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Neurotoxicity assessment of oleic acid-coated iron oxide nanoparticles in SH-SY5Y cells

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ABSTRACT

Iron oxide nanoparticles (ION) awaken a particular interest for biomedical applications due to their unique physicochemical properties, especially superparamagnetism, and ability to cross the blood-brain barrier. ION surface can be coated to improve their properties and facilitate functionalization. Still, coating may affect toxicity. The aim of this work was to evaluate the possible effects of oleic acid-coated ION (O-ION) on human neuronal cells (SH-SY5Y). A set of assays was conducted in complete and serum-free culture media for 3 and 24 h to assess O-ION cytotoxic effects – cell membrane disruption, cell cycle alteration and cell death induction –, and genotoxic effects – primary DNA damage, H2AX phosphorylation and micronuclei induction –, considering also DNA repair competence and iron ion release. Results obtained show that O-ION exhibit a moderate cytotoxicity related to cell membrane impairment, cell cycle disruption and cell death induction, especially notable in serum-free medium. Iron ion release was only observed in complete medium, indicating that cytotoxicity observed was not related to the presence of ions in the medium. However, O-ION genotoxic effects were limited to the induction of primary DNA damage, not related to double strand breaks, and this damage did not become fixed in cells in most conditions. Alterations in repair ability (DNA repair competence assay) were observed when cells where treated with O-ION before or during the challenge with H_2O_2, but not during the repair period. Further investigation is needed to clarify the possible role of oxidative stress and protein corona on observed O-ION toxicity.

1. Introduction

Among the different types of nanomaterials, iron oxide nanoparticles (ION) awaken a particular interest due to their unique properties, including superparamagnetism and high biocompatibility (Elsaesser and Howard, 2012). These features make them very suitable for a broad variety of uses, mostly in biomedical applications, namely magnetic resonance imaging, targeted drug delivery, and tumor location and magnetic hyperthermia, among others (reviewed in Revia and Zhang, 2016). In particular, over the last decade, ION are being used for diagnosis and treatment of several central nervous system (CNS) diseases, such as Alzheimer´s, Parkinson´s, multiple sclerosis, and primary brain tumors (Kanwar et al., 2012). This is mainly because the reduction in particle size gives ION the ability to cross the blood-brain barrier (BBB) and gain access to the brain (Halamoda Kenzaoui et al., 2012).

Recent investigations indicated that ION can not only reach the brain (Liu et al., 2013), but also cause a certain degree of neurotoxicity (Migliore et al., 2015). Several previous *in vitro* studies have described the possible toxicity associated with exposure to ION in human SH-SY5Y cells (Imam et al., 2015; Kiliç et al., 2015), rat PC12 cells (Wu and Sun, 2011; Wu et al., 2013; Deng et al., 2014), mouse c17.2 neural progenitor cells (Soenen et al., 2012), chick cortical neurons (Rivet et al., 2012), and primary rat cerebellar granule neurons (Petters and Dringen, 2015), among other nerve cell types. Despite these works, knowledge on the possible risk of human brain cells exposed to ION is very limited and conflicting so far. This is, at least in part, due to the great variety of ION, bare or with different coatings, tested. Besides, most studies were focused on addressing cytotoxic effects, *e.g.*, decrease

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in viability, cytoskeleton alterations, or reactive oxygen species (ROS) generation (Suh et al., 2009; Soenen and De Cuyper, 2010). However, their possible effects on other different cellular functions, on the genetic material, or on the DNA repair ability have been hardly addressed on nerve cell types (Valdiglesias et al., 2016). Furthermore, results of toxicity assays available are not always comparable since they are influenced by several factors such as the cell type tested (Ding et al., 2010; Kunzmann et al., 2011), experimental conditions assessed (Pisanic et al., 2007), and physicochemical properties of ION (Thorek and Tsourkas, 2008).

Iron oxide can exist in different chemical compositions, including magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3). Both oxides have very similar physical properties due to their nearly identical crystalline structure (Estelrich et al., 2015), whereas magnetization is higher for magnetite than for maghemite (Turcheniuk et al., 2013). Naked ION have high chemical activity and oxidation capacity, resulting in loss of magnetism and dispersibility. In order to avoid this and to improve their properties, ION surface can be coated with different natural or synthetic polymers and/or numerous biological molecules (Mahdavi et al., 2013). Nevertheless, surface modifications may also alter their biocompatibility, stability, aggregation state, size, and toxicity (Mahmoudi et al., 2011b). The type of coating employed usually depends on the functionalization needed for each particular ION application. Thus, ION coated with an oleic acid bilayer (O-ION) are a good option for biomedical purposes since oleic acid coating stabilizes the nanoparticles in organic solvents (Sahoo et al., 2001). This mono-unsaturated fatty acid is commonly used as high affinity surfactant agent to modify the surface of ION through the formation of strong chemical bonds between the carboxylic acid and ION, resulting in highly uniform, monodispersed and biocompatible nanoparticles (Soares et al., 2016). Moreover, the lipid-soluble and non-ionic oleic acid coating improves the nanoparticle ability to cross BBB and reach the brain, being particularly useful in specific applications directed to this organ, as targeted drug delivery or hyperthermia (Dilnawaz and Sahoo, 2015). However, studies regarding the potential toxic effects of ION on CNS are still scarce, and their potential health effects on human brain cells have raised concern (Braeuer et al., 2015).

In vitro studies are essential to initially evaluate the potential risk for the different CNS cells, from neurons to glial cells, associated with the use of ION (Dwane et al., 2013; Migliore et al., 2015). Human SH-SY5Y neuroblastoma cells are frequently used as cell model of dopaminergic neurons (Xie et al., 2010; Kovalevich and Langford, 2013). They are specially suitable for neurobiological, neurochemical and neurotoxicological evaluation (Wu et al., 2011; Yusuf et al., 2013) due to their ability to differentiate into a functionally mature neuronal phenotype and proliferate in culture for long periods, and they also possess many biochemical and functional features of primary neurons (Xie et al., 2010).

In a previous study conducted by our research group (Costa et al., 2016), effects induced by O-ION on viability of SH-SY5Y neuron-like cells were evaluated. Results showed that high concentrations of O-ION significantly decreased cell viability; besides, a serum-protective effect was observed. Moving forward, the aim of the present work was to evaluate the possible effects of O-ION on human neuronal cells (SH-SY5Y), in order to obtain an overall view of the potential risk, mainly at molecular and cellular levels, associated with their exposure. With this main purpose, a complete set of toxicological assays was carried out for assessing O-ION cytotoxic effects (cell membrane impairment, cell cycle disruption and cell death induction), and genotoxic effects (primary DNA damage, H2AX phosphorylation and micronuclei (MN) induction), considering also alterations in DNA repair competence and iron ion release capacity. Besides the abovementioned reasons, undifferentiated SH-SY5Y were chosen to carry out this study because inducing differentiation has been reported to confer tolerance on these cells and, therefore, the role of toxicity or protection cannot be evaluated in differentiated cells (Xie et al., 2010).

2. Material and methods

2.1. Chemicals

Bleomycin (BLM) (CAS no. 9041-93-4) and Triton X-100 (CAS no. 9002-93-1) were purchased from Panreac AppliChem (Barcelona, Spain), and mitomycin C (MMC) (CAS no. 50-07-7), camptothecin (Campt) (CAS no. 7689-03-4), dimethyl sulfoxide (DMSO) (CAS Number: 67-68-5), hydrogen peroxide (H_2O_2) (CAS no. 7722-84-1), and propidium iodide (PI) (CAS no. 25535-16-4) were purchased from Sigma-Aldrich Co. (Madrid, Spain), BLM, MMC, and PI were dissolved in sterile distilled water, and Campt was dissolved in DMSO prior to use.

2.2. Preparation and characterization of nanoparticles

O-ION were synthesized and prepared as stable water stock suspensions (19 mg/ml) as described by Maity and Agrawal (2007). Particle size and morphology were previously studied by transmission electron microscopy, surface chemistry was analyzed by photoelectron spectroscopy, while average hydrodynamic size and zeta potential of nanoparticles in suspension were determined by dynamic light scattering in deionized water, complete and serum-free SH-SY5Y culture medium (see composition below) (Costa et al., 2016). Prior to each treatment, a stock suspension of O-ION (1 mg/ml) was prepared in serum-free or complete SH-SY5Y culture medium, and was ultrasonicated in a water bath (Branson Sonifier, USA) for 5 min. Serial dilutions were carried out to obtain the different nanoparticle concentrations tested and were ultrasonicated in the water bath for an additional 5 min period.

2.3. Dissolved iron quantification in the cell culture medium

In order to determine the iron ions released from O-ION, suspensions of 10, 50, 100 and 200 μg/ml were prepared in complete or serum-free cell culture medium (see composition below) and incubated for 3, 6 and 24 h at 37 °C in a humidified 5% CO_2 environment. After centrifugation at 14,000 rpm for 30 min, the liquid medium over the O-ION solid phase was collected. Flame atomic absorption spectroscopy (FAAS) (Thermoelemental Solaar S4 v.10.02) was used to quantify the iron content in the supernatant. Complete or serum-free cell culture media without nanoparticles subjected to the same experimental conditions were used as negative controls.

2.4. Cell line, culture conditions and nanoparticle treatments

Human neuroblastoma SH-SY5Y cell line was obtained from the European Collection of Cell Cultures and grown in a nutrient mixture composed of DMEM/F12 (1:1) medium with 1% non-essential amino acids, 1% antibiotic and antimycotic solution, and supplemented with 10% heat-inactivated fetal bovine serum (FBS) (all from Gibco), in a humidified atmosphere at 37 °C with 5% CO_2. Before carrying out the experiments, SH-SY5Y cells (passage number below 20 in all experiments) were seeded in 96-well plates (5–9 × 10^4 cells per well), and allowed to adhere for 24 h at 37 °C. For each experiment, cells were incubated with four different O-ION concentrations (10, 50, 100 and 200 μg/ml) at 37 °C for 3 or 24 h, in complete or serum-free cell culture medium.

Complete and serum-free cell culture media were used as negative control in all experiments. The following chemicals were employed as positive controls: Triton X-100 (1%) for lactate dehydrogenase (LDH) assay, MMC (1.5 μM) for cell cycle analysis and MN test, Campt (10 μM) for apoptosis, H_2O_2 (100 μM) for comet assay and DNA repair competence assay and BLM (1 μg/ml) for γH2AX analysis.