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Toxicological interactions of silver nanoparticles and non-essential metals in human hepatocarcinoma cell line

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ABSTRACT

Toxicological interaction represents a challenge to toxicology, particularly for novel contaminants. There are no data whether silver nanoparticles (AgNPs), present in a wide variety of products, can interact and modulate the toxicity of ubiquitous contaminants, such as nonessential metals. In the current study, we investigated the toxicological interactions of AgNP (size = 1–2 nm; zeta potential = −23 mV), cadmium and mercury in human hepatoma HepG2 cells. The results indicated that the co-exposures led to toxicological interactions, with AgNP + Cd being more toxic than AgNP + Hg. Early (2–4 h) increases of ROS (DCF assay) and mitochondrial $O_2^{\bullet -}$ levels (Mitosox® assay) were observed in the cells co-exposed to AgNP + Cd/Hg, in comparison to control and individual contaminants, but the effect was partially reverted in AgNP + Hg at the end of 24 h-exposure. In addition, decreases of mitochondrial metabolism (MTT), cell viability (neutral red uptake assay), cell proliferation (crystal violet assay) and ABC-transporters activity (rhodamine accumulation assay) were also more pronounced in the co-exposure groups. Foremost, co-exposure to AgNP and metals potentiated cell death (mainly by necrosis) and Hg^{2+} (but not Cd^{2+}) intracellular levels (ICP-MS). Therefore, toxicological interactions seem to increase the toxicity of AgNP, cadmium and mercury.

1. Introduction

Silver nanoparticles (AgNP) are present in about 24% of all products listed in the Woodrow Wilson Database (Vance et al., 2015), such as food products, fabrics, cosmetics and medical devices (Seltenrich, 2013; Gaillet and Rouanet, 2015). Due to the extensive production and applications of AgNP, increased amounts of nanowaste will be generated and released into the environment (Bystrzejewska-Piotrowska et al., 2009; Cleveland et al., 2012). It has been already estimated that the predicted environment concentrations (PEC) of AgNP in surface waters is 0.088–10,000 ng l^{-1} and the maximum estimated PEC in wastewater treatment plant effluent is 17 μg l^{-1} (Kim et al., 2015; Maurer-Jones et al., 2013), representing a potential danger to the biota and human health. AgNP can cause adverse effects on a variety of biological models (Morones et al., 2005; Wu et al., 2010; Gliga et al., 2014; Monfared et al., 2015). However, it is not clear whether they represent a threat to the health of vertebrates (Fabrega et al., 2011; Della Torre et al., 2015), particularly when the interactions of AgNP and other environmental contaminants are considered. For example, AgNP, cadmium and mercury can disrupt cell antioxidant defense and induce the production of ROS, DNA damage, apoptosis and promote cell proliferation (Aguado et al., 2013; Chen et al., 2014; Vergilio et al., 2014; Lee et al., 2014; Kim et al., 2015; Kumar et al., 2015), but the effects of the combination of these metals and AgNP are unknown.

Cadmium and mercury are non-essential metals and ubiquitous contaminants of natural environments and dietary products (Monroe and Halvorsen, 2009; Guo et al., 2013; Arbuckle et al., 2016). These metals enter the environment through different anthropogenic sources. Cadmium is used in battery production, fertilizers, paints and plastic stabilizers (Capaldo et al., 2016) and mercury applications include soda chlorine production, coal combustion, paints and seed treatment (Sahu et al., 2014; Syversen and Kaur, 2012). Some studies reported

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that nanoparticles can modulate the toxicity of environmental contaminants, such as metals, polycyclic aromatic hydrocarbons (PAH) and organochlorinated pesticides (OCP), leading to unexpected results (Guo et al., 2013; Ferreira et al., 2014; Kim et al., 2015; Glinski et al., 2016). Guo et al. (2013), for instance, observed positive synergetic interaction of SiNP + $CdCl_2$ for hepatic biochemical and histopathological parameters, as well as the distribution of $CdCl_2$ in the liver and kidneys of mice. Likewise, Kim et al. (2015) reported that co-exposure with citrate coated-AgNP increased the bioaccumulation of Cd in *Daphnia magna*.

Considering the increasing use of AgNP, the widespread metal contamination and therefore, the possibility of these contaminants coexist in natural environments, we investigated the biological effects of combination of AgNP, Cd and Hg in human hepatoma (HepG2) cells through viability, metabolism, proliferation, efflux transporters activity, reactive oxygen species production and cell death parameters, to answer whether the co-exposure increase toxicity. HepG2 cells were selected, since liver is an important target organ of the three contaminants (Fowler, 2009; Stacchiotti et al., 2009; Elsaesser and Howard, 2012). Also, this cell line has been routinely used to investigate the toxicity of several compounds, because it preserves most of the phenotypic characteristics of normal hepatocytes (Knasmüller et al., 2004; Mersch-Sundermann et al., 2004; Urani et al., 2005).

2. Materials and methods

2.1. Silver nanoparticles synthesis and characterization

We synthesized silver nanoparticles through laser ablation in liquid medium (water as solvent) and determined the concentration of the stock solution through flame absorption spectrometry. Shape, diameter, size distribution, zeta potential and spectral properties of AgNP were determined by electron transmission microscopy JEOL 1200 EXII, *Zeta-sizer* (MALVERN®), Dynamic light scattering (DLS) and UV-vis, respectively.

2.2. HepG2 cell culture

Human hepatoma cells HepG2 (Rio de Janeiro Cell bank - Brazil) were cultured as a monolayer in high glucose DMEM medium supplemented with 10% inactivated fetal bovine serum (FBS) and antibiotics (10 U ml^{-1} penicillin and 10 μg/ml streptomycin), at 37 °C and 5% CO_2. Cells at passages 100–110 were utilized in the current study.

2.3. Selection of the concentrations of AgNP, Hg and Cd

Initially, toxicity-screening tests (MTT metabolism and neutral red uptake) were performed, in different concentrations and periods of exposure, for AgNP (0.005–6.66 μg/ml), Hg^{2+} (20–640 μM) and Cd^{2+} (0.5–50 μM) to determine two test concentrations of each contaminant: one that did not induce toxicity and one that induced pronounced toxicity. Based on these results, two concentrations for each contaminant at 24 h time-point were selected (Table 1; data of screenings are not shown).

2.4. Contaminants preparation and exposure protocol

AgNP were synthesized in H_2O and both metals ions stock solutions were prepared in 0.01 M HCl to avoid adsorption to the glass. For the exposure, the contaminants were added to fresh culture medium. In the case of co-exposures, first one contaminant was added to the medium, mixed and then the other contaminant was added. At final, all groups (control, AgNP, metal ions and mixtures) have the same concentration of HCl (buffered by culture medium) and water. Metal ions concentrations are expressed in μM and AgNP concentration is expressed in μg/ml.

Cells were seeded onto 96-well microplate (2×10^5 cells $well^{-1}$) for cytotoxicity, proliferation, reactive oxygen species production and multidrug efflux transporters assays, and onto 6-well plates (1.2×10^6 cells $well^{-1}$) for metal uptake assays. After 24 h, the medium was replaced by fresh DMEM medium with antibiotics and 2% fetal bovine serum containing AgNP ((I) 0.35 μg/ml; (II) 3.5 μg/ml), $CdCl_2$ ((I) 0.15 μM; (II) 1.5 μM) and $HgCl_2$ ((I) 2.8 μM; (II) 28 μM) or the combination of AgNP and each metal. Cells were exposed to these contaminants for 4 and 24 h, and an appropriate control group was kept in parallel. 'I' and 'II' stand, respectively, for the lowest and highest concentrations of AgNP and metals.

Table 1
Experimental design used in the evaluation of the toxicity of AgNP, metals and its association in HepG2 cells.

Groups	Concentration
Control	–
AgNP (I)	0.35 μg/ml
AgNP (II)	3.5 μg/ml
$CdCl_2$ (I)	0.15 μM
$CdCl_2$ (II)	1.5 μM
$HgCl_2$ (I)	2.8 μM
$HgCl_2$ (II)	28 μM
AgNP (I) + $CdCl_2$ (I)	0.35 μg/ml + 0.15 μM
AgNP (I) + $CdCl_2$ (II)	0.35 μg/ml + 1.5 μM
AgNP (II) + $CdCl_2$ (I)	3.5 μg/ml + 0.15 μM
AgNP (II) + $CdCl_2$ (II)	3.5 μg/ml + 1.5 μM
AgNP (I) + HgC_{l2} (I)	0.35 μg/ml + 2.8 μM
AgNP (I) + $HgCl_2$ (II)	0.35 μg/ml + 28 μM
AgNP (II) + $HgCl_2$ (I)	3.5 μg/ml + 2.8 μM
AgNP (II) + $HgCl_2$ (II)	3.5 μg/ml + 28 μM

2.5. Cytotoxicity and proliferation assays

Neutral red (NR) uptake assay was determined after incubation of cells with 50 μg/ml of neutral red for 3 h. MTT assay was determined after incubation of cells with 0.5 mg 1^{-1} of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 2 h.

Cell proliferation was determined after DNA staining with 50 μl $well^{-1}$ of 0.25 mg ml^{-1} of violet crystal solution, according to previously published protocols for HepG2 cells (Liebel et al., 2015).

2.6. Reactive oxygen species (ROS) levels

Intracellular ROS levels were evaluated with H_2DCF-DA (2′, 7′-dichlorodihydro-fluorescein diacetate) and MitoSOX™ Red (Invitrogen). After exposure, cells were incubated with either 10 μM of H_2DCF-DA or 5 μM of MitoSOX™, in fresh culture medium, (15 min, 37 °C, protected from light), washed with PBS and suspended in 250 μl of PBS. Fluorescence was measured at 488/530 nm (H_2DCF-DA) and 514/580 nm (MitoSOX; Benov et al., 1998).

2.7. Multidrug efflux transporters activity

The activity of ABC transporters was determined by Rhodamine 123 efflux assay (Pessatti et al., 2002, with modifications for cell culture). Culture medium was replaced by 200 μl of PBS containing 1 μM of rhodamine B, and cells were incubated. Cells were incubated (30 min, 37 °C, protected from light), washed twice with PBS and frozen at −76 °C in 250 μl $well^{-1}$ of PBS. Then, cells were thawed and 200 μl of the lysate were transferred to a black microplate for fluorescence quantification (540/580 nm). Verapamil (20 μM) was utilized as a positive control.

2.8. Cell death

Apoptotic and necrotic cells were detected using the FITC-Annexin V/propidium iodide (PI) Apoptosis Detection Kit (BD Biosciences, Heidelberg, Germany) and analyzed by time-lapse confocal microscopy (during 0–24 h exposure), according to the manufacturer's instructions.