Copper Oxide Nanoparticles Stimulate Cytotoxicity and Apoptosis in Glial Cancer Cell Line

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ABSTRACT: Due to cytotoxic potential, Copper Oxide Nanoparticles (CuO NPs) have recently been studied in various *in vivo* and in culture cell line. Also, CuO has received much attention in cancer therapy. We aimed to evaluate the cytotoxicity of CuO NPs on glial cancer (B92) cell line. B92 cancer cells were cultured with CuO NPs at different concentrations (5, 10, and 20 µg/ml) with 30 and 60 nm particle size. Then, cancer cells were incubated for 24 hrs. The apoptosis and cytotoxicity of cells were estimated by acridine orange/propidium iodide staining and MTT assay, respectively. Both sizes of CuO NPs had cytotoxic effect. Even with the lowest concentration, the cytotoxic impact accommodated 32% of cell apoptosis with 30 nm size. When the concentration of CuO NPs increased, viability decreased and apoptosis increased. However, these amounts have no significant changes in the concentration of 10 to 20 µg/ml between two particle sizes (30 and 60 nm). The IC_{50} was decreased as the size of particles increased, but there was no significant change. This finding suggests that exposure to CuO NPs had significant cytotoxic effect with the sizes tested when compared to unexposed control in a way that the smaller size and higher concentration exerted the maximum cytotoxic effects. It seems that augmentation may not have any impact on their *in vitro* cytotoxicity.

Key words: B92 cancer cells, Copper Oxide Nanoparticles, Cytotoxicity, Apoptosis, Glial.

INTRODUCTION

Nanoparticles (NPs) are usually a group of nanoscale materials with a range from 1 to 100 nm. Inorganic NPs consisting of metal oxides have been manufactured on a large scale for applications in medicine like drug delivery, bio-sensing, cell imaging and cancer therapy in organs such as brain which is protected by specialized brain barrier.[1-4] Chemotherapeutic researches based on the use of metals for new anti-cancer drugs with the potential of being less toxic and exhibiting more anti-proliferative activity against tumors have been developed.[5] NPs in comparison to large biological molecules (enzymes, receptors, etc.) can easily display specific interactions with biomolecules and may be useful in cancer diagnosis and treatment.[6,7] Due to their antimicrobial properties, copper oxide (CuO) NPs are intentionally widely manufactured and used in plastics, paints, food containers.[8]

Various studies have reported an increased cytotoxic and genotoxic potential as well as an intense pro-inflammatory responses of CuO NPs *in vivo*[9,10] and in cultured cell lines.[11-15] Oxidative stress is often used to describe the toxicity of cells associated with particle exposure. Because of their unique features such as size, morphology, solubility, surface area as well as their ability to associate with biomolecules from the environment to form a protein and interactions of the particles with cellular membranes, uptake and intracellular bioavailability, can finally lead to elevated levels of reactive oxygen species (ROS).[5] CuO NPs are able to generate oxidative stress and significantly decrease cell viability in tumor cells.[16-18]

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Glioma is a type of brain and central nervous system tumors, which starts in supportive glial cells that surround nerve cells and help them function.[19] Cell division is a normal process to build new cells and tissues but it is stopped whenever there is no longer need for more cells or is a threat for the organisms to be destroyed by apoptotic cell death. The ability of cancer cells to continue reproducing, spreading and avoiding apoptosis are the basic properties of tumors, therefore, inducing apoptosis in cancer cells is a major desire for researchers in the field of tumor treatments.[20] Even several recent studies evaluated cytotoxic effects of metal and metal-containing material components on glioma cancer cell lines,[15,21,22] the consequences of exposure of CuO NPs with B92 cancer cells have not yet been reported.

Therefore, we designed to determine the concentration and size-dependent effects of CuONPs exposure in B92 cancer cells.

MATERIALS AND METHODS

Synthesis and characterization of CuO nanoparticles. To synthesis colloidal suspension of CuO NPs (30 and 60 nm), we used an alcothermal method with minor modifications. To 100 ml of distilled water, we added 0.25 g of $CuSO_4$ and then 5 g of polyvinyl pyrrolidone K30 (PVP-K30) and was dissolved in this solution. For reduction of CuO, 0.25 g $NaBH_4$ was poured into the solution. The solution of $CuSO_4$ turned from blue to green immediately and finally a brown colored product was obtained. After 30 min, required quantities of ascorbic acid was added and kept in a water bath at 60 °C for 30 min. The precipitates were washed with ethanol and centrifuged (4000 g, 15 min) and finally, CuO NPs colloid powder was obtained and stored for further use. A Different amount of ascorbic acid was used to produce CuO NPs with different sizes. Structural studies of CuO NPs were done via scanning electron microscopy (SEM) (TESCAN 510 Czech Republic) and dynamic light scattering (DLS) (Nano-zetasizer MALVERN. England).

Cell culture and treatment. B92 rat cancer cell line was purchased from National Cell Bank of Iran (NCBI, Tehran, Iran) and was cultured in 75 cm^2 flask at the density of 6×10^4 cells/cm^2 in RPMI 1640, supplemented with 100ug/mL penicillin and 100 µg/mL streptomycin and 10% heat-inactivated FBS at 37°C and 5% CO_2 to reach 80% confluency. Cells were harvested using 0.25% trypsin and were sub-cultured into 75 cm^2 flasks, 6-well plates or 96 well plate; they were allowed to recover for 24–hrs prior to treatment. Particles were suspended in cell culture medium by pulse sonication for 5 min to avoid particle agglomeration. A serial dilution was established by mixing equal volumes of particle suspension and cell culture medium.

Cell cytotoxicity assay. The viability of B92 cancer cells was assessed by the MTT assay as described before.[23] Briefly, 1×10^4 cells/well were seeded in 96-well plates and exposed to CuO NPs at the concentrations of 0 (as a control), 5, 10, and 20 µg/mL for 24 hrs. Twenty microliters of a 5 mg/mL solution of MTT in PBS was added to each well, and the plates were incubated at 37°C in 5% CO_2 for 4 hrs. The medium was then carefully removed, and the purple products were dissolved in 0.1 mL dimethyl sulfoxide (DMSO). The plates were shaken for 10 min, and the optical density (OD) of the dissolved solute measured by an ELISA reader (Dynatech, Denkendorf, Germany) at 570 nm.

Detection of apoptosis by acridine orange and propidium iodide. Cells were cultured in a 6 well plates at a concentration of 1×10^6 cells/well and allowed for 24 hrs adherence. Cells were treated with different concentrations of CuONPs (0, 5, 10 and 20 µg/ml) for 24 hrs. The suspension was discarded and the cells were washed twice with phosphate buffered saline (PBS). Staining with 10 µl of Acridine Orange (AO, Sigma Aldrich 10 µg/ml) was performed for 15 min at room temperature in dark and immediately before fluorescence microscopic studies, 10 µl Propidium Iodide (PI) (Sigma Aldrich) was added to the cellular pellet. Apoptotic modification in B92 cancer cells was determined in an improved Neubauer rhodium hemocytometer under fluorescent

microscopy and the percentage of cells exhibiting apoptosis was counted and calculated.

Statistical analysis. Statistical significance was performed by Student's t-test and one-way analysis of variance. Data were presented as mean ± SD and $p < 0.05$ was considered as the level of significance for all analyses performed.

RESULTS AND DISCUSSION

Characterization of CuO NPs. As shown in figure 1, the SEM images of synthesized CuO NPs confirmed - both powders were aggregated and the apparent average sizes were 30 and 60 nm. Also, the zeta sizer of CuO NPs by DLS confirmed their sizes as shown in figure 2.

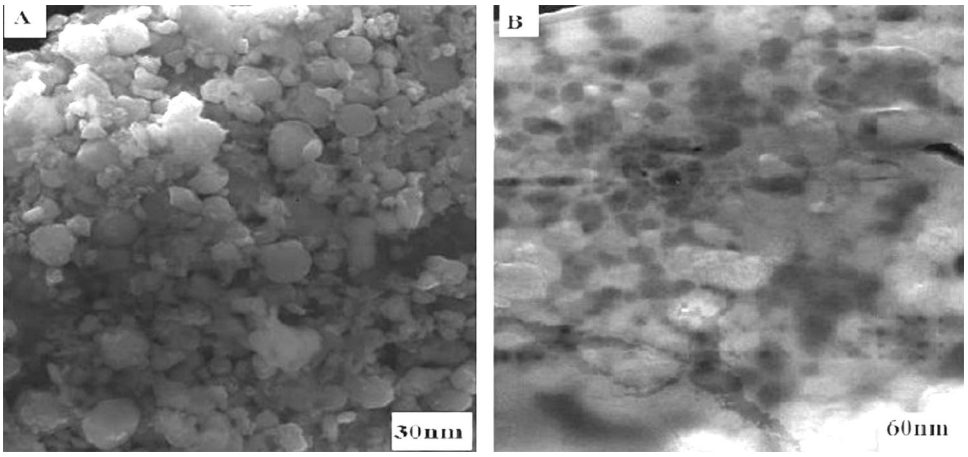

Figure 1. Structural characterization of synthesized CuO NPs by field emission scanning electron. A) CuO NPs with 30 nm sizes. B) CuO NPs with 60 nm sizes.

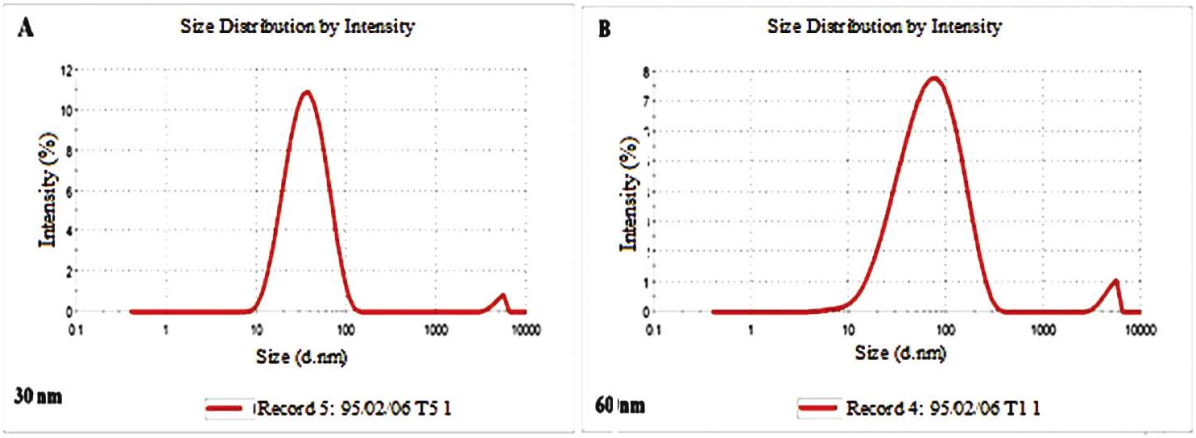

Figure 2. Zeta sizer of synthesized CuO NPs (DLS). A) CuO NPs with 30 nm sizes. B) CuO NPs with 60 nm sizes.

Concentration-dependent cytotoxicity of CuO NPs on B92 cells. Cell cytotoxicity in response to different concentration and two sizes of CuO nanoparticles was evaluated by MTT assay. B92 cancer cells were cultured in media containing 0, 5, 10 and 20 µg/ml CuO NPS for 24 hrs. As the concentration of CuO NPs increased from 5 to 20 µg/ml in medium, the viability of cells decreased in both sizes of CuO NPs. However, there was no significant decrease between 30 and 60 nm sizes in any concentration ($p > 0.05$) as are shown in figure 3. On the other hand, there were slight changes in the cell viability at 30 and 60 nm sizes of synthesized CuO NPs. The half maximal inhibitory concentration

(IC_{50}) was counted as the concentration required to inhibit the growth of B92 cancer cells in the culture medium by 50% compared to the untreated cells. The IC_{50} of nanoparticles with the size of 30 nm were 12.01 ±1.9 and for nanoparticles with 60 nm was 9.17 ± 3.3. The IC_{50} decreased by increasing of particle size, but the decrease was non-significantly ($p > 0.05$).

Quantification of apoptosis by double staining using acridine orange and propidium iodide. The fluorescence microscopic study was used to determine whether the cytotoxic effect of CuONPs was related to the induction of apoptosis. In cell populations, the viable cell possesses uniform bright green nucleus with diffused chromatin (exclude of PI), whereas early apoptotic cells showed bright green areas of condensed chromatin in the nucleus, and necrotic cells in a uniform bright red nucleus with non-condensed chromatin (include of PI). However, cells exposed to CuONPs at 20 µg/ml concentration exhibited more condensed chromatin, fragmented nuclei, and appearance of apoptotic bodies as displayed in figure 4. The percentages of the apoptotic cell were evaluated at different concentrations of CuO NPs treated samples (0, 5, 10 and 20 µg/ml). The percentage of apoptotic cells showed concentration-dependent manner, in a way that, the possible induction of apoptosis enhanced as the concentration of treatment increased (Figure 5). Results showed that morphological identification of apoptosis and apoptotic DNA fragmentation has been developed with the use of CuO NPs.

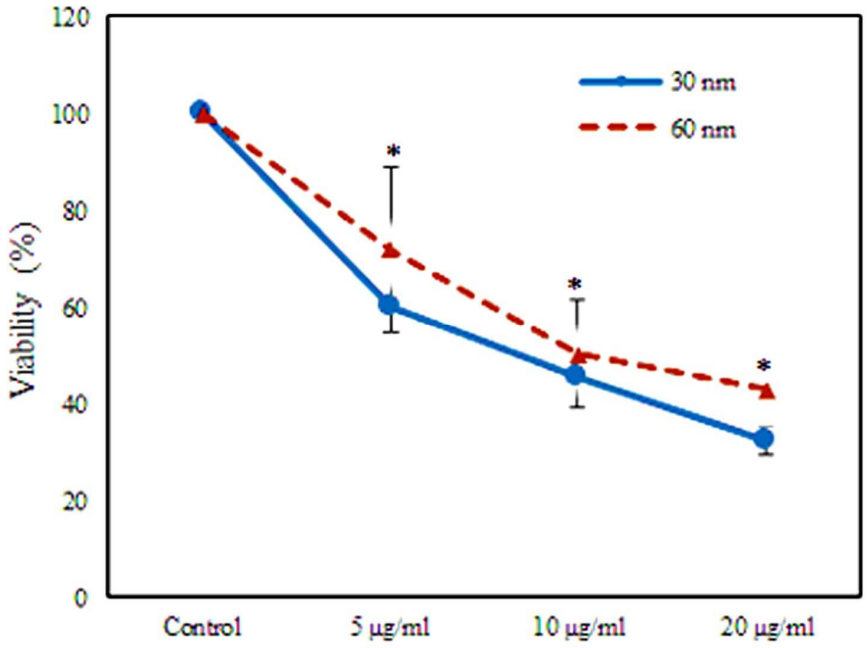

Figure 3. Cytotoxicity in different concentrations of CuO NPs with the sizes of 30 and 60 nm by MTT assay. represents a significant difference from the control ($p < 0.05$).

Nanoparticles have some physicochemical properties through their size which make them chemically more reactive in possessing biological activities that can be either desirable as drug delivery or undesirable by induction of oxidative stress or cellular dysfunction.[24] A glioma is an aggressive form of brain cancer that starts from transformed of glial cells.[19] Limitations of current therapies necessitate an effective, inexpensive and non-toxic treatments with slight side effects that are accepted

by people. The ability of cancer cells to avoid apoptosis and continue to propagate to make metal oxide nanoparticles as a demand for continuing new anti-cancer drugs researches[20]. Due to antimicrobial properties, CuO NPs are a right choice for being used in products such as textiles, intrauterine devices, food contact materials and wood preservation, therefore, these are widely manufactured and used.[8] Earlier studies showed potent cytotoxic, genotoxic and toxicological activities of CuNPs and even, toxic effects of various

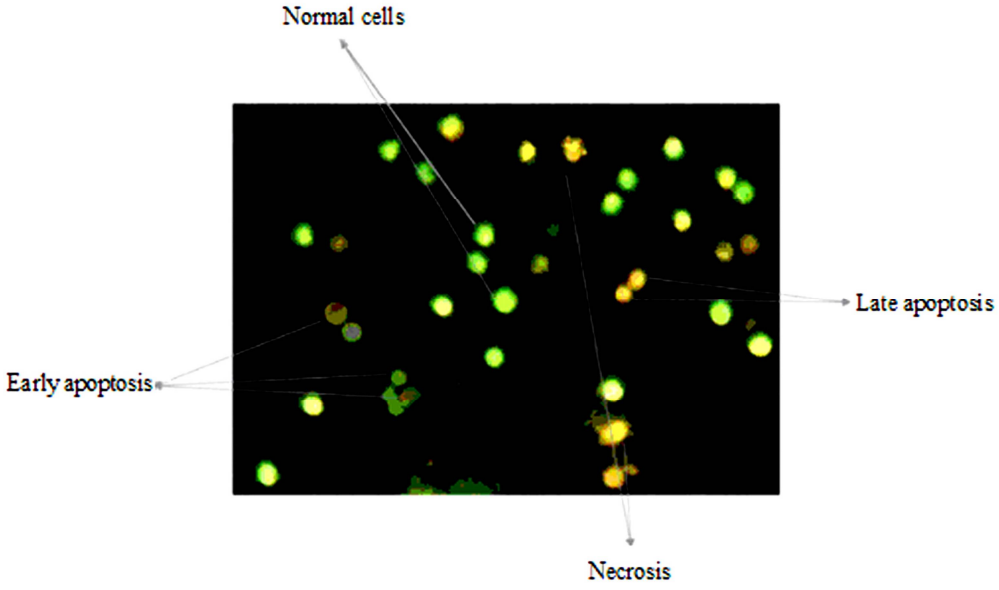

Figure 4. Apoptotic morphological observation at 20 µg/ml concentration with acridineorange/ propidium iodide staining by fluorescence microscope (40x). The cells were seeded in 6-well plates and were treated with CuO NPs (0, 5, 10 and 20 µg/ml) for 24 ~~hours~~ hrs.

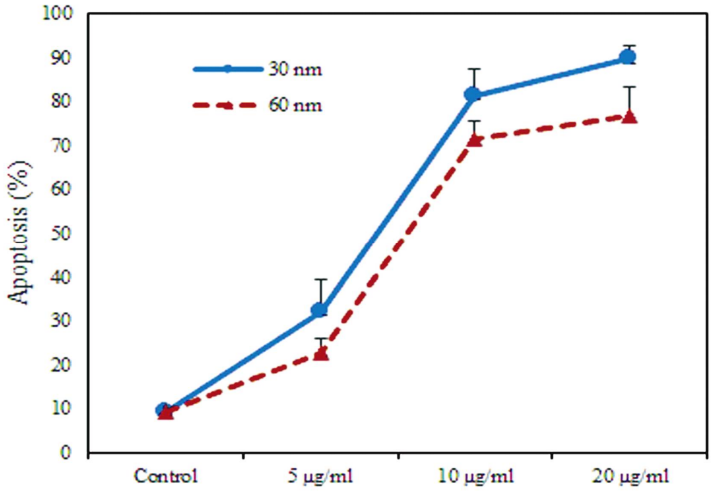

Figure 5. Determination of apoptosis in different concentrations of CuO NPs treated samples with 30 and 60 nm sizes of NPs.

types of nanoparticles in C6 glioma cancer cells.[15,22,25] So, in the present study, two sizes of CuO NPs (30 and 60 nm) were synthesized by an alcothermal method and were used in different concentration to evaluate their anticancer activity in B92 glial cancer cells. Characterization of

nanoparticles is essential before assessing the cytotoxicity since any changes in their characterization can have an effect on cellular responses upon exposure.[26,27] Thus, we first characterized the synthesized CuO NPs for better interpretation of results. The SEM images and zeta size reconfirmed the 30 and 60 nm sizes of CuO NPs, respectively.

The results of MTT assay was used for studying the cytotoxicity of two sizes of CuO NPs and showed that both CuO NPs (30 and 60 nm) induces cytotoxicity in B92 cancer cells after 24 hrs of exposure in a concentration-dependent manner. In a way that exposure to CuO NPs had a significant cytotoxic effect with the sizes tested when compared to unexposed control and the smaller size and higher concentration exerted the maximum cytotoxic effects. Our findings are in agreement with previous studies.[13-15,28] Although, there is a significant variation in the ability of both particles to alter cell cytotoxicity, the differences in cytotoxicity of each concentration of CuO-NPs is not certain as previously reported for somato sensory neurons of a rat in cell culture.[29] The IC_{50} decreased by increasing particle size, however, the decrease was non-significant. This suggests that augmentation may not have any impact on their cytotoxicity *in vitro* state.

Apoptosis and necrosis are two distinct mechanisms responsible for cell death, but induction of apoptosis in tumor cells is a vital and useful anti-cancer mechanism. Apoptotic cells are distinguished by altering morphological, molecular and biochemical properties and necrosis produces a leakage of cell content.[30,31] A research illustrated that CuO NPs induces the typical appearance of characteristic modifications for apoptosis in cells.[13] To quantitate apoptosis, AO staining was done to measure apoptosis induced by various concentrations of CuO NPs. AO/PI was used to distinguishing apoptosis from necrosis. The fluorescence microscopic study showed the condensation of chromatin and reduction in cell volume follows CuO NPs treatment. Apoptotic features are characterized by aggregated chromatin and condensed cytoplasm and occurred in the CuO NPs-treated rat cancer cells.

In conclusion, cellular apoptosis was affected by the presence of CuO NPS. By increasing the particle size, cellular apoptosis was developed. Although, exposure to 30 nm and 60 nm sizes had cytotoxic effects in cancer cells, it seems that augmentation may not have any impact on their *in vitro* cytotoxicity. Further investigations are required to better assess the biocompatibility and biosafety of CuO NPs for *in vivo* and *in vitro* conditions.

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