Global Journal of Medical Research: B
Pharma, Drug Discovery, Toxicology & Medicine
Volume 16 Issue 3 Version 1.0 Year 2016
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4618 & Print ISSN: 0975-5888

Copper (II) Oxide Nanoparticles Induce High Toxicity in Human Neuronal Cell

By Abudayyak Mahmoud, Guzel E. Elif & Özhan Gül
Istanbul University

Abstract- Copper (II) oxide nanoparticles (CuO-NPs) are widely used in industry, cosmetics and medicine. People have increasingly been exposed to these active materials. Several studies indicate that CuO-NPs could be taken up by different organs and cause toxicities. However, there is still a lack of data on the toxicological effects of CuO-NPs in neuronal system. In the present study, the toxic potentials of CuO-NPs were investigated in human SH-SY5Y neuroblastoma cells. After assessment of their cellular uptake potential, cytotoxicity by MTT and neutral red uptake (NRU) and genotoxicity by comet assay were evaluated. Enzyme-Linked Immune Sorbent Assays (ELISA) determination of malondialdehyde (MDA), 8-hydroxy-deoxyguanosine (8-OHdG), protein carbonyl (PC), and glutathione (GSH) levels for oxidative damage, and Annexin V-FITC with propidium iodide (PI) for apoptosis were used. In conclusion, CuO-NPs were found to accumulate in the cells and induced significant cytotoxic and genotoxic, and oxidative and apoptotic effects. CuO-NPs are hypothesized to dangerously affect human health, especially neuronal system.

Keywords: *copper oxide; nanoparticle; neurotoxicity; cellular uptake; genotoxicity; apoptosis.*

GJMR-B Classification : *NLMC Code: QV 600*

Strictly as per the compliance and regulations of:

Copper (II) Oxide Nanoparticles Induce High Toxicity in Human Neuronal Cell

Abudayyak Mahmoud [α], Guzel E. Elif [σ] & Özhan Gül [ρ]

Abstract- Copper (II) oxide nanoparticles (CuO-NPs) are widely used in industry, cosmetics and medicine. People have increasingly been exposed to these active materials. Several studies indicate that CuO-NPs could be taken up by different organs and cause toxicities. However, there is still a lack of data on the toxicological effects of CuO-NPs in neuronal system. In the present study, the toxic potentials of CuO-NPs were investigated in human SH-SY5Y neuroblastoma cells. After assessment of their cellular uptake potential, cytotoxicity by MTT and neutral red uptake (NRU) and genotoxicity by comet assay were evaluated. Enzyme-Linked Immune Sorbent Assays (ELISA) determination of malondialdehyde (MDA), 8-hydroxy-deoxyguanosine (8-OHdG), protein carbonyl (PC), and glutathione (GSH) levels for oxidative damage, and Annexin V-FITC with propidium iodide (PI) for apoptosis were used. In conclusion, CuO-NPs were found to accumulate in the cells and induced significant cytotoxic and genotoxic, and oxidative and apoptotic effects. CuO-NPs are hypothesized to dangerously affect human health, especially neuronal system. However, further studies should be done to elucidate their toxic mechanism.

Keywords: copper oxide; nanoparticle; neurotoxicity; cellular uptake; genotoxicity; apoptosis.

I. Introduction

CuO-NPs are widely used in gas sensors, catalysts, high temperature conductors, solar energy converters and antimicrobial agents owing to their high temperature conductivity, electron correlation effects, antimicrobial activity and special physicochemical properties in various fields (Chang et al., 2012; Huang et al., 2010). Indeed, as it is well known, nanoparticles exist as contaminants in water, air and food products as outputs of natural phenomena or due to the high increase in the anthropogenic activity (Ahamed et al., 2013; Elsaesser et al., 2011; Kim et al., 2010). CuO-NPs caused changes in different organs like lung, kidney, renal tubular, liver, spleen, gastrointestinal tract and stomach tissue (Barceloux, 1999; Cho et al., 2012; Lei et al., 2008; Manna et al., 2012). Acute death, abnormalities in the embryo and gill damage were observed in Zebra fish exposed to CuO-NPs (Griffitt et al., 2007; Yeo et al., 2009). The toxicity studies of CuO-NPs have been focused more generally on the pulmonary system and to a lesser extent on skin, breast, intestine and liver (Ahamed et al., 2010; Akhtar et al., 2012; An et al., 2012; Cuillel et al., 2014; Laha et al., 2014; Piret et al., 2012; Siddiqui et al., 2013; Sun et al., 2011; Wang et al., 2011). However, there are few reports on the nervous system (An et al., 2012; Chen et al., 2008; Perreault et al., 2012). Therefore, it was aimed to evaluate the toxicity and possible mechanism of action of CuO-NPs in neuroblastoma cells following their cellular uptake potential.

Author α: Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Istanbul University, Istanbul-Turkey, Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Karadeniz Technical University, Trabzon-Turkey. e-mail: abudiak1987@yahoo.com
Author σ: Department of Histology and Embryology, Cerrahpasa Medical Faculty, Istanbul University, Istanbul-Turkey.
e-mail: elifguzelctf@yahoo.com
Author ρ: Department of Pharmaceutical Toxicology, Faculty of Pharmacy, Istanbul University, Istanbul-Turkey, Istanbul University, Faculty of Pharmacy, Department of Pharmaceutical Toxicology, Beyazit 34116, Istanbul, Turkey. e-mail: gulozhan@istanbul.edu.tr

II. Materials and Methods

Chemicals: Eagle's minimum essential medium (EMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS, 10X), antibiotic solutions and ethylene diamine tetraacetic acid (EDTA) were purchased from Multicell Wisent (Quebec, Canada). Triton X-100 and MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) were purchased from Biomatik (Ontario, Canada). GSH, 8-OHdG, MDA and PC ELISA kits were purchased from Yehua Biological Technology Co., Ltd. (Shanghai, China). Annexin V-FITC apoptosis detection kit with PI and dye reagents for protein assay were obtained from Exbio (Vestec, Czech Republic) and Bio-rad (Munich, Germany), respectively. All other chemicals were obtained from Merck (NJ, USA).

CuO-NPs were obtained from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). The CuO-NPs suspensions in milli-Q water and cell culture medium with 10% FBS, were measured by Transmission Electron Microscopy (TEM) (Jem-2100 HR, Jeol, USA) (Abudayyak et al. 2016; 2016a). The average diameter was calculated by measuring over 100 particles in random fields of TEM view.

Copper release into cell medium: Copper release from CuO-NPs into the cell culture medium was determined using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (Thermo Elemental X series 2, USA) method (Abudayyak et al. 2016; 2016a). The released amount of copper was analyzed by ICP-MS. Cu content of the cell culture medium was also measured.

Cell culture conditions: Human neuronal cell line (SH-SY5Y) was obtained from the American Type Culture

Collection (CRL-2266™, ATCC, VA, USA). The cells were incubated in EMEM medium supplemented with FBS 10% and antibiotics at 5% CO_2, 90% humidity and 37°C for 24 h (60-80% confluence). Cell densities were in the range from 1×10^5 to 1×10^7 cells/mL for all assays (Abudayyak et al. 2016; 2016a). Exposure occurred for 24 h.

Cellular uptake and morphology examinations: It was evaluated by ICP-MS and TEM (Abudayyak et al. 2016; 2016a). The cells were washed several times with equal volumes of PBS and cell culture medium with 10% FBS and counted via Luna cell counter (Virginia, USA) following exposure to two different concentrations of the particle suspension (2.5 and 25 μg/mL). Ultra-thin sections (50-60 nm) were cut by an ultra-microtome (Reichert UM 3, Austria). Sections were analyzed and photographed using a TEM (Jeol-1011, Tokyo, Japan) with attached digital camera (Olympus-Veleta TEM Camera, Tokyo, Japan).

Cytotoxicity assays: Cytotoxic activities of CuO-NPs on SH-SY5Y cells were determined by MTT and NRU assays based on different cellular mechanisms (Abudayyak et al. 2016; 2016a; Repetto et al., 2008; Van Meerloo et al., 2011). Optical density (OD) values were read at 590 and 540 nm for MTT and NRU, respectively, using a microplate spectrophotometer system (Epoch, Germany). In every assay, unexposed cells were served as a negative control. The inhibition of enzyme activity was calculated as compared to a negative control. The half-maximal inhibitory concentration (IC_{50}) was then expressed as the concentration of the sample causing a 50% inhibition of enzyme activity in cells. The CuO-NP concentrations were 2.5-60 μg/mL in the cytotoxicity assays.

Genotoxicity assay: Genotoxic activities of CuO-NPs were determined by comet assay (Abudayyak et al. 2016; 2016a; Collins et al., 2004; Speit et al., 1999). Hydrogen peroxide (H_2O_2) (100 μM) and PBS were used as positive and negative controls, respectively. The number of DNA breaks was scored under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) at 400X magnification using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). DNA damage to individual cells was expressed as a percentage of DNA in the comet tail (tail intensity %). The CuO-NP concentrations were 5-50 μg/mL in the comet assay.

Oxidative damage assays: The oxidative damage potentials of CuO-NPs were measured by human GSH, MDA, 8-OHdG, or PC ELISA kits with different endpoints according to the manufacturer's instructions. The OD value was read at 450 nm using a microplate spectrophotometer system. In every assay, the unexposed cells served as a negative control. The protein amount in 10^6 cells was measured according to Bradford (1976). Results were expressed as μmol, μmol, μg, and μg per g protein for GSH, MDA, 8-OHdG, and PC, respectively, using a standard calibration curve. The CuO-NP concentrations were 5-25 μg/mL in the oxidative damage assays.

Apoptosis assay: The cellular apoptosis or necrosis was determined by Annexin V-FITC apoptosis detection kit with PI (Abudayyak et al. 2016; 2016a). In every assay, the untreated cells served as a negative control. The results were expressed as a percentage of the total cell amount. The CuO-NP concentrations were 10-80 μg/mL in the apoptosis assay.

Statistical analysis: The assays were done in triplicate and repeated four times. Data were expressed as mean±standard deviation (SD). Significant differences between untreated and treated cells were calculated by one-way ANOVA Dunnett t-test using SPSS version 17.0 for Windows. *p* values of less than 0.05 were considered significant.

III. Results and Discussion

Particle size and distribution: According to the X-ray diffraction results supplied by the manufacturer (Sigma Chemical Co. Ltd., USA), the surface area of CuO-NPs was 29 m^2/g (Figure 1). The average size was observed to be 34.9 nm with a narrow size distribution (ranging from 16.7-64.2 nm) after suspending in water. When suspending in the culture medium, the size of the particles was found to be slightly agglomerated and/or aggregated with 38.8 nm (ranging from 18.8-73.8 nm) (Figure 2). The copper ion release of CuO-NPs was evaluated in the cell culture medium. Although the concentration was 3.1 ± 0.322 μg/mL, which represented 15.5% of the nanoparticles, in the CuO-NPs cell culture suspension, there was no observed copper ions in the cell culture medium. Based on that, the observed toxicological endpoints and morphological changes were mainly due to CuO-NPs.

Cellular uptake: ICP-MS revealed that the particles were taken up by SH-SY5Y cells in the range of 0.390-0.917 $\mu g/10^5$ cells in concentration dependent manner following exposure to CuO-NPs at 5-25 μg/mL concentrations (Table 1). Some researchers reported iron oxide and two different types of titanium dioxide nanoparticles to enter SH-SY5Y cells in concentration dependent manner (Kilic et al., 2016; Valdiglesias et al., 2013).

Cellular morphology by TEM: The particles were observed in the cytoplasmic vacuoles. Mitochondria were visible in few of the cells exposed to both 2.5 and 10 μg/mL CuO-NPs. Some cells exposed to 2.5 μg/mL CuO-NPs revealed nuclear fragmentation. The electron-lucent cytoplasmic vacuoles lead to complete disruption of the cytoplasm in few of the cells (Figure 3).

Cytotoxicity: IC_{50} values of CuO-NPs were 25.49±2.06 and 7.27±0.843 μg/mL by MTT and NRU assay,

respectively. The reduction in cell viability was concentration-dependent (Figure 4). The CuO-NPs were found to cause cytotoxic effects to HaCaT keratinocytes, BALB3T3 embryonic fibroblasts (Akhtar et al., 2012; Kilic et al., 2016), HepG2 (Siddiqui et al., 2013; Wang et al., 2011), A549 lung epithelial (Karlsson et al., 2008; Wang et al 2012), HEp-2 airway epithelial (Wang et al 2012), Caco-2 intestinal (Piret et al., 2012), cardiac microvascular endothelial cells (Sun et al., 2011) and primary culture of channel catfish hepatocytes (Wang et al., 2011). Perreault et al. (2012) found mouse N2A neuroblastoma cell viability decreased to 63% at 400 μg/mL for 24 h. Chen et al. (2008) reported CuO-NPs showed the cytotoxic effect in SH-ST5Y neuroblastoma and H4 neuroglioma cells were dose-dependent. It caused a drop of 60 and 40% in live cell percentages in SH-ST5Y and H4 cells, respectively, at 100 μM concentration.

Genotoxicity: In positive controls (100 μM H_2O_2), the tail intensity was 21.43%. The results revealed that CuO-NPs significantly induced DNA damage in all exposure concentrations (2.57-7.09 fold) and generally in dose dependent manner ($p \leq 0.05$). The highest tail intensity was 24.09 observed at a concentration of (15 μg/mL). The cell death was ≤50% in all concentrations (Figure 5). CuO-NPs induced genotoxic responses in A549 (Ahamed et al., 2010; Akhtar et al., 2016; Cronholm et al., 2011; Wang et al., 2012) and BEAS-2B lung epithelial cells (Cronholm et al., 2011). Perreault et al. (2012) found CuO-NPs significantly induced DNA damage in mouse N2A neuroblastoma cells at 12.5 μg/mL. Researchers suggested CuO-NPs induced DNA damage significantly correlated with reactive oxygen species (ROS) (Akhtar et al., 2016). Also, it could be via disruption of cell membrane integrity (Cronholm et al., 2011). However, there was no study about genotoxicity on SH-SY5Y cells.

Oxidative damage: The oxidative damage potential of CuO-NPs was evaluated by measuring cellular levels of GSH, MDA, 8-OHdG, and PC (Table 2). CuO-NPs induced oxidative damage resulting in significant decrease in the GSH levels (≤46.1%). Although an increase on the levels of MDA (≤1.33 fold) was observed it was not significant. On the other hand, the levels of PC and 8-OHdG protein and DNA oxidative damage biomarkers did not change. In previous studies, it was observed that CuO-NPs induced oxidative damage in HaCaT keratinocytes (Alarifi et al., 2013), BALB3T3 fibroblasts (Akhtar et al., 2012), A549, (Ahamed et al., 2010; Akhtar et al., 2013; Karlsson et al., 2008; Kim et al., 2010), HEp-2 (Fahmy and Cormier, 2009), and HepG2 cells (Piret et al., 2012; Siddiqui et al., 2013). The reduction in cell viability observed could be due to an increase in oxidative stress after CuO-NPs exposure.

Apoptosis: Death in SH-SY5Y cells was significantly induced by CuO-NPs, with a maximum percentage of 73.4 and 40.0% for apoptosis and necrosis, respectively. According to our results, apoptosis was seen to be the main pathway for cell death in the SH-SY5Y cell line. At the highest exposure concentration (40 μg/mL), the apoptosis percentage was 79.2% of the dead cells (Figure 6). The previous studies showed CuO-NPs could induce apoptosis in the following cells: MCF7 breast cancer (Laha et al., 2014), HepG2 (Siddiqui et al., 2013), and Caco-2 cells (Piret et al., 2012). In rats, CuO-NPs induced apoptosis via increased cleaved caspase-3 levels (An et al., 2012). Siddiqui et al. (2013) observed CuO-NPs induced apoptosis via a decrease in mitochondrial membrane potential with a concomitant increase in the gene expression ratio of Bax/Bcl2, up-regulation of p53 tumour suppressor and caspase-3 apoptotic genes. Also, the researchers showed apoptosis could be induced by reduction of BAD phosphorylation and an increase in cleaved caspase-3 products (Laha et al., 2014). An et al. (2012) indicated that the apoptosis and cognitive impairment could be via increased cleaved caspase-3 levels on hippocampal CA1 neuron in rats.

IV. Conclusion

Generally, the studies about Cu based nanoparticles and CuO-NPs were focused on the pulmonary system. However, very few researchers were concerned about the possible toxicity over other systems. In the present study, it was observed that CuO-NPs taken up by the neuronal cells could produce cytotoxic, genotoxic, and apoptotic effects, as well as oxidative damage in the neuronal cells *in vitro*. Their commercial and industrial applications should be carefully evaluated because of their potential hazardous effects on human health. Further *in vivo* studies are needed to fully understand the toxicity mechanisms of CuO-NPs.

V. Acknowledgement

This work was supported by the Research Fund of Istanbul University (Project No: 52253). Dr. M. Abudayyak carried out cell culture and exposure conditions, the toxicological assays and the particle characterisation. Prof. Dr. G. Özhan participate the toxicological assays and carried out the evaluation of the results. Dr. E. Guzel carried out the uptake and morphological changes in the cells. All authors wrote, read and approved the manuscript. Also, the authors declare there is no conflict of interest.

References Références Referencias

1. Ahamed M, Ali D, Alhadlaq HA, et al. 2013. Nickel oxide nanoparticles exert cytotoxicity via oxidative

stress and induce apoptotic response in human liver cells (HepG2). *Chemosphere* **93**: 14-22.
2. Abudayyak M, Altıncekic T, Özhan G. 2016. In vitro toxicological evaluation of cobalt ferrite nanoparticles. *Biol. Trace Element Res*. Doi: 10.1007/s 12011-016-0803-3.
3. Abudayyak M, Guzel EE, Özhan G. 2016a. Copper (II) oxide nanoparticles induced nephrotoxicity in vitro conditions. *Appl. Vitro Toxicol*. Doi:10.1089/aivt. 2016.0008.
4. Ahamed M, Siddiqui MA, Akhtar MJ, et al. 2010. Genotoxic potential of copper oxide nanoparticles in human lung epithelial cells. *Biochem. Biophys. Res. Commun.* **396**: 578-583.
5. Akhtar MJ, Ahamed M, Fareed M, et al. 2012. Protective effect of sulphoraphane against oxidative stress mediated toxicity induced by CuO nanoparticles in mouse embryonic fibroblasts BALB 3T3. *J. Toxicol. Sci.* **37**(1):139-148.
6. Akhtar MJ, Kumar S, Alhadlaq HA, et al. 2016. Dose-dependent genotoxicity of copper oxide nanoparticles stimulated by reactive oxygen species in human lung epithelial cells. *Toxicol. Ind. Health* **32**(5):809-821.
7. Alarifi S, Ali D, Verma A, et al. 2013. Cytotoxicity and genotoxicity of copper oxide nanoparticles in human skin keratinocytes cells. *Int. J. Toxicol.* **32**(4): 296-307.
8. An L, Liu S, Yang Z, et al. 2012. Cognitive impairment in rats induced by nano-CuO and its possible mechanisms. *Toxicol. Lett.* **213**(2): 220-227.
9. Barceloux DG. 1999. Copper. *J. Toxicol. Clin. Toxicol.* **37**: 217-237.
10. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **7**: 248-254.
11. Chang YN, Zhang M, Xia L, et al. 2012. The toxic effects and mechanisms of CuO and ZnO nanoparticles. *Materials* **5**: 2850-2871.
12. Chen J, Zhu J, Cho H-H, et al. 2008. Differential cytotoxicity of metal oxide nanoparticles. *J. Exp. Nanosci.* **3**: 321-328.
13. Cho WS, Duffin R, Poland CA, et al. 2012. Differential pro-inflammatory effects of metal oxide nanoparticles and their soluble ions in vitro and in vivo; zinc and copper nanoparticles, but not their ions,recruit eosinophils to the lungs. *Nanotoxicol.* **6**: 22-35.
14. Collins AR. 2004. The comet assay for DNA damage and repair principles, applications, and limitations. *Mol. Biotechnol.* **26**: 249-261.
15. Cronholm P, Midander K, Karlsson HL, et al. 2011. Effect of sonication and serum proteins on copper release from copper nanoparticles and the toxicity towards lung epithelial cells. *Nanotoxicol.* **5**(2): 269-281.
16. Cuillel M, Chevallet M, Charbonnier P, et al. 2014. Interference of CuO nanoparticles with metal homeostasis in hepatocytes under sub-toxic conditions. *Nanoscale* **6**(3): 1707-1715.
17. Elsaesser A, Howard CV. 2011. Toxicology of nanoparticles. *Adv. Drug Deliv Rev* **64**: 129-37.
18. Fahmy B, Cormier SA. 2009. Copper oxide nanoparticles induce oxidative stress and cytotoxicity in airway epithelial cells. *Toxicol. in Vitro* **23**(7): 1365-1371
19. Griffitt RJ, Weil R, Hyndman KA, et al. 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (Danio rerio). *Environ. Sci. Technol.* **41**: 8178-8186.
20. Huang YW, Wu CH, Aronstam RS. 2010. Toxicity of transition metal oxide nanoparticles: Recent insights from in vitro studies. *Materials* **3**: 4842-4859.
21. Karlsson HL, Cronholm P, Gustafsson J, et al. 2008. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. *Chem. Res. Toxicol.* **21**(9): 1726-1732.
22. Kilic G, Costa C, Fernández-Bertólez N, et al. 2016. In vitro toxicity evaluation of silica-coated iron oxide nanoparticles in human SHSY5Y neuronal cells. *Toxicol. Res.* **5**: 235-247.
23. Kim YJ, Yu M, Park HO, et al. 2010. Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by silica nanomaterials in human neuronal cell line. *Mol. Cell. Toxicol.* **6**: 337-344.
24. Laha D, Pramanik A, Maity J, et al. 2014. Interplay between autophagy and apoptosis mediated by copper oxide nanoparticles in human breast cancer cells MCF7. *Biochim. Biophys. Acta* **1840**: 1-8.
25. Lei R, Wu C, Yang B, et al. 2008. Integrated metabolomic analysis of the nano-sized copper particle-induced hepatotoxicity and nephrotoxicity in rats: a rapid in vivo screening method for nanotoxicity. *Toxicol. Appl. Pharmacol.* **232**(2): 292-301.
26. Manna P, Ghosh M, Ghosh J, et al. 2012. Contribution of nano-copper particles to in vivo liver dysfunction and cellular damage: role of IκBα/NF-κB, MAPKs and mitochondrial signal. *Nanotoxicol.* **6**: 1-21.
27. Perreault F, Melegari SP, de Costa CH, et al. 2012. Genotoxic effects of copper oxide nanoparticles in Neuro 2A cell cultures. *Sci. Total Environ.* **441**: 117-124.
28. Piret JP, Jacques D, Audinot JN, et al. 2012. Copper (II) oxide nanoparticles penetrate into HepG2 cells, exert cytotoxicity via oxidative stress and induce pro-inflammatory response. *Nanoscale* **4**: 7168–7184.

29. Repetto G, del Peso A, Zurita JL 2008. Neutral red uptake assay for the estimation of cell viability/ cytotoxicity. *Nature Protocols* **3**: 1125-1131.
30. Siddiqui M, Alhadlaq HA, Ahmad J, et al. 2013. Copper oxide nanoparticles induced mitochondria mediated apoptosis in human hepatocarcinoma cells. *PloS One* **8**(8): e69534.
31. Speit G, Hartmann A. 1999. The comet assay (single-cell gel test): A sensitive genotoxicity test for the detection of DNA damage and repair. *DNA Repair Protocols* **113**: 203-212.
32. Sun J, Wang S, Zhao D, et al. 2011. Cytotoxicity, permeability, and inflammation of metal oxide nanoparticles in human cardiac microvascular endothelial cells. *Cell Biol. Toxicol.* **27**(5): 333-342.
33. Valdiglesias V, Costa C, Sharma V, et al. 2013. Comparative study on effects of two different types of titanium dioxide nanoparticles on human neuronal cells. *Food Chem. Toxicol.* **57**: 352-61.
34. Van Meerloo J, Kaspers GJ, Cloos J. 2011. Cell sensitivity assays: The MTT assay. *Methods Mol. Biol.* **731**: 237-245.
35. Wang Y, Aker WG, Hwang H, et al. 2011. A study of the mechanism of in vitro cytotoxicity of metal oxide nanoparticles using catfish primary hepatocytes and human HepG2 cells. *Sci. Total Environ.* **409**: 4753-4762.
36. Wang Z, Li N, Zhao J, et al. 2012. CuO nanoparticle interaction with human epithelial cells: cellular uptake, location, export, and genotoxicity. *Chem. Res. Toxicol.* **25**(7): 1512-1521.
37. Yeo MK, Kang M. 2009. Effects of CuxTiOy nanometer particles on biological toxicity during zebrafish embryogenesis. *Korean J. Chem. Eng.* **26**: 711-718.

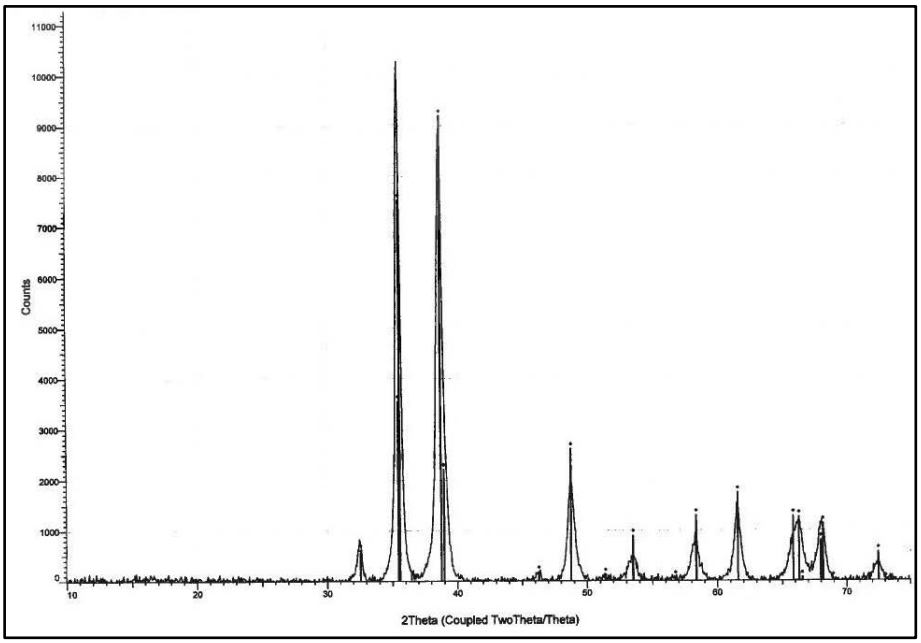


Figure 1 : The X-ray diffraction analysis of CuO-NPs.

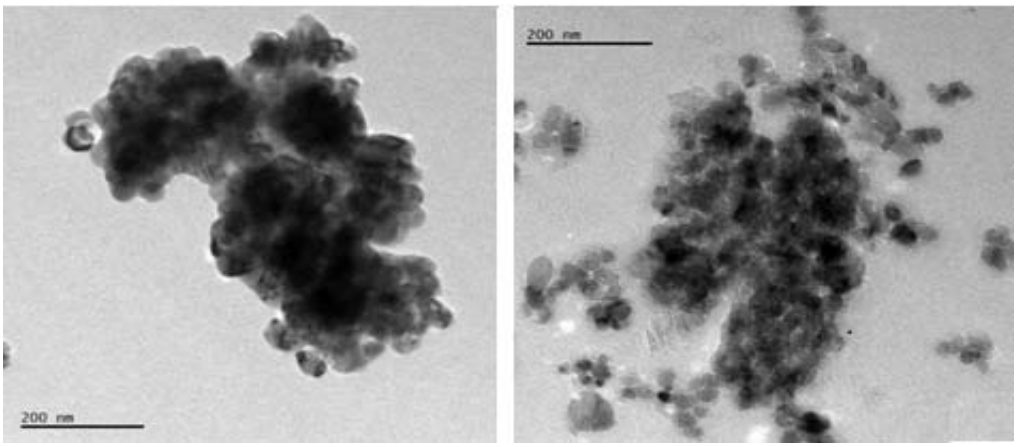


Figure 2 : The TEM images and size distributions of CuO-NPs after dissolution in water (a) and cell culture medium (b).

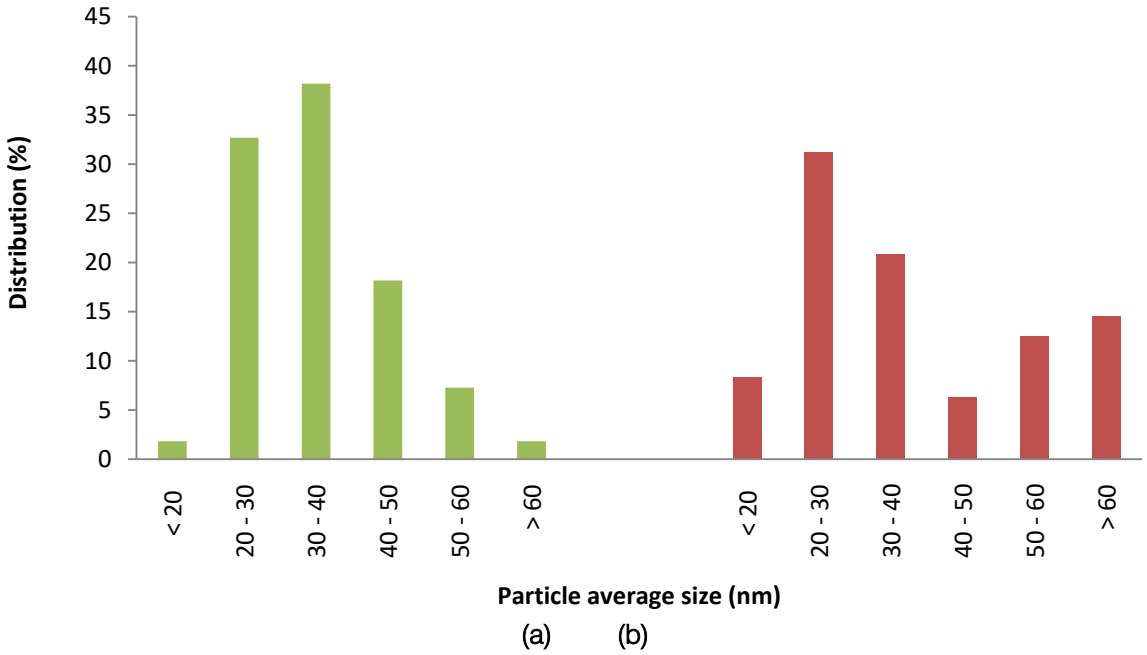


Figure 3 : TEM observations of SH-SY5Y cells after exposure to CuO-NPs. (a) cells exposed to CuO-NPs at 2.5 μg/mL; (b) cells exposed to CuO-NPs at 10 μg/mL; (c) unexposed cell (negative control).

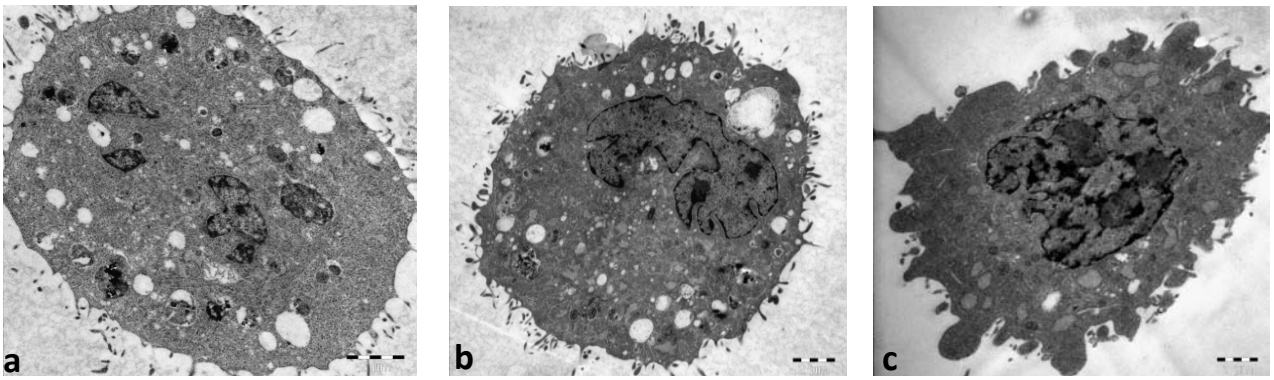


Figure 4 : Effects of CuO-NPs on SH-SY5Y cell viability.

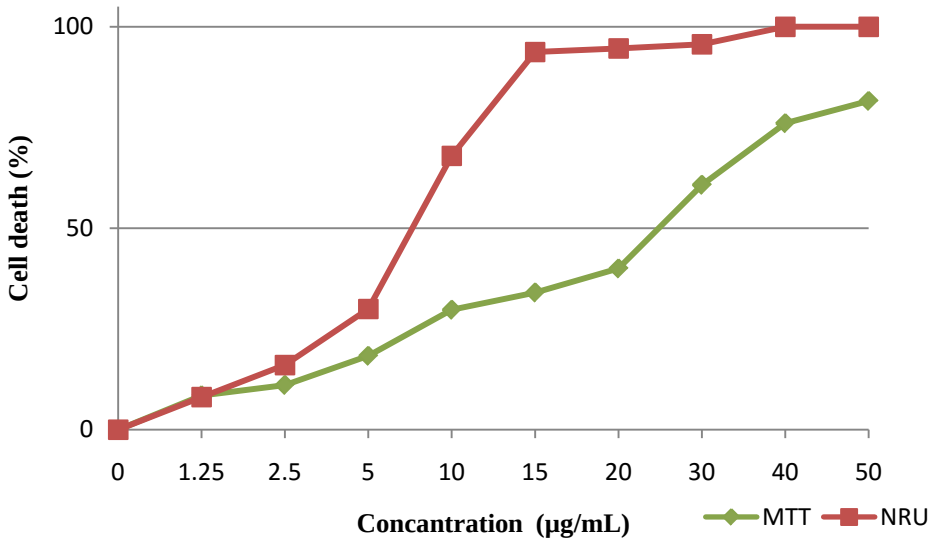


Figure 5 : Evaluation of DNA damage potential of CuO-NPs in SH-SY5Y cells.
All experiments were done in triplicate and each assay was repeated four times.
The results were expressed as the mean cell death (%) compared to negative control (unexposed cell).

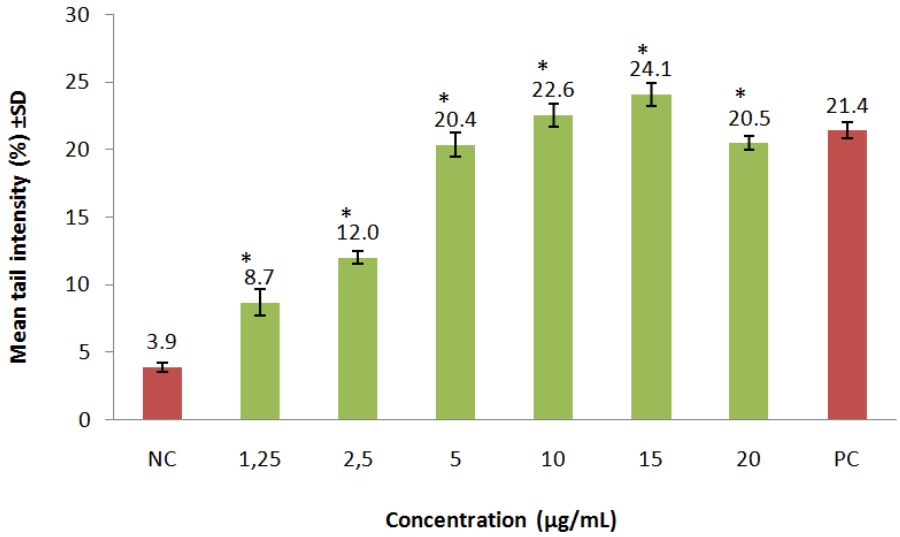
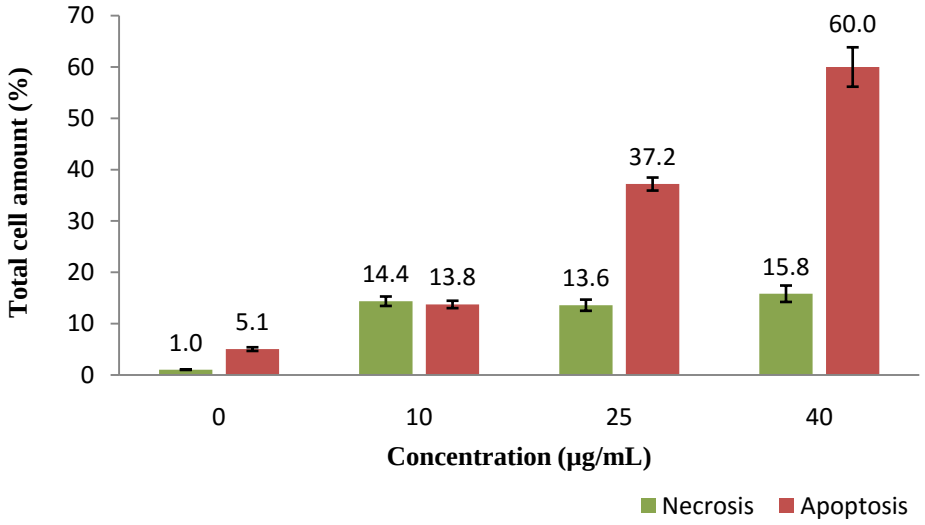


Figure 6 : Evaluation of the apoptosis- and necrosis-inducing potential of CuO-NPs in SH-SY5Y cells.

All experiments were done in triplicate and each assay was repeated four times.

The results were presented as mean tail intensity (%) with ± SD, NC and PC mean negative and positive controls, respectively.

*****$p \leq 0.05$ were selected as the levels of significance by one-way ANOVA Dunnett t-test.



All experiments were done in triplicate and each assay was repeated four times.

The results were presented as percentages of the total cell amount.

Table 1 : Evaluation of the cellular uptakes of CuO-NPs from SH-SY5Y cells.

Exposure concentration ($\mu g/mL/10^5$ cells)	Cu amount ($\mu g/10^5$ cells)
Negative control	0.0137±0.002
5	0.390±0.051
10	0.378±0.076
15	0.641±0.062
25	0.917±0.980

Cu content of the negative control (unexposed cell) was also measured. Every assay was repeated four times. The results were expressed as mean ± SD.

Table 2 : Evaluation of oxidative damage potentials of CuO-NPs in SH-SY5Y cells.

Exposure concentration (µg/mL)	GSH (µmol /g protein)	MDA (µmol/g protein)	8-OHdG (µg/g protein)	PC (µg/g protein)
0	46.796±0.952	0.320±0.086	6.777±0.0988	0.916±0.019
5	30.514±1.319*	0.280±0.092	5.870±0.529	0.747±0.057
10	25.247±1.072*	0.350±0.064	6.287±0.418	0.807±0.120
15	32.160±1.491*	0.416±0.108	6.576±0.246	0.866±0.109
25	34.884±1.220*	0.425±0.156	6.836±0.098	0.805±0.088

The protein amount calculated for $4x10^4$ cells in every assay according to Bradford (1976).

The results were expressed as µmol, µmol, µg and µg per g protein for GSH, MDA, 8-OHdG and PC, respectively, using standard calibration curve.

*$p \leq 0.05$ were selected as the levels of significance.