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Article *in* Toxicology mechanisms and methods · May 2015

DOI: 10.3109/15376516.2015.1045654 · Source: PubMed

Toxicology Mechanisms and Methods

http://informahealthcare.com/txm
ISSN: 1537-6516 (print), 1537-6524 (electronic)

Toxicol Mech Methods, Early Online: 1–6
 DOI: 10.3109/15376516.2015.1045654

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RESEARCH ARTICLE

Zinc oxide nanoparticles induced cyto- and genotoxicity in kidney epithelial cells

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Abstract

The wide uses of zinc oxide nanoparticles (nano-ZnO) in industrial, cosmetics, medicine, food production and electronics associate with increase in occupational and public exposure. Although, toxicity of nano-ZnO has been extensively studied on many different cell types and animal systems, there is a significant lack of toxicological data focus on nephrotoxic potential of nano-ZnO. In this study, the cyto- and genotoxic effects of nano-ZnO on rat kidney epithelial cells (NRK-52E) were investigated by using different assays. Nano-ZnO (10–50 nm of sizes) were synthesized by sol–gel method. For the cytotoxic effect of nano-ZnO, mean of inhibition concentration (IC_{50}) values in cell line was evaluated by MTT, Trypan Blue (TB) and Neutral Red Uptake (NRU) assays at 25.0–100.0 μg/mL exposure concentrations. Nano-ZnO showed cytotoxic activity by acting on different targets in renal cells, with $IC_{50} \geq 73.05$ μg/mL. Comet assay was used to evaluate the genotoxicity of nano-ZnO (12.5–50.0 μg/mL). Nano-ZnO caused statistically significant DNA damage. Our results highlight the important risk of cyto- and genotoxic effects of nano-ZnO over the kidney.

Keywords

Cytotoxicity, genotoxicity, nanoparticle, nephrotoxicity, zinc oxide

History

Received 14 January 2015
Revised 12 April 2015
Accepted 20 April 2015
Published online 18 May 2015

Introduction

Nano-sized metal oxide particles, whose structures exhibit unique physical, chemical and biological properties, have gained increased interest in recent years (Cui et al., 2001; Fubini, 1997). Zinc oxide nanoparticles (nano-ZnO) are one of the most commonly used materials in diverse industrial fields, such as dyes, paints, pigments, metallurgy additives, rubber, alloys, ceramics, chemical fibers, electronics, catalyst, medical diagnosis, sunscreens, cosmetics, personal care products and food additives (EPA, 2007; Wang, 2004). The wide range of applications of nano-ZnO is attributed to their unique characteristics, including semiconducting, electrical, optical, catalytic, magnetic, antimicrobial and ultraviolet light absorption properties (Fan & Lu, 2005; Kumari & Li, 2010; Qian, 2011; Su et al., 2009).

ZnO is generally considered to be a material with 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 (Wang et al., 2008). It is well known that the nano-sized particles have a higher proportion of atoms on their surfaces than do bulk-sized particles, so the former is more reactive and responsive than the latter (Oberdorster et al., 2005). Similarly, toxicological studies indicated that nano-ZnO had adverse impacts on human health and environmental species. Nano-ZnO induce toxicity leading to damage of lipids, proteins and DNA, increased release of lactate dehydrogenase, induce inflammation and death by either necrosis or apoptosis (Deng et al., 2009; Huang et al., 2010; Rasmussen et al., 2010; Sharma et al., 2009). Despite the existing studies on the toxicity of nano-ZnO, the underlying molecular mechanism leading to toxicity remains largely unclear (Sharma et al., 2012a).

Human body may be intentionally or unintentionally exposed to nanoparticles *via* several possible routes, including oral ingestion, inhalation, intravenous injection and dermal penetration (Revell, 2006). According to the pharmacokinetics studies, the distribution of nano-ZnO was observed in lung, liver, kidney, spleen, mammary tissue, heart, pancreas and bone and nano-ZnO accumulated especially in the liver, lung and kidney (Baek et al., 2012; Esmaeillou et al., 2013; Hillyer & Albrecht, 2001; Jo et al., 2013; Lee et al., 2012; Li et al., 2012; Sharma et al., 2012a; Wang et al., 2008). By *in vivo* studies, it was observed that respiratory exposure to nano-ZnO caused strong oxidative stress in the lung and increased lipid peroxidation, inflammogenic effect, while oral exposure to nano-ZnO decreased the wet weights of spleen, kidney and liver, inflammation in pancreas, injury in liver

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(Cho et al., 2010; Esmaeillou et al., 2013; Fukui et al., 2012; Li et al., 2012; Seok et al., 2013). Although, nano-ZnO were shown to distribute to the kidney which is one of the target organ for accumulation, there are very few studies evaluating nano-ZnO toxicity in kidney (Baek et al., 2012; Esmaeillou et al., 2013; Jo et al., 2013; Lee et al., 2012; Sharma et al., 2012a,b; Wang et al., 2008). Nano-ZnO caused necrosis in the epithelial cells in the tubules, swelling in the epithelial cells of proximal tubules causing sever toxic effects on the kidney (Esmaeillou et al., 2013). Sharma et al. (2012a) observed that nano-ZnO caused cystic dilation of tubules and hypertrophied in the kidney, even though the results were not statistically significant with the potential of oxidative stress or genotoxicity. According to Jo et al. (2013), nano-ZnO could pose health risk to pregnant rats and fetus in rats exposed to nano-ZnO during pregnancy and lactation period because nano-ZnO were present in the liver and kidney of pups of exposed rats.

The aim of this study was to investigate the cyto- and genotoxic effects of nano-ZnO on kidney epithelial cells (NRK-52E) by using MTT, TB and NRU assays and comet assay.

Materials and methods

Chemicals

Neutral red dye, triton X-100, ethylenediaminetetraacetic acid (EDTA), trypsin, MTT and trypan blue (TB) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO). Phosphate buffered saline (PBS), penicillin–streptomycin solution and Dulbecco's modified Eagle's medium (DMEM)/F12 from Multicell Wisent (Quebec, Canada). Fetal bovine serum (FBS) and ethidium bromide (EtBr) were purchased from Gibco (Carlsbad, CA) and from Merck (Kenilworth, NJ), respectively. Low and normal melting agaroses were purchased from Himedia (Mumbai, India). All other chemicals were obtained locally in Turkey and were of analytical reagent grade.

Synthesis of nano-ZnO

Nano-ZnO were synthesized using zinc acetate dihydrate (99.5%), sodium hydroxide (NaOH), ethanol and *n*-heptane (99%) with sol–gel method (Ba-Abbad et al., 2013; Kumar et al., 2014). Zinc acetate dihydrate (0.46 g) was dissolved in 30 mL ethanol at 70 °C for 20 min with magnetic stirrer. In the same way, we dissolved 0.2 g NaOH in 10 mL ethanol for 30 min at 70 °C. Zinc acetate dehydrate solution was added to the NaOH aqueous solution under string with magnetic stirrer in an ice for 10 min. Then, *n*-heptane was added in the admixture with the aid of magnetic stirrer for 20 min at room temperature. After centrifugation, the resulting precipitated nano-ZnO were cleaned several times with ethanol to remove the unwanted elements which were used in synthesizing and dried in the oven at about 55 °C. Lastly, nano-ZnO were obtained as white-powder.

The morphologies and chemical compositions of nano-ZnO (10–50 nm) were determined by Scanning Electron Microscopy (SEM) (FEI-Quanta, OR) and Energy Dispersive Spectroscopy (EDS) (FEI-Quanta, OR), respectively. UV–Vis absorption spectroscopy measurement was carried out for optical characterization with using UV-spectrophotometer (Shimadzu, Columbia, MD).

The size and distribution of nano-ZnO were also obtained with Zetasizer Dynamic Light Scattering (DLS) (Malvern 2000 ZetaSizer®, Malvern, UK) in both bidistilled water and cell medium. Nano-ZnO were also blocked by pre-treatment with FBS according to Horie et al. (2009) with some modifications.

Cell culture

Rat kidney epithelial cell line (NRK-52E) was obtained from the American Type Culture Collection (CRL-1571™, ATCC, Manassas, VA). The cells were cultured in DMEM-F12 containing 5.5 mmol/L D-glucose supplemented with 10% FBS and 100 U/mL penicillin–streptomycin at 5% CO_2, 90% humidity and 37 °C.

Nano-ZnO were suspended in cell culture medium with FBS and sonicated at room temperature for 5 min to avoid nanoparticle agglomeration prior to cell exposure and diluted to appropriate concentrations (25.0–100.0 μg/mL for cytotoxicity assays and 12.5–50.0 μg/mL for genotoxicity assay).

Cytotoxicity

The cytotoxic potential of nano-ZnO was investigated by using several methods based on different cellular mechanisms depending on damaged region of cells. MTT, TB and NRU assays were obtained for mitochondrial, membrane and lysosomal damage, respectively. In each assay, 1% PBS is used as negative and triton X-100 is used as positive controls with the concentrations of 0.1, 1.0 and 10.0 μg/mL. For all concentrations, it was tested in triplicates and each assay was repeated twice. The half maximal inhibitory concentration (IC_{50}) was expressed as the concentration of sample that caused an inhibition of 50% in enzyme activities in cells.

MTT assay

MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide, is a water soluble yellow colored salt reduced by the mitochondrial succinate dehydrogenase to insoluble purple formazan product. Mitochondrial succinate dehydrogenase is only active in viable cells, thus, color changes by the activity of the enzyme can be used as a cytotoxicity endpoint (Alley et al., 1988; Van Meerloo et al., 2011). NRK-52E cells were seeded at 10^4 cells into each well of 96-well plates. After 24 h, the cells were exposed to nano-ZnO. After 24 h incubation period, 20 μL MTT was added and incubate for 1 h. Optical densities (ODs) of each well were determined at 590 nm (against a reference wavelength of 670 nm) using a microplate spectrophotometer system (Epoch, Germany).

Neutral red uptake (NRU) assay

In NRU assay, lysosomal integrity can be used as an indicator of cell viability by up taking neutral red dye into cells (Borenfreund & Puerner, 1984; Repetto et al., 2008). After 24 h of exposure in 96-well plates, the medium was discarded. Neutral red dye (100 μL) in serum free medium (100 μg/mL) was added to each well and incubated for 3 h at 37 °C. Cells were washed three times with warm 1 × PBS. The dye taken

up by cells was then dissolved in a medium containing 50% ethanol and 1% acetic acid in Milli-Q water. OD was taken at 540 nm using a microplate spectrophotometer system (Epoch, Germany).

Trypan blue (TB) assay

TB assay is based on the principle that viable cells do not take up certain dyes whereas non-viable cells do (Midander et al., 2009). 5×10^4 cell per well were allow to attach for 24 h in 24-well plate before exposed to nano-ZnO. After 24 h of incubation, the medium was discharged. Cells were washed with PBS (500 µL). The cells were collected by trypsination. The removed PBS, discharged exposure medium and trypsinated cells were collected in tubes and centrifuged for 3 min at 1000 rpm. After the supernatant was removed, the remaining cell suspension (30 µL) was mixed with TB (30 µL) and incubated for 3 min. The percentage of viable cells was counted by Luna cell counter (Annandale, VA).

Genotoxicity

In each assay, 1% PBS and 100 µM hydrogen peroxide (H_2O_2) were used as negative and positive controls, respectively. For all concentrations, it was tested in triplicates. DNA damage to individual cells was expressed as a percentage of DNA in the comet tail (% TDNA, tail intensity).

Comet assay

The exposed cells were washed with PBS, trypsinized, centrifuged at 1000 rpm for 3 min and re-suspended in 0.5 mL 1 × PBS. The viability of cells was determined with TB test and cells viability was >80% in all concentrations. Briefly, 100 µL of cells was mixed with 100 µL pre-warmed low-melting point agarose (0.65% in 1 × PBS), layered on normal-melting point agarose (1.5% in distilled water) pre-coated microscope slides then covered with cover slip. Slides were placed in refrigerator for solidification, then cover slips were removed and slides were incubated for 1 h at 4 °C in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris-HCl, pH 10), added with 10% DMSO and 1% triton X-100. DNA was unwinded for 20 min in cold-fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) at 4 °C and electrophoresis was performed at 4 °C for 20 min (20 V/300 mA) (Speit & Hartmann, 1999). After electrophoresis, slides were neutralized with 0.4 M Tris-HCl buffer (pH 7.5) three times for 5 min. Hundred cells were scored per concentration under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) at 400 magnification by using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK) just after DNA was stained with ethidium bromide (20 mg/mL). At least 100 cells were scored per concentration.

Statistical analysis

The significance of differences between the control and nano-ZnO-treated cells was calculated by one-way ANOVA Dunnett *t*-test using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL) and expressed as mean ± standard deviation (SD) and standard error (SE). *p* Values of less than 0.05 were selected as the levels of significance.

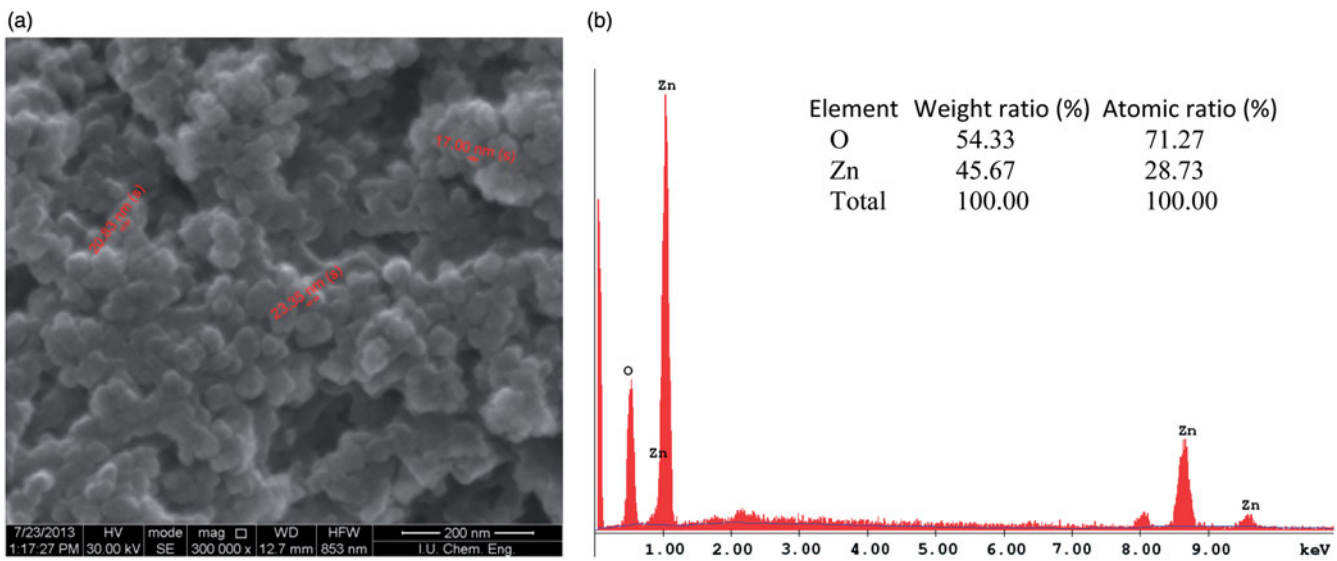
Result

Structural and optical characterization results

According to SEM results, we obtained the nanoparticles having 10–50 nm of size (Figure 1a). In EDS analysis, the sample was only composed of Zn and O elements (Figure 1b).

The typical absorption spectra of the synthesized nano-ZnO and bulk ZnO are given in Figure 2. The absorption peaks were at 375 and 359 nm for bulk ZnO and nano-ZnO, respectively.

As it can be seen in Figure 1, the sample exhibits the homogenous nanoparticle distribution. Also, the size and distribution of nano-ZnO were obtained with Zetasizer DLS in both bidistilled water and exposure medium. The results obtained were compatible with SEM (Table 1).



Element	Weight ratio (%)	Atomic ratio (%)
O	54.33	71.27
Zn	45.67	28.73
Total	100.00	100.00

Figure 1. (a) SEM image and (b) EDS image of nano-ZnO.

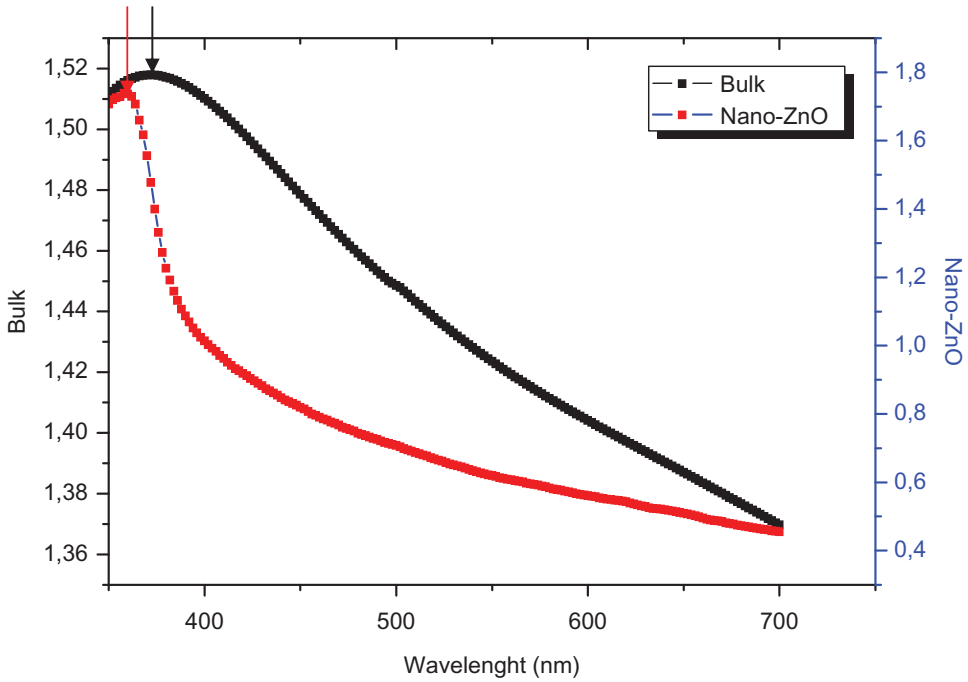


Figure 2. UV–VIS absorption spectra of synthesized bulk ZnO and nano-ZnO.

Table 1. Size and distribution of nano-ZnO with Zetasizer DLS.

Media	Average particle size (nm)	Frequency (%)
Bidistilled water	9.6	43.1
	43.0	52.8
	68.0	3.1
Medium	13.4	57.2
	62.8	38.9
	122	3.6
FBS-blocking[a]	20.9	7.0
	53.4	82.0
	97.1	10.0

[a]According to Horie et al. (2009) with some modifications.

Table 2. Cytotoxicity assessment by MTT, NRU and TB assays in NRK-52E cells following the exposure to various concentrations of nano-ZnO for 24 h.

	% Cell death[a] (±SD)		
Concentration (µg/mL)	MTT	NRU	TB
25	8.0 (±1.8)	9.9 (±0.8)	15.5 (±1.2)
50	29.0 (±2.6)	43.7 (±3.3)	34.8 (±3.5)
75	55.6 (±3.2)	50.2 (±7.1)	44.9 (±2.4)
100	71.0 (±6.6)	55.4 (±3.4)	61.9 (±5.8)
IC_{50}	73.05	75.39	80.46

[a]The viability value was 95% for negative control (1% PBS). % Cell death was ≤ 80 for positive control.

Cytotoxicity

Nano-ZnO were disturbed on membrane permeability, mitochondrial and lysosomal functions in NRK-52E cells. IC_{50} values of nano-ZnO were found to be 73.05, 75.39 and 80.47 µg/mL by using MTT, NRU and TB assays, respectively. As shown in Table 2, the cytotoxicity observed was concentration dependent for all assays. Also, NRU assay showed that kidney cells were less sensitive in lysosomal pathways than membrane permeability and mitochondrial pathway in exposed nano-ZnO.

Genotoxicity

Nano-ZnO caused increase of tail intensity at the concentration range of 12.5–50.0 µg/mL and induced DNA damage even in less than %30 deaths according to TB linear equation (Table 3). Significant differences were found in tail intensity in the treated NRK-52E cells when compared to the control group ($p < 0.05$). In the highest concentration (50.0 µg/mL), the tail intensity was 18.02 which being approximately 6.35-fold of the non-exposed cell. The results revealed the induction of DNA damage by nano-ZnO's in NRK-52E cells.

Table 3. The mean tail intensity (%TDNA) with ± SE obtained from comet assay in NRK-52E cells following the exposure of various concentrations of nano-ZnO for 24 h.

Concentration (µg/mL)	% Tail intensity (±SE)	*p* Value
Negative control	2.8 (±0.3)	
12.5	5.0 (±0.1)	0.248
25.0	4.4 (±0.7)	0.347
32.5	5.3 (±0.7)	0.155
50.0	18.1 (±0.8)	0.000*
Positive control	17.0 (±0.7)	

**p* Values of less than 0.05 were selected as the levels of significance.

Discussion

The increasing use of nano-ZnO in commercial, industrial and medical fields has brought attentions to their potential toxicity and health risks. It is well established that, under specific conditions, nano-ZnO result toxic to a variety of mammalian and human cells and to animals (De Berardis et al., 2010; Osman et al., 2010; Sharma et al., 2012a,b). However, there is a lack of information on the effects of nano-ZnO on kidney (Esmaeillou et al., 2013; Jo et al., 2013; Sharma et al., 2012a).

Nanoparticles are capable of inducing oxidative stress by overproduction of reactive oxygen species. Oxidative stress causes damage to cellular macromolecules and is also involved in the intracellular signaling mechanisms which determine the cell's final fate (Abdollahi et al., 2004; Kohen & Nyska, 2002). Lipid peroxidation has been suggested to be one of the molecular mechanisms involved in the cytotoxicity of nano-ZnO (Deng et al., 2009). In the present study, nano-ZnO were disturbed on mitochondrial and lysosomal functions as well as membrane permeability in NRK-52E cells. Similar to our results, a concentration-depended decrease in cellular viability after nano-ZnO exposure was previously described for several cell types, including hepatocarcinoma HepG2 (Sharma et al., 2012a), lung carcinoma A549 (Fukui et al., 2012) and bronchial epithelial BEAS-2B (Akhtar et al., 2012), colon carcinoma LoVo (De Berardis et al., 2010), myeloblastic leukemia HL60 (Premanathan et al., 2011) and osteoblast (Nair et al., 2009) cells. Yu et al. (2013) nano-ZnO induced autophagic cell death and mitochondrial damage *via* reactive oxygen species generation in normal skin cells. By Ahamed et al. (2011), nano-ZnO were observed to induce cytotoxicity, reactive oxygen species generation, oxidative stress and activities of caspase-3 and caspase-9 in a dose- and time-dependent manner in A549 cells.

As to genotoxicity of nano-ZnO, it is of great concern that long-term nano-ZnO exposure caused certain types of cancer by their carcinogenic and mutagenic properties with the capacity of causing DNA damage. However, there has been no investigation about the effects of nano-ZnO in kidney cell.

By some studies, nano-ZnO have been demonstrated to cause DNA damages (in Comet assay) in Chinese hamster ovary CHO-K1 and human epidermal A431 cells (Dufour et al., 2006; Hackenberg et al., 2010; Sharma et al., 2009). Also, a mutagenic potential has been detected in the presence of metabolic activation (Kumar et al., 2011). Gerloff et al. (2009) found that nano-ZnO decreased the metabolic activity and increased the DNA strand break and oxidative DNA damage. Gopalan et al. (2009) found that high concentration of nano-ZnO caused genotoxicity in the exposed cells. On the contrary, nano-ZnO showed negative mutagenic responses (Li et al., 2012; Sawai et al., 1998; Yoshida et al., 2009). We observed that nano-ZnO caused statistically significant DNA damage at 12.5–50.0 µg/mL even in higher than 75% cell viability. Osman et al. (2010) observed that genotoxic effect of nano-ZnO (50 and 100 µg/mL) was clearly associated with an increase in tyrosine phosphorylation in human negroid cervix carcinoma HEp-2 cell line. Yang et al. (2009) observed an enhanced DNA damage in the comet assay for a ZnO-NP concentration of 5 µg/mL in mouse embryo fibroblast cells. These contradictions may be due to the difference in the sensitivity and reactivity of the analytic approaches.

In the study, we have demonstrated that nano-ZnO induced cell death and DNA damage by different pathways on kidney. The results highlight the existence of different mechanisms in the nano-ZnO toxicity, and point to protective strategies to overcome nanoparticle-induced harmful side effects is the cellular level. The study could be useful in determining their toxicological effects and taking precautions regarding their consumption, however it needs to be investigated in a future study.

Declaration of interest

The authors declare that there are no conflicts of interest.

The study was supported by the Research Fund of Istanbul University (UDP17307/UDP6550).

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