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Evaluation of in vitro cytotoxicity and genotoxicity of copper–zinc alloy nanoparticles in human lung epithelial cells

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ABSTRACT

In the present study, in vitro cytotoxic and genotoxic effect of copper–zinc alloy nanoparticles (Cu–Zn ANPs) on human lung epithelial cells (BEAS-2B) were investigated. XTT test and clonogenic assay were used to determine cytotoxic effects. Cell death mode and intracellular reactive oxygen species formations were analyzed using M30, M65 and ROS Elisa assays. Genotoxic effects were evaluated using micronucleus, comet and γ-H2AX foci assays. Cu–Zn ANPs were characterized by transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential measurements. Characterization of Cu–Zn ANPs showed an average size of 200 nm and zeta potential of −22 mV. TEM analyses further revealed the intracellular localization of Cu–Zn ANPs in cytoplasm within 24 h. Analysis of micronucleus, comet and γ-H2AX foci counts showed that exposure to Cu–Zn ANPs significantly induced chromosomal damage as well as single and double stranded DNA damage in BEAS-2B cells. Our results further indicated that exposure to Cu–Zn ANPs significantly induced intracellular ROS formation. Evaluation of M30:M65 ratios suggested that cell death was predominantly due to necrosis.

1. Introduction

In the last decade, significant progress has been achieved in the nano-scale science and technology. Today, nanomaterials and nanoparticles are widely used in different areas such as health, cosmetics, clothing, food, energy, space exploration, and defense industries (Arora et al., 2012; Ferreira et al., 2013). Among different types of nanoparticles, metals constitute one of the most widely studied groups due to their widespread usage. Most widely studied metal nanoparticles include gold, silver, titanium, and iron particles (Hussain et al., 2005; Schrand et al., 2010; Love et al., 2012). The widespread production and utilization of nanoparticles also makes them available for humans and the environment. Chemical, physical, and functional properties of nano-sized particles often differ compared with particles of larger size or dissolved species of the same element (Roduner, 2006). Similarly they can show different effects on biological systems then they exhibit on a macroscale (Nel et al., 2006; Midander et al., 2009). It is known that, nanoparticles may lead the cell death via damaging DNA or organelles (Buzea et al., 2007; Manke et al., in press). Thus the evaluation of potential effects of nanoparticles is crucial.

In the recent years Copper and zinc containing nanoparticles also received considerable attention due to their unique antibacterial, antifungal, UV filtering and semiconductor properties as well as their high catalytic and photochemical activities (Meruvu et al., 2011; Trickler et al., 2012). In vivo studies demonstrated the toxicity of Cu and Zn containing nanoparticles on different organisms such as fish (Ateş et al., in press), drosophila (Han et al., 2014), amphipods (Hanna et al., 2013), rats (Amara et al., in press), mice (Adamcakova-Dodd et al., 2014) and bacteria (Rousk et al., 2012). In vitro toxicity of Cu Zn nanoparticles have also been shown by several authors (Akhtar et al., 2012; Chang et al., 2012; Sahu et al., 2013). In general, Cu and zinc Zn containing nanoparticles were found to be comparatively more toxic than other metal nanoparticles (Karlsson et al., 2008, 2013). For instance, Lanone et al. (2009) compared 24 different nanoparticles on the toxicity in the human lung cell line (A549) and macrophage cell line (THP-1) and found that copper and zinc containing nanoparticles were the most toxic ones, whereas titanium, aluminum, cerium, and zirconium containing nanoparticles were moderately toxic and tungsten carbide nanoparticles were non-toxic.

Metals can be mixed in different proportions to create new metallic systems with different characteristics (Ferrando et al., 2008). In this manner, combination of copper and zinc elements constitute a well-known example of metal alloys (Singh et al., 2009). Cu–Zn ANPs are one of the most commonly used alloys

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especially in biomedical applications and in consumer products, due to their specific properties (Tripathi et al., 2012; Bondarenko et al., 2013; Karlsson et al., 2013). They are also used during production stages of microbateries, microelectronic circuits, nanocables and nanoliquids (Tripathi et al., 2012). It is known that alloys can possess different effecs than their components (Bardack et al., 2014). Numerous genotoxicity studies have demonstrated DNA damaging potential of copper and zinc containing nanoparticles on human skin keratinocytes (Alarifi et al., 2013), lung epithelial cells (Ahamed et al., 2010), peripheral blood lymphocytes (Gumus et al., 2014) and kidney cells (Wahab et al., 2013). However, Cytotoxicity and genotoxicity data on the Cu–Zn ANPs is scarce.

In the present study, we aimed to evaluate the in vitro cytotoxic and genotoxic effects of Cu–Zn ANPs on human lung epithelium cells (BEAS-2B). We used XTT and clonogenic assays to assess cytotoxicity and comet, micronucleus (MN) and gamma H2AX foci formation assays to evaluate genotoxicity. Additionally, we measured the intracellular production of reactive oxygen species (ROS) by the oxidation sensitive fluoroprobe 2,7-dichlorofluorescin diacetate (DCFH-DA) and applied M30 and M65 assays to evaluate apoptotic and necrotic responses, respectively.

2. Materials and methods

2.1. Chemicals

Cu–Zn ANPs (40%Zn–60%Cu: <150 nm) at >99% purity were purchased from Sigma–Aldrich. Other chemicals and their sources were as follows: RPMI-1640 medium and supplemental growth factors (Lonza, Basel, Switzerland); PBS, cytochalasin-B, giemsa, low melting point agarose, normal melting point agarose, EDTA and Triton X-100 (Sigma–Aldrich, St. Louis, MO); anti phospho γ-H2AX primary and aleuxafuor 488 labeled secondary antibodies (Thermo Scientific and Invitrogen). Hydrogen peroxide (H_2O_2) was used as positive control at a single concentration of 147 μM. Sterile distilled water was used as solvent control at a maximum concentration of 0.5% (v/v).

2.2. Characterization of nanoparticles

A stock suspension of Cu–Zn ANPs (final concentration 1 mg/ml) was prepared in distilled water. Prior to each treatment, it was ultrasonicated (Bandelin Sonifier) for 20 min and diluted to prepare the desired ANPs concentrations. Particle morphology, size, and agglomeration states were characterized by using transmission electron microscopy (TEM), dynamic light scattering (DLS), and zeta potential analyses. Average hydrodynamic size, size distribution, and zeta potential of particles in suspension were determined by dynamic light scattering (DLS) using a Malvern Zetasizer.

2.3. Cell culture

Since inhalation is considered as one of the main routes of exposure to copper and zinc, healthy human lung epithelial cell line (BEAS-2B) was selected for out studies. Suitability of this cell line for genotoxicity assessment of different types of chemicals including nanoparticles has also been previously demonstrated (Capasso et al., 2014; Kim et al., 2011). BEAS-2B cells were kindly provided by Dr. E. Ulukaya (University of Uludağ). The cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), penicillin–streptomycin (50 μg/ml), 2 mM L-glutamine, and 1% sodium pyruvate. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 and grown in 75 cm^2 flasks and subcultured once a week.

2.4. Selection of the doses

BEAS-2B cells were exposed to serial concentrations of Cu–Zn ANPs (0.1, 0.5, 1, 5, 8, 12, 16, 20, 25, 50 and 100 μg/ml) to determine cytotoxicity and IC50 value. According to determined IC50 value, non-cytotoxic and low cytotoxic doses (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml) of Cu–Zn ANPs were selected for genotoxicity experiments. Furthermore, two additional toxic doses (6.4 and 12.8 μg/ml) were also used to obtain and analyze cell death pathways in M30 and M65 tests.

2.5. Cellular uptake

BEAS-2B cells exposed to 2 μg/ml of ANPs solution for 24 h were examined under TEM to evaluate possible cellular internalization of Cu–Zn ANPs.

2.6. Cytotoxicity

XTT and clonogenic assays were used to test the potential effects of Cu–Zn ANPs on viability of BEAS-2B cells. To determine the cytotoxicity of Cu–Zn ANPs, BEAS-2B cells were exposed to serial concentrations of Cu–Zn ANPs. Untreated cells served as a control group.

XTT assay was performed by using a Cell Viability Assay Kit, which detects the cellular metabolic activities due to colorimetric features. During the assay, the yellow tetrazolium salt XTT is reduced to a highly colored formazan dye by dehydrogenase enzymes in metabolically active cells. This conversion only occurs in viable cells and thus, the amount of formazan produced is proportional to viable cells in the sample (Scudiero et al., 1988; Berridge et al., 2005). Five thousand cells were seeded in into a flat-bottom 96-well plate (which contains 100–200 μl/well culture medium) in triplicate and allowed to grow for 24 h. Cells were than treated with serial concentrations of Cu–Zn ANPs (0.1, 0.5, 1, 5, 8, 12, 16, 20, 25, 50 and 100 μg/ml) for 24 h. Three wells containing 100 μl of growth medium were used for blank absorbance readings. Then 50 μl of the Activated-XTT Solutions were added to each well. Finally, the plate was incubated for 2 h and absorbance was read at 450 nm. Experiments were repeated for three times.

Clonogenic assay, which measures the reduction in plating efficiency in the treatment and control groups, was also used to assess cytotoxicity (Wise et al., 2010). Fifty thousand cells were seeded in T25 tissue culture treated flasks and allowed to grow for 48 h. The cultures were then treated for 24 h with serial concentrations (0.1, 0.5, 1, 5, 8, 12, 16, 20, 25, 50 and 100 μg/ml) of Cu–Zn ANPs. Following exposure period, the treatment medium was collected, the cells were rinsed with PBS; and then removed with 0.25% trypsin/1 mM EDTA solution. Cells were centrifuged at 1000 rpm, 4 °C for 5 min. The pellet was re-suspended in 5 ml of medium, counted with Cedex XS (Roche) cell counter, and re-seeded at colony forming density (500 cells per well) into four pieces of a 60 × 15 mm petri dishes. Colonies were allowed to grow for 7 days, fixed with 100% methanol, stained with crystal violet, and counted. Experiments were repeated for three times.

2.7. Cytokinesis-block micronucleus test

Cytokinesis-block micronucleus test was used to detect chromosomal damages occurred due aneugenic or clastogenic effects (Fenech, 1993). BEAS-2B cells were seeded in T25 tissue culture treated flasks at a density of 3×10^4 cells/flask and allowed to grow for 48 h. Cells were then treated with different concentrations (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml) of Cu–Zn ANPs and H_2O_2 for 24 h. After treatment, cells were further cultured with cytochalasin-B for 24 h. Then, cells were trypsinized centrifuged and resuspended in 0.075 M KCl, and incubated for 2 min. Cells were then fixed 2 times in freshly prepared methanol: glacial acetic acid (3:1). Afterwards, the cell solution was dropped onto pre-cleaned slides and the nucleus was stained by 5% giemsa for 10 min. Slides were investigated under light microscope and the numbers of binucleated (BNC) cells with micronuclei (MNBNC) were recorded based on observation of 2.000 cells per treatment group.

Cytotoxicity was further evaluated by analysis of the nuclear division index (NDI) values. The numbers of cells with one to four nuclei were determined in 1.000 cells. NDI was calculated using the following formula: NDI = (1 × M1 + 2 × M2 + 3 × M3 + 4 × M4)/1.000, where M1 through M4 represent the number of cells with one to four nuclei.

2.8. Alkaline comet assay

Alkaline comet assay was used to detect single stranded DNA damage (Cavas, 2010). BEAS-2B cells were seeded on T25 tissue culture treated flasks at a density of 3×10^4 cells/flask and allowed to grow for 48 h. Cells were then treated with different concentrations (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml) of Cu–Zn ANPs and H_2O_2 for 24 h. After treatments with Cu–Zn ANPs, the alkaline comet assay was performed according to Singh et al. (1988), with some modification (Costa et al., 2008). BEAS-2B cells were harvested and embedded in 0.8% low melting agarose on slides precoated with normal melting point agarose. Slides were then placed in precooled lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris base, pH 10) with 1% Triton X for 1 h at 4 °C. Cells were then denatured in alkaline buffer (0.3 M NaCl, 1 mM EDTA) for 30 min in the dark at room temperature (RT). Electrophoresis was performed at 25 V and 300 mA for 20 min. The slides were immersed in neutralization buffer (0.5 M Tris–HCl, pH 7.5) for 10 min followed by dehydration in 70% ethanol. The slides were air dried and stained with ethidium bromide (EtBr). Slides and comets were examined under Nikon epifluorescence microscope equipped with a digical camera (Kameram 21) using an image processing software (Arganit Mikrosistem Comet Assay).

2.9. Immunofluorescence for γ-H2AX foci formation

γ-H2AX foci were used to identify the presence of Cu–Zn ANPs induced double strand DNA breaks (Leopardi et al., 2010) according to the method of Tanaka et al. (2009) with some modifications (Valdiglesias et al., 2011). The cells grown on 8 well chamber slides were exposed to different concentrations (0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 μg/ml) of Cu–Zn ANPs for 24 h. The cells were then fixed in 4%

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