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Toxicity of aluminium oxide nanoparticles demonstrated using a BY-2 plant cell suspension culture model

Zuzana Poborilova[a], Radka Opatrilova[b], Petr Babula[a,*]

[a] Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho 1/3, CZ-61242 Brno, Czech Republic

[b] Department of Chemical Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences Brno, Palackeho 1/3, CZ-61242 Brno, Czech Republic

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ABSTRACT

Aluminium oxide nanoparticles have been applied in many branches of industry. They are also used in personal care products, such as cosmetics. Because of these uses, their impact on the environment must be considered and investigated. Almost nothing is known about the effects of aluminium oxide nanoparticles on plants at the cellular level; the objective of this work was thus to study the effects of nanoparticles on the plant cell model tobacco BY-2 cell suspension culture, which serves as a model comparable with the HeLa cells used for animal cell studies. We observed the impact of these nanoparticles at different levels. The inhibitory effect on growth was observed in both time- and concentration-dependent studies. In addition, the ability of the nanoparticles to generate reactive oxygen (hydrogen peroxide, superoxide anion radical) and nitrogen species (nitric oxide) has been established. The principal part of the work was focused on the ability of aluminium oxide nanoparticles to induce the processes of programmed cell death. Changes observed in the permeability of the plasma membrane are connected with the effects of the reactive oxygen species and lipid peroxidation. In addition, the loss of mitochondrial potential, the enhancement of the caspase-like activity and the fragmentation of DNA determined in both time- and concentration dependent studies are closely connected with the execution of the programmed cell death. Our results indicate the ability of aluminium oxide nanoparticles to induce programmed cell death in plant cells and may explain the toxic effect of these nanoparticles on plants.

1. Introduction

Aluminium is the third most abundant element in the Earth's crust and the most abundant metallic element. It has a very wide range of uses, especially in the form of different types of alloys, where it improves the mechanical properties (Johnson and Sanders, 2012; Ruan and Schuh, 2012). However, elemental aluminium is not the only chemical form used. Because aluminium compounds demonstrate relatively low toxicity, they are found in significant and sometimes large-scale applications. Aluminium oxide, Al_2O_3, is usually used for the reduction to aluminium, but it has also been applied in industry, especially as a catalyst or catalyst support, in gas purification, and as an abrasive (Buzdugan and Beckman, 2007; Platonov et al., 2007). Aluminium oxide plays an important part in cosmetics. Ceramics based on Al_2O_3 have high mechanical strength, hardness, wear resistance, and chemical inertness with good biocompatibility, so, they are attractive for use in dental and bone implants (Lukin et al., 2001). Carbon-doped aluminium oxide film can be used to measure luminiscence signals optically stimulated by irradiation, so a possible application in the film dosimetry used in radiotherapy may be expected (Schembri and Heijmen, 2007). Aluminium oxide is among the chemicals most abundantly produced in nano-sized particles. These are used by the military and by commercial industries in many applications including coatings, thermites, and propellants. Generally, nanoparticles have very high surface reactivity; this means that they may have a negative impacts on health or the environment (Handy et al., 2008). Whereas the toxicity of aluminium is relatively well known and has been discussed in many publications, the toxicity of aluminium oxide nanoparticles remains almost unknown and is still an object of experimental work. The possibility of an impact of these nanoparticles on the environment has been demonstrated by their bioaccumulation in various organisms, including *Tubifex tubifex*

Abbreviations: Ac-DEVD-pNA, acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide; DHE, dihydroethidium; DW, dry weight; FDA, fluorescein diacetate; FW, fresh weight; H_2DCFDA, 2′,7′-dichlorodihydrofluorescin diacetate; MDA, malondialdehyde; MS, Murashige and Skoog; NPs, nanoparticles; PI, propidium iodide; RNS, reactive nitrogen species; ROS, reactive oxygen species.

* Corresponding author. Tel.: +420 776 206027; fax: +420 541 562835.
E-mail address: petr-babula@email.cz (P. Babula).

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Müller, *Hyalella azteca* Saussure, *Lumbriculus variegates* Müller, and *Corbicula fluminea* Müller (Stanley et al., 2010). The results of a study by Coleman et al. indicate that nano-sized Al_2O_3 can accumulate and impact the reproduction and behaviour of *Eisenia fetida* Savigny, although at high levels that are unlikely to be found in the natural environment (Coleman et al., 2010). This fact means that aluminium oxide nanoparticles may possibly enter the food chain and be responsible for toxicity in animals. In light of these facts the further impact of nanoparticles on various organisms must be considered. The oral exposure of rats to aluminium oxide nanomaterial has demonstrated the potential to cause genotoxic damage (Balasubramanyam et al., 2009). Work by Chen et al. using the model of the microvascular endothelial cells of the human brain indicates that the nano-alumina affects the cerebral vasculature in time- and concentration-dependent ways (Chen et al., 2008). Sprague-Dawley rats injected intraperitoneally with nano-sized aluminium oxide showed an effect on the innate immune system of the brain (Li et al., 2009). A cytotoxic effect of these nanoparticles on a model of UMR 106 cells has been revealed by Di Virgilio et al. (2010), and their transport and accumulation in L929 and BJ cells (Radziun et al., 2011) and L5178Y and BEAS-2B cells (Kim et al., 2009) have been described. Possible mechanisms for the cytotoxicity of nano-sized Al_2O_3 particles are still being discussed, but oxidative stress (Prabhakar et al., 2012) and DNA damage (Kim et al., 2009) may be responsible for their cytotoxic effect. In conclusion, some work focused on the possible cytotoxicity of aluminium oxide nanoparticles on cell models and animals has been published, but, their toxic effects on plants, *e.g.* phytotoxicity, remain almost entirely unknown. Possible interactions between these nanoparticles and the cell walls of algae (*Scenedesmus* sp., *Chlorella* sp.) have been shown in the work of Sadiq et al. (2011). These authors observed a decrease in the chlorophyll content. In light of the above-mentioned facts, further studies focused on the possible toxic effects of aluminium oxide nanoparticles on plants, especially as the particles accumulate, are highly necessary. We sought to determine the toxicity of aluminium oxide nanoparticles on the plant cell model, cell suspension culture BY-2, focusing on growth parameters, stress markers, and polyphenols. In addition, the content of aluminium in the form of soluble Al was determined using simple but sensitive spectrophotometric methods and visualized by the method of fluorescence microscopy. The principal goal of the work was to determine the ability of nanoparticles to induce the processes of programmed cell death.

2. Material and methods

2.1. Chemicals

All of the chemicals used were obtained from Sigma–Aldrich, USA, unless otherwise noted. They were stored in accordance with the manufacturer's recommendations. Working solutions were prepared immediately before use.

2.2. BY-2 cell suspension culture and determination of growth

Nicotina tabacum L. cv. Bright Yellow-2 suspension-cultured cells (BY-2) were grown in liquid MS medium (Murashige and Skoog, 1962) as modified by Nagata et al. (1992) under constant shaking (130 rpm) at 27 °C in the dark in 250 ml Erlenmeyer flasks. The pH of the cultivation media was adjusted to 5.6. Cells in the exponential growth phase were transferred into fresh cultivation media and aluminium oxide nanoparticles (<50 nm particle size, characterized by TEM, 20 wt.% in H_2O, Sigma–Aldrich, USA) were added to create concentrations of 0, 10, 20, 50 and 100 μg mL^{-1}. To investigate possible impact of size of Al_2O_3 nanoparticles, aluminium oxide microparticles (5 μm mean particle size, Sigma–Aldrich, USA) were applied at he same concentrations. Cells were subsequently cultivated for 96 h, with samples being collected at strictly defined time intervals of 12, 24, 36, 48, 72, and 96 h. All experiments were carried out in triplicate. The cell density (growth of the cell suspension culture) was determined using a Fuchs-Rosenthal haemocytometer (Fisher Scientific, Czech Republic). The cells were counted at the above-defined time intervals as viewed in white light under an Axioscop 40 microscope (Zeiss, Germany). Ten random fields were evaluated for each series, and the cell numbers were determined in triplicate.

2.3. Observations with the microscope

2.3.1. Cell viability and visualization of soluble Al

The cell viability was measured using fluorescein diacetate (FDA, Sigma–Aldrich, USA) and the propidium iodide (PI, Sigma–Aldrich, USA) as described by Babula et al. (2012). A 20 μL sample of the cell suspension culture was diluted to 50 μl by fresh MS cultivation medium and incubated for 5 min at 25 °C with FDA (final concentration 2.4 μmol L^{-1}) and PI (30 μmol L^{-1}). PI, a nucleic acid stain, penetrates through damaged cell membranes and intercalates the DNA of the cell, so PI positive cells are dead or dying. Living cells metabolize FDA to fluorescein which emits green light upon excitation. The percentages of viable and dead cells were evaluated by counting using a fluorescent microscope (Axioscop 40, Zeiss, Germany) equipped with broad spectrum UV excitation. Ten random fields (minimally 100 cells per field, totally minimally 1000 cells) from each series were evaluated in the microscope and the viability was determined in triplicate. Soluble aluminium was visualized using fluorescence microscopy (Axioscop 40, Zeiss, Germany) in accordance with the work of Vitorello and Haug, which was based on morin staining and the formation of highly fluorescent Al–morin complexes (Vitorello and Haug, 1997). NIS elements software (Nikon, Japan) was used to process of images and to evaluate the resultant pictures.

2.3.2. Visualization of ROS

Dihydroethidium (DHE, Invitrogen, USA) was used for the fluorescent visualization of the reactive oxygen species, respectively oxidative stress (Garnczarska, 2005; Goto et al., 1993). Thrice-washed BY-2 cells were incubated for 1 h in 10 μM DHE in fresh MS cultivation medium in darkness to avoid possible light-accelerated DHE oxidation. They were then washed three times with MS medium and observed with the microscope using the appropriate excitation filter (Axioscop 40, Zeiss, Germany). In a similar manner, 2′,7′-dichlorodihydrofluorescin diacetate (H_2DCFDA, Invitrogen, USA) was used to determine the oxidative stress (Garnczarska, 2005). Thrice-washed BY-2 cells were incubated for 1 h in 10 μM H_2DCFDA in fresh MS cultivation medium in darkness to avoid possible light-accelerated H_2DCFDA oxidation. They were then washed three times with MS medium and observed with the microscope using the appropriate excitation filter (Axioscop 40, Zeiss, Germany).

2.3.3. Nuclear architecture and programmed cell death

In order to observe nuclei and detect signs of programmed death, a 20 μL sample of cells was treated with 20 μL of PEM-buffer (100 mM PIPES, 10 mM EGTA, 10 mM $MgCl_2$, pH 6.9, all chemicals obtained from Sigma–Aldrich, USA) containing formaldehyde (4%, w/w; Sigma–Aldrich, USA). A Hoechst 33258 fluorescent probe (Sigma–Aldrich, USA) was used for fluorescence microscopy. One thousand nuclei in each preparation were observed using a fluorescence microscope (Olympus AX 70, Germany) equipped with broad-spectrum UV excitation. Ten random fields (minimally 1000