sizes of ZnO-NP for 14 consecutive days as described by Sharma et al. (2012)(n=90). Mice of this group were then divided into 6 subgroups, 15 mice each:

Sub G1-G3: Animals were administered ZnO NPs (50 mg/ Kg.b.wt) with sizes 14, 30, 50nm respectively.

Sub G4-G6: Animals were administered ZnO NPs (150 mg/ Kg.b.wt) with sizes 14, 30, 50nm.

Blood sampling and tissue preparation:

Mice were fasted after 24 h of the last dose administration and then weighed, slightly anesthetized and blood samples were collected from the sublingual vein. Sera were separated by centrifugation at 4000 rpm for 10 min and were kept at −80◦C for subsequent estimation of amino transferases activities. Liver, kidney and brain tissues were carefully separated blotted dry and weighed. Liver tissue was divided into four portions. The first portion was homogenized in 4 volumes of phosphate buffer, pH 7.4, using Teflon homogenizer (Glass-Col homogenizer, Terre Haute, USA). An aliquot of this homogenate (20% w/v) was centrifuged at 4000 rpm at 4◦C for15 min and the supernatant was used for MDA analysis. Another aliquot was mixed with 7.5% sulfosalicylic acid, centrifuged at 3000 rpm for 15 min, and the resulting protein free supernatant was used for the estimation of reduced glutathione (GSH), nitric oxide and total antioxidant capacity.

The second portion of the liver was used for the estimation of caspase 3, Bax and Bcl-2 mRNA expression levels, whereas the third portion was used for comet assay. The remaining portion was kept in 10% formaldehyde, and then embedded in paraffin for histopathological examinations.

Brain tissue was homogenized in normal physiological saline solution (0.9N NaCl) (1:9 w/v). The homogenate was centrifuged at 4ºC for 5 min at 3000 r.p.m and the supernatant was used for estimation of brain functions and marker enzyme parameters.

Biochemical and physiological characterization of treated mice:

Coefficient of liver, Kidney and Brain:

After weighing the body, liver, kidney and brain the coefficient of these organs to bodyweight was calculated as the ratio of tissue (wet weight, mg) to body weight (BW, g) (Peters et al., 2006).

ALT, AST and ALP activities:

Serum ALT and AST activities were spectro photometrically estimated by the method of Reitman and Frankle, (1957). ALP activity was estimated in serum by the method of Belfield and Goldberg (1971).

Hepatic total protein:

Hepatic total protein was assayed by the method of Bradford (1976), where Coomassie Brilliant Blue dye reacted with Bradford reagent and gave a blue complex at 595 nm.

MDA and NOx level in liver:

MDA, as an index of lipid peroxidation, was measured. MDA reacts with thiobarbituric acid (TBA) in acid medium giving a pink-colored complex that can be measured spectrophotometrically at 520 nm and 535 nm, using1,1,3,3-tetramethoxy propane as standard (Ohkawa et al., 1979). Total Nitrite/Nitrate was measured according to the method of Miranda et al. (2001). The method employs the reduction of any nitrate to nitrite by vanadium chloride followed by detection of total nitrite by Griess reagent at 540 nm.

GSH, TAC and CAT level in liver:

GSH level was estimated. In brief, GSH content was estimated using 5, 5-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent), which produces a stable yellow color that can be measured colorimetrically at 412 nm (Moron et al., 1979). Total antioxidant capacity (TAC) was estimated according to manufacturer's instructions using Randox kits (Ou BX., et al, 2002). Catalase

(CAT) activity was estimated as the decomposition rate of hydrogen peroxide (H_2O_2) according to Wang et al.(2001**).**

Determination of Nor-adrenaline, Dopamine and Serotonin:

Nor-adrenaline (NA), Dopamine (DA) and Serotonin (5-HT) were determined in brain of mice using high performance liquid chromatography (HPLC) technique according to the method of Zagrodzka et al., (2000).

Expression analysis of apoptosis related genes:

-Isolation of total RNA:

Total RNA was isolated from liver tissues of treated mice by the standard TRIzol® Reagent extraction method (cat#15596-026, Invitrogen, Germany).. Purity of total RNA was assessed by the 260/280 nm ratio (between 1.8 and 2.1). Additionally, integrity was assured with ethidium bromide-stain analysis of 28S and 18S bands by formaldehyde-containing agarose gel electrophoresis. Aliquots were used immediately for reverse transcription (RT).

-Reverse transcription (RT) reaction:

The complete Poly(A) + RNA isolated from liver tissues was reverse transcribed into cDNA in a total volume of 20 µl using RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany). An amount of total RNA (5 µg) was used with a reaction mixture, termed as master mix (MM). The MM was consisted of 50 mM $MgCl_2$, 5x reverse transcription (RT) buffer (50 mMKCl; 10 mMTris-HCl; pH 8.3; 10 mM of each dNTP, 50 µM oligo-dT primer, 20 U ribonuclease inhibitor (50 kDa recombinant enzyme to inhibit RNase activity) and 50 U M-MuLV reverse transcriptase. The RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and the reaction was stopped by heating for 5 min at 99 °C. Afterwards the reaction tubes containing RT preparations were flash cooled in an ice chamber until being used for DNA amplification through quantitative real time-polymerase chain reaction (qRT-PCR).

-Quantitative real time-polymerase chain reaction (qRT-PCR):

PCR reactions were set up in 25 µL reaction mixtures containing 12.5 µL 1 × SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template. The reaction program was allocated to 3 steps. First step was at 95.0 °C for 3 min. Second step consisted of 40 cycles in which each cycle divided to 3 sub-steps: (a) at 95.0 °C for 15 sec; (b) at 55.0 °C for 30 sec; and (c) at 72.0 °C for 30 sec. The third step consisted of 71 cycles which started at 60.0 °C and then increased about 0.5 °C every 10 sec up to 95.0 °C. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers. Each experiment included a distilled water control.

The quantitative values of RT-PCR (qRT-PCR) of Bax (Bax-F: 5′- CGA GCT GAT CAG AAC CAT CA-3′, Bax-R: 5′-CTC AGC CCA TCT TCT TCC AG-3′, NCBI: NM-017059.2); Bcl2 (Bcl2-F: 5′-CTC AGT CAT CCA CAG GGC GA-3′, Bcl2-R: 5′-AGA GGG GCT ACG AGT GGG AT-3′, Khalil and Booles, 2011); caspase 3 (Casp3-F: 5′-GGA CCT GTG GAC CTG AAA AA-3′, Casp3-R: 5′-GCA TGC CAT ATC ATC GTC AG-3′, NCBI: NM- 012922.2) genes were normalized on the bases of β-actin (β -actin- F: 5′-CAC GTG GGC CGC TCT AGG CAC CAA-3′, β-actin-R: 5′-CTC TTT GAT GTC ACG CAC GAT TTC-3′, Khalil and Booles, 2011) expression. At the end of each qRT-PCR a melting curve analysis was performed at 95.0 °C to check the quality of the used primers.

Calculation of gene expression:

The relative quantification of the target to the reference was determined by using the $2^{-\Delta\Delta CT}$ method as follows:

$\Delta CT_{(test)} = CT_{(target, test)} - CT_{(reference, test)}$,

$\Delta CT_{(calibrator)} = CT_{(target, calibrator)} - CT_{(reference, calibrator)}$,

$\Delta\Delta CT = \Delta CT_{(Test)} - \Delta CT_{(calibrator)}$.

The relative expression was calculated by $2^{-\Delta\Delta CT}$(EL-Baz et al., 2015).

Comet assay:

Isolated liver tissues of all groups of mice were subjected to the modified single-cell gel electrophoresis or comet assay (Fairbairn et al., 1995). In brief, the protocol for electrophoresis involved embedding of the isolated cells in agarose gel on microscopic slides and lysing them with detergent at high salt concentrations overnight (in the cold). The cells were treated with alkali for 20 min to denature the DNA and electrophoresis under alkaline conditions (30 min) at 300 mA, 25 V. The slides were stained with ethidium bromide and examined using a fluorescence microscope with a green filter at × 40

magnification. For each animal about 100 cells were examined to determine the percentage of cells with DNA damage that appear like comets. The non-overlapping cells were randomly selected and were visually assigned a score on an arbitrary scale of 0–3 (i.e., class 0 = no detectable DNA damage and no tail; class 1 = tail with a length less than the diameter of the nucleus; class 2 = tail with length between 1× and 2× the nuclear diameter; and class 3 = tail longer than 2× the diameter of the nucleus) based on perceived comet tail length migration and relative proportion of DNA in the nucleus (Collins et al., 1997).

Histopathological examination:

After deparaffinization and dehydration, sections of liver of 4mm thickness were stained with hematoxylin and eosin (H&E) and examined under the light microscope (Bancroft and Stevens, 1996). All histopathologic processing and assessment of specimens were performed by an experienced observer unaware of the identity of the sample being examined to avoid any bias.

Statistical analysis:

All data were expressed as mean ± SD of 15 mice in each group. Statistical analysis was carried out by one-way analysis of variance (ANOVA), Cost at Software Computer program.

RESULTS

ZnO NPs (14, 30 and 50 nm) were used in this study. Physical characterizations of these NPs were examined by transmission electron microscopy (TEM) to determine particle size as shown in Fig (1).Results showed the accuracy of NPs preparation relatively close to 14 nm (a), (b) close to 30nm and (c) close to 50 nm.

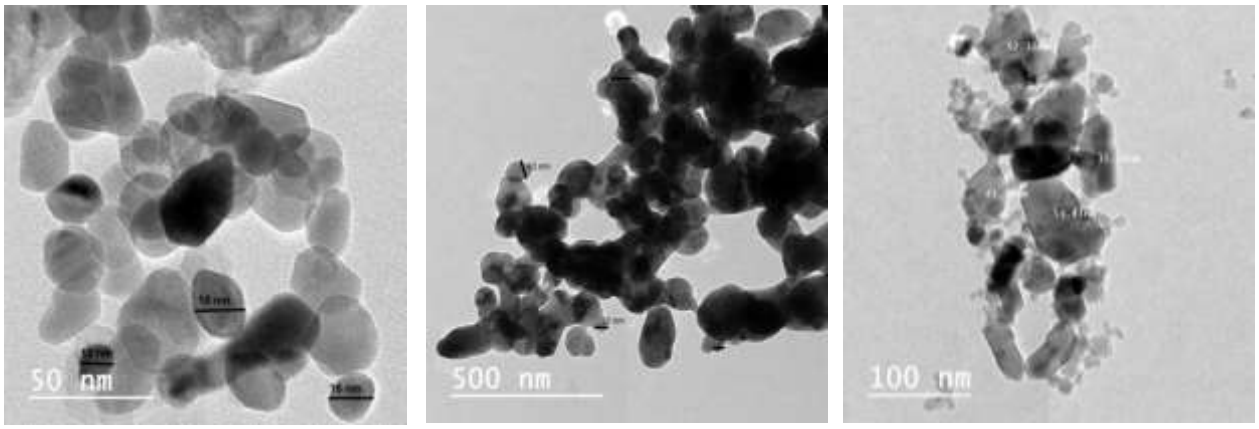
Physiological and biochemical characterization of serum pretreated with different size of ZnO NPs:

The results of the present study showed no abnormality behavior within treated groups compared to control group. Data listed in table (1) showed that treatment with ZnO NPs affects the growth rate of mice causing loss of body weight and elevation in liver coefficient range (48-57 mg/g), while no difference in weight of brain were noticed during treatment with ZnO NPs.

Table (2) showed that ZnO NPs with size 30nm and dose 150mg/kg cause the highest significant elevation in ALT (72 U/L), AST (71.2 U/L) and ALP 236.46 U/L. On the other hand, the same size and dose induced the lowest significant reduction in total Protein (32 µg).

Toxicity of ZnO NPs on hepatic levels of MDA, NOx , GSH , CAT and TAC in mice:

Data listed in table (3) showed highest significant elevation in MDA (52.18 nmol/mg protein), TAC reaching 1.59mmol/g tissue and NOx reaching 266.3 µmol/g.tissue using size30nm and dose 150 mg/kg.



ZnO NPs 14 nm **ZnO NPs 30 nm** **ZnO NPs 50 nm**

Figure 1: Characterization of ZnO NPs by transmission electron microscopy (TEM

Table 1: Toxicity of ZnO NPs on liver, kidney and brain in treated mice

Parameters Groups		Body weight (g)		Liver wt. *(g)*	Liver coefficient *(mg/g)*	Kidney wt (g)	Kidney coefficient *(mg/g)*	Brain wt. (g)	Brain coefficient *(mg/g)*
		Initial	After (14 days)						
Control		24.5±0.5^{c}	27.2±1.2^{a}	1.31±0.01^{a}	48.5± 1.6^{d}	0.41±0.01^{a}	15.3±0.3^{abc}	0.33±0.01^{a}	12.3±0.3^{a}
14 nm	**50mg/kg**	24.7±0.9^{c}	26.7±0.5^{a}	1.31±0.01^{ab}	49 ± 0.4^{d}	0.41±0.01^{a}	15.4 ±0.1^{ab}	0.34±0.01^{a}	12.7±0.4^{a}
	150mg/kg	25.7±2.5^{bc}	21.5±2^{c}	1.20±0.1^{d}	53.4± 3.2 ^{cd}	0.33±0.02^{c}	15.7±0.6^{a}	0.27±0.01^{c}	12.6±0.8^{a}
30 nm	**50mg/kg**	28±1.4^{a}	25±0.8^{ab}	1.28±0.02^{abc}	51.5± 1^{c}	0.37±0.01^{b}	14.9±0.2^{bcd}	0.27±0.01^{c}	12.5±0.4^{a}
	150mg/kg	25.7±0.5^{bc}	22.5±1.7^{c}	1.20±0.02^{c}	54 ± 2.9^{bc}	0.34±0.02^{bc}	15.2 ±0.2^{ab}	0.3±0.01^{ab}	12.2±0.2^{a}
50 nm	**50mg/kg**	26.5±1.7^{abc}	23±2.1^{bc}	1.24±0.05^{bc}	54 ± 1.5^{a}	0.34±0.01^{c}	14.6±0.6^{d}	0.29±0.01^{bc}	12.8±0.2^{a}
	150mg/kg	27.2±1.5^{ab}	23±1.1^{bc}	1.24±0.02^{bc}	54.2 ± 2^{ab}	0.34±0.02^{c}	14.8±0.3^{cd}	0.29±0.02^{bc}	12.7±0.4^{a}

Values are expressed as mean ± S.D, (n=15). Statistical analysis is carried out using SPSS computer program coupled with Co-Stat computer program (version 8), where unshared letters between groups are the significance value at $p \leq 0.05$

Table 2: Toxicity of ZnO NPs on liver function in serum of treated mice

Parameters Groups		ALT *(U/L)*	AST *(U/L)*	ALP *(U/L)*	Total Protein *(µg)*
Control		23.0± 3.5^{e}	28.2±0.9^{g}	38.0± 0.54^{d}	50±1.2^{a}
14nm	**50mg/kg**	32.75 ± 1.2^{d}	37.5 ± 1.7^{f}	77.19 ± 9.85^{c}	41.2±1.2^{b}
	150mg/kg	59.75± 2.3^{b}	59± 0.5^{c}	131.8 ± 6.3^{b}	37±1.2^{c}
30nm	**50mg/kg**	43.75 ± 3.3^{c}	45.7± 0.9^{d}	119.49 ± 10.67^{b}	39±1.2^{b}
	150mg/kg	72.0 ± 9.9^{a}	71.2± 0.9^{a}	236.46 ± 31.2^{a}	32±1.2^{d}
50nm	**50mg/kg**	43.25 ± 0.5^{c}	42± 1.1^{e}	78.67 ± 24.53^{c}	40.2±1.2^{b}
	150mg/kg	59.0 ± 0.82^{b}	66.7± 0.5^{b}	218.8± 11.15^{a}	36±0.8^{c}

Values are expressed as mean ± S.D, (n=15). Statistical analysis is carried out using SPSS computer program coupledwith Co-Stat computer program (version 8), where unshared letters between groups are the significance value at p ≤ 0.05.

Table 3: Biochemical parameter of untreated (control) & treated mice with ZnO NPs

Parameters Groups		MDA *(nmol/mg protein)*	NO_x *(µmol/g tissue)*	GSH *(mmol/g tissue)*	TAC *(mmol/g. tissue)*	CAT *(unit/g.tissue)*
Control		5.7± 0.26^{e}	169.3 ± 1.5^{g}	317.57 ± 7.9^{a}	0.67 ± 0.06^{f}	44.8±0.5^{a}
14 nm	**50mg/kg**	11.72 ± 1.73^{de}	176.6 ± 0.5^{f}	310.08 ± 0.9^{a}	0.92 ± 0.03^{e}	37±1.4^{b}
	150mg/kg	14.37±3.22^{cd}	209 ± 0.5^{c}	263.06± 12.72^{c}	1.25 ± 0.03^{c}	25.7±0.9^{d}
30 nm	**50mg/kg**	19.76 ± 4.6^{bc}	188 ± 1^{d}	274.17 ± 17.48^{bc}	1.21 ± 0.09^{c}	23±0.8^{e}
	150mg/kg	52.18 ±8.1^{a}	266.3 ± 1.5^{a}	224.79± 8.58^{e}	1.59± 0.03^{a}	14±0.8^{g}
50 nm	**50mg/kg**	17.31± 1.07^{bcd}	184 ± 1^{e}	285.42 ±11.98^{b}	1.06 ± 0.07^{d}	34.7±1.8^{c}
	150mg/kg	22.01 ± 4.39^{b}	240± 1^{b}	244.45± 2.98^{d}	1.37 ± 0.16^{b}	19±0.95^{f}

Values are expressed as mean ± S.D, (n=15). Statistical analysis is carried out using SPSS computer program coupledwith Co-Stat computer program (version 8), where unshared letters between groups are the significance value at p ≤ 0.05

Table 4: Toxicity of different size and dose of ZnO NPs on neurotransmitters parameters in brain of mice:

Parameters Groups		Dopamine *(ng /mg tissue)*	Serotonin *(ng /mg tissue)*	Noradrenaline *(ng /mg tissue)*
Control		61.11 ± 0.85^{a}	431 ± 0.82^{a}	550 ± 1.6^{g}
14 nm	**50mg/kg**	56 ± 0.82^{b}	410 ± 1.63^{b}	560 ± 0.8^{f}
	150mg/kg	45± 0.82^{e}	390 ± 1.63^{d}	595 ± 2.4^{e}
30 nm	**50mg/kg**	47 ± 1.2^{d}	370 ± 0.5^{e}	620 ± 1.6^{c}
	150mg/kg	41± 0.81^{f}	345 ± 3.8^{g}	660 ± 1.6^{a}
50 nm	**50mg/kg**	52 ±0.82^{c}	395 ± 1.63^{c}	615 ± 0.8^{d}
	150mg/kg	44 ± 0.82^{e}	364± 2.4^{f}	645 ± 0.8^{b}

Values are expressed as mean ± S.D, (n=15). Statistical analysis is carried out using SPSS computer program coupled with Co-Stat computer program (version 8), where unshared letters between groups are the significance value at p ≤ 0.05.

Toxicity of ZnO NPs on brain of mice:

Dopamine, seratonine and noradrenaline were measured in brain of mice pretreated with the different sizes and dosesof ZnO NPs.

Table (4) showed highest significant elevation in noradrenalin with size 30nm and dose 150 mg/kg reaching 660 ng /mg tissue. On the other hand, highest significant reduction were noticed in dopamine and serotonin reaching 41 ng /mg tissue and 345 ng /mg tissue respectively using the same dose.

Toxicity of ZnO nanoparticle on hepatic levels of Bax, Bcl2 and caspase 3 levels in mice:

Expression of apoptosis related genes (Caspase-3, Bcl-2, and Bax) in liver tissues of mice exposed to ZnO NPs with different doses and sizes is summarized in Fig (2). The results revealed that the highest increase in gene expression levels of caspase 3 and Bax in liver tissues was in the group treated with ZnO Nps (30 nm ,150 mg/kg) compared with the other treated groups and with the control group. On the other hand, the gene expression levels of Bcl-2 in liver tissues was the lowest in the group treated with ZnO NPs (30 nm,150 mg/kg) compared to all other groups.

Effect of ZnO NPs on percentage of DNA damage in liver tissue in mice:

Table (5) show the oxidative DNA damage in the liver cells of mice that was evaluated by the Fpg-modified Comet assay after exposure to different doses and sizes of ZnO NPs. Damage levels were significantly increased with various values recording the highest damage (23.8 %) and Tail length (58.9±8.7) with 30 nm and dose 150 mg/kg.

Histopathological Findings:

The histopathological study was performed on mice treated with ZnO NPs (50 and 150 mg/kg) and sizes 14, 30 and 50 nm for 14 consecutive days.

The results showed that the histopathological disorders in liver were gradually revealed in a dose dependent manner. However NPs with size 30 nm and dose 150 mg/kg induced the most adverse effect as shown in figure (3).

Fig. 3(A&B) showed hepatic tissue with preserved (intact) lobular hepatic architecture with normal morphological appearance and mild hydropic degeneration (black arrow) respectively (H and E,x200, x400). (C): showed preserved (intact) lobular hepatic architecture, hepatocyte with ballooning (black arrow) and bi nucleated hepatocytes (yellow arrow), collection of lymphocytes (yellow arrow), (H&E,x200, x400). (D):showed preserved (intact) lobular hepatic architecture, hepatocyte with ballooning (red arrow), hydropic degeneration of hepatocytes (black arrow) and bi nucleated hepatocytes (green arrow), congested blood vessels (green arrow) and 10 % mild necrosis .(H&E,x100,x200, x400). (E): showed hepatic tissue with preserved (intact) lobular hepatic architecture and collection of interlobular lymphocytes (black arrow) (H&E,x200, x400).(F): showed hepatic tissue with preserved (intact) lobular hepatic architecture and insignificant pathological changes (H&E,x200, x400). (G): liver section from control group showed hepatic tissue with preserved (intact) lobular hepatic architecture and normal morphological appearance (H&E,x200, x400).

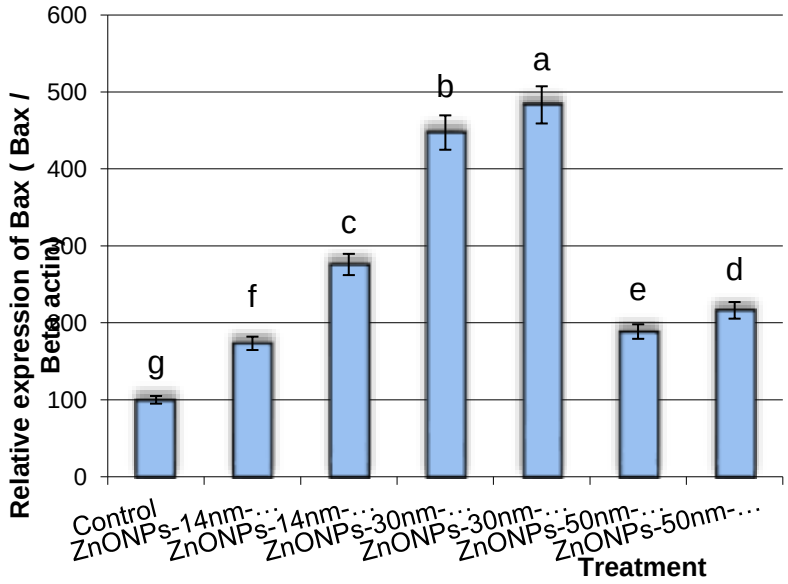


Figure .2A: Effect of ZnO NPs on mRNA expression of Bax. Values of each bar were means ±S.D. β-Actin was used as an internal control for calculating mRNA fold changes.

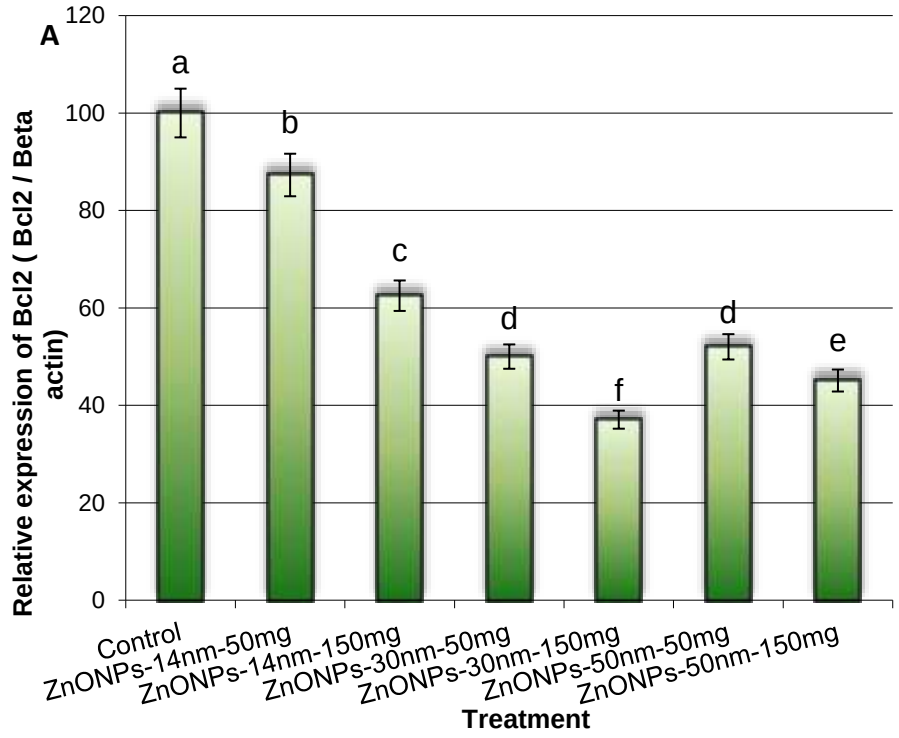


Figure 2B: Effect of ZnO NPs on mRNA expression of Bcl2. Values of each bar were means ±S.D. β-Actin was used as an internal control for calculating mRNA fold changes.

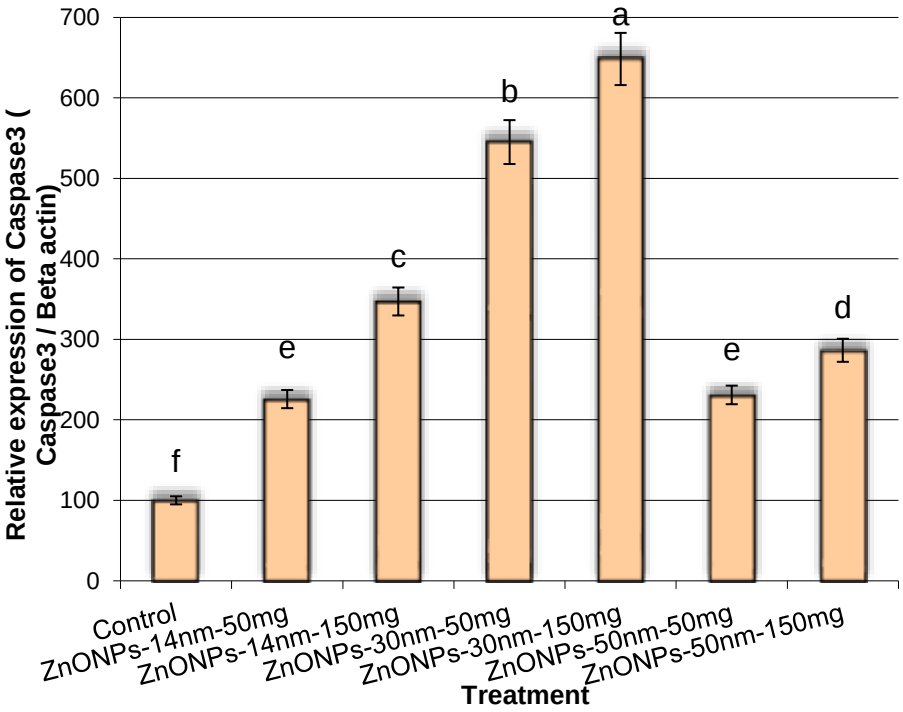


Figure 2c: Effect of ZnO NPs on mRNA expression of caspase 3. Values of each bar were means ±S.D. β-Actin was used as an internal control for calculating mRNA fold changes.

Table 5: Visual score of DNA damage in liver tissues from different treated groups with ZnO NPs

Treatment		No. of animals	No. of cells		Class**				DNA Damaged cells %	Tail length (Mean ± SEM)
			Cell Analyzed*	No of comets	0	1	2	3		
Control		5	500	31	469	23	8	0	6.2	14.3±4.2
14nm	50mg/kg	5	500	41	459	21	14	6	8.2	17.6±3.6
	150mg/kg	5	500	78	422	28	31	19	15.6	28.4±5.1
30nm	50mg/kg	5	500	96	404	26	42	28	19.2	43.6±8.2
	150mg/kg	5	500	119	381	33	52	34	23.8	58.9±8.7
50nm	50mg/kg	5	500	49	451	23	17	9	9.8	21.1±6.2
	150mg/kg	5	500	62	438	28	21	13	12.4	26.4±3.4

*: Number of cells examined per a group, **: Class 0= no tail; 1= tail length < diameter of nucleus; 2= tail length between 1X and 2X the diameter of nucleus; and 3= tail length > 2X the diameter of nucleus .Number of examined mice in each group=5

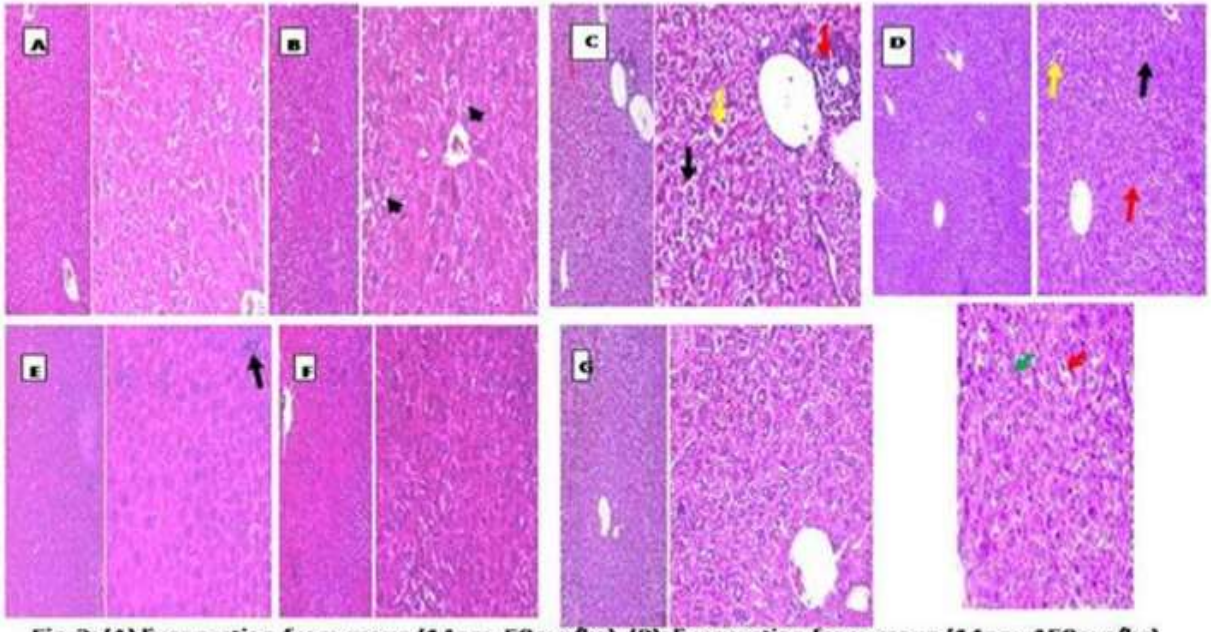


Fig. 3: (A) liver section from group (14nm, 50mg/kg), (B): liver section from group (14nm, 150mg/kg) (C): Liver section from group (30nm, 50mg/kg), (D): Liver section from group (30nm, 150mg/kg) (E): liver section from group (50nm, 50mg/kg), (F): liver section from group (50nm, 150mg/kg) (G): liver section from control group

DISCUSSION

Although Nanotechnology is currently applied in industrial and therapeutic applications, it could induce risk factors with regards to their neuropathological and toxicological effects. Human body may be intentionally or unintentionally exposed to nanoparticles via several possible routes including oral ingestion, inhalation, intravenous injection, and dermal penetration. Because of their small size, they can penetrate physiological barriers, and travel within the circulatory systems (Wang et al., 2007). They can cross the systemic circulation and further distribute into the blood, brain, lung, heart, kidney, spleen, liver, intestine and stomach (Hillyer and Albrecht, 2001).

Biological effects of nano-scaled particles might contribute to their physicochemical properties, such as size, surface area and surface charge, as demonstrated by Long et al., (2006).

In the present study, the oral toxicity of ZnO NPs with size ranges (14, 30 and 50 nm) was investigated in mice using two different doses (50,150 mg/kg) after an exposure for 14 consecutive days. ZnO NPs with dose (150 mg/kg) and particle size (30 nm) revealed the highest toxic effects which may accumulate in the liver leading to oxidative stress, DNA damage and apoptosis Which gives some support to the findings of Sharma et al., (2012).

In the same field of nanoparticle, research scientist demonstrated that gold nanoparticles were mainly accumulated in liver followed by lung, spleen and kidney. Interestingly, 15 and 50 nm gold NPs was able to pass blood brain barrier as evidented from gold concentration in brain (Sonavanea et al., 2008).

Studies have demonstrated that the pathogenic mechanisms initiated by NPs are dominated by inflammation driven effects such as oxidative stress, apoptosis and DNA damage (Asuku et al., 2011).

The current study showed that serum ALT and AST activities were increased significantly post ZnO NPs intoxication (30 nm,150 mg/kg) compared to the control value. These results were supported by the finding of Pasupuleti et al., (2011) who reported that ZnO NPs fed to Sprague Dawley rats for 14 days showed an elevation in ALT and AST activities which was correlated with liver damage and cellular leakage. Another study by Wang et al. (2007) used TiO_2 particles with primary size of 25 nm and 80 nm via gastrointestinal demonstrated changes in serum activities of ALT, AST, and LDH. These data were supported by the recent findings of Almansour et al., (2017).Nano-particles were observed to produce free radicals leading to oxidative stress and thus can disturb the antioxidant defense system in mice (Simko and Mattsson, 2010). Oxidative stress was shown to be one of the predominant ways of nanoparticle toxicity and stress in various cell lines. Lipid peroxides (LPO) can be defined as the oxidative deterioration of cell membrane lipids and has been used extensively as a marker of oxidative stress (Sayeed et al., 2003). The over accumulation of MDA can damage cells and trigger apoptosis (Kong et al., 2007).

The present study showed that ZnO NPs with particle size (30 nm) administered in a dose of 150 mg/kg caused the most significant elevation in hepatic MDA, NOx and TCA levels and depletion in hepatic total protein. These results may be explained as a result of NPs accumulation, which due to their catalytic properties reactive oxygen species (ROS) may be generated causing break down in the balance of the oxidative/antioxidative system in the liver. These results in lipid peroxidation via ROS and MDA production leading to hepatocyte apoptosis associated with diminution in the antioxidative enzymes(Jeon et al., 2013).The increase in lipid peroxidation was accompanied with depletion in total protein level leading to tissue injury and liver damage which was in line with the previous observation of Bheeman et al. (2014). These findings were also in accordance with Fu et al. (2014)that TiO_2NPs produce ROS due to their catalytic properties. NO is a chemical mediator involved in the maintenance of physiological homeostasis due to its regulatory and protective functions. Besides its known antioxidant property, NO which is produced by inducible nitric oxide synthase (iNOS) can be cytotoxic, especially at higher local concentrations. Also, it can react with reactive oxygen species (ROS) or oxygen yielding reactive nitrogen species (RNS), which causes damage on biological molecules such as enzymes, lipids and DNA by nitrosation, oxidation and nitration.

On the other hand, the level of hepatic GSH revealed the most significant reduction post ZnO NPs (30 nm, 150 mg/kg) intoxication. A previous study by Shokouhian et al., (2013) showed that GSH levels had been reported to be reduced significantly post oral administration of ZnO NPs in lung tissue. In addition, Attia et al.(2013)found a significant increase in MDA, CAT and NOx levels and a decrease in GSH levels in liver tissues after

TiO_2NPs intoxication. Also, Azim et al., (2015)demonstrated that the oral administration of NPs caused a significant elevation in the hepatic levels of MDA and TAC.

The present study showed that DNA damage in the liver cells of mice after exposure to different doses and sizes of ZnO NPs showed a significant increase with various values recording the highest damage with 30 nm and dose 150 mg/kg. These data were reinforced by the study of Xiong et al., (2011), which suggested that NPs-induced DNA damage is oxidative stress-dependent. NPs may cause several biological responses including DNA strand breaks (Vinzents et al., 2005). Virtually, ROS react with DNA molecule, causing damage to purine and pyrimidine bases of DNA backbone, apoptosis and there by cell death (Trouiller et al., 2009).Apoptosis maintains the cellular homeostasis between cell division and cell death. Apoptosis is triggered via two principal signaling pathways; the death receptor-mediated extrinsic apoptotic pathway and the mitochondrion-mediated intrinsic apoptotic pathway. In this latter, cells respond to various stress full stimuli by prompting apoptosis through Bax /Bcl-2/ Caspase-9 cascade where Caspase-9 cleaves and activates the apical effect or caspases such as Caspase-3. Caspase-3 is thus regarded as one of the key executioners of apoptosis, being responsible either partly or totally for the proteolytic cleavage of many key proteins (Kandasamy et al., 2003). NPs were also found to be potent inducers of Bax and inhibitors of Bcl-2 (Yao et al., 2008).

In the current study, oral administration of ZnO NPs caused a significant elevation in caspase-3 activity and Bax levels and a down-regulation in Bcl-2 level in mice treated with ZnO Nps (30nm, 150 mg/kg) compared with all other groups.

Dopamine and serotonin are neurotransmitters, which are found in neurons of all animals. Alteration in the human neurological can cause disorders such as Parkinson's disease and depression (Dawson and Dawson, 2003). Serotonin has been reported to have an inhibitory action in the brain (Daw et al., 2002). Dopamine is a neurotransmitter involved in modulating aggressive behavior in animals (Tidey and Miczek, 1996). In current study, ZnO NPS showed a significant decrease in serotonin and dopamine but significant increase in noradrenaline with size 30 nm and dose 150mg/kg compared with all other groups. This was declared by Hussain et al.,(2006)that manganese (Mn) NPs (40nm) induce the depletion of dopamine and its metabolites in a dopaminergic neuronal cell line (PC12); and such depletion is accompanied by an increase in reactive oxygen species (ROS) production. The same study also demonstrated that Ag-NPs (15nm) moderately decrease dopamine content with an increase in ROS production.

Our results demonstrated that the histopathological changes were highly affected in ZnO NPs (30 nm, 150 mg/kg) in liver tissues. Liver sections showed intact lobular hepatic architecture, hepatocyte with ballooning, hydropic degeneration of hepatocytes and binucleated hepatocytes, congested blood vessels and 10 % mild necrosis. This is in agreement with the previous report indicating that sub-acute oral exposure to ZnO nanoparticles (300 mg/kg) for 14 consecutive days induced hepatocellular necrosis (Soheili et al., 2013). Histopathologic alterations, including hydropic degeneration around the central vein and the spotty necrosis of hepatocytes (Wang et al., 2007), Moreover, previous report showed inflammation; hepatic necrosis and degeneration induced by 35 nm ZnO NPs (Almansour et al., 2017).

CONCLUSION

To summarize the scientific impact of the present study, it could be concluded that, although ZnO NPs are previously reported to have diverse toxic effects, the data in this research helped to attain a safe dose correlated with a small particle size of these NPs which may be used in biomedical applications in further research. The achieved results revealed the biosafety of these NPs on certain biochemical indices in liver and brain using the smallest particle size and lowest dose. Also their safety on other organs is demonstrated histo pathologically. This could be of a significant scientific benefit to be applicable in medical and /or therapeutic fields for management of diseases affecting the society such as cancer by killing target cells. This effect may be attributed to the ability of antioxidant defense mechanisms to overcome the oxidative stress induced by these nanoparticles.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

Nahla Nabil kamel performed the experimental part and participated in writing the manuscript and submission. Prof. MahaZakiRizk contributed in suggesting the research point and interpretation of data. DrWalaa Gamal Hozayen participated in scientific design and scientific consultations. Drwagdikhalil supervised molecular part. Dr Abdel hamidZaki Abdel Hamid took part in suggesting the topic and in scientific consultations. All authors revised the manuscript.

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