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In vitro developmental toxicity test detects inhibition of stem cell differentiation by silica nanoparticles

Margriet V.D.Z. Park [a,b,*], Wijtske Annema [a], Anna Salvati [c], Anna Lesniak [c], Andreas Elsaesser [d], Clifford Barnes [d], George McKerr [d], C. Vyvyan Howard [d], Iseult Lynch [c], Kenneth A. Dawson [c], Aldert H. Piersma [a,e], Wim H. de Jong [a]

[a] *Laboratory for Health Protection Research, National Institute for Public Health and the Environment, 3720 BA, Bilthoven, The Netherlands*
[b] *Department of Health Risk Analysis and Toxicology, Maastricht University, 6200 MD Maastricht, The Netherlands*
[c] *Centre for BioNano Interactions, School of Chemistry and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland*
[d] *Centre for Molecular Bioscience, University of Ulster, Coleraine, BT52 1SA, UK*
[e] *Institute for Risk Assessment Sciences, University of Utrecht, 3508 TD, Utrecht, The Netherlands*

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ABSTRACT

While research into the potential toxic properties of nanomaterials is now increasing, the area of developmental toxicity has remained relatively uninvestigated. The embryonic stem cell test is an *in vitro* screening assay used to investigate the embryotoxic potential of chemicals by determining their ability to inhibit differentiation of embryonic stem cells into spontaneously contracting cardiomyocytes.

Four well characterized silica nanoparticles of various sizes were used to investigate whether nanomaterials are capable of inhibition of differentiation in the embryonic stem cell test. Nanoparticle size distributions and dispersion characteristics were determined before and during incubation in the stem cell culture medium by means of transmission electron microscopy (TEM) and dynamic light scattering.

Mouse embryonic stem cells were exposed to silica nanoparticles at concentrations ranging from 1 to 100 μg/ml. The embryonic stem cell test detected a concentration dependent inhibition of differentiation of stem cells into contracting cardiomyocytes by two silica nanoparticles of primary size 10 (TEM 11) and 30 (TEM 34) nm while two other particles of primary size 80 (TEM 34) and 400 (TEM 248) nm had no effect up to the highest concentration tested.

Inhibition of differentiation of stem cells occurred below cytotoxic concentrations, indicating a specific effect of the particles on the differentiation of the embryonic stem cells. The impaired differentiation of stem cells by such widely used particles warrants further investigation into the potential of these nanoparticles to migrate into the uterus, placenta and embryo and their possible effects on embryogenesis.
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Introduction

There is an ongoing debate whether current risk governance systems are appropriate for nanomaterials, including the nanoforms of common materials such as silica (SiO_2) (Renn and Roco, 2006). To facilitate a faster risk assessment procedure for promising nanotechnology products, the use of *in vitro* studies has been suggested as a rapid approach to distinguish between low and high toxicity nanomaterials (Nel et al., 2006; Service, 2008; Shaw et al., 2008). Numerous *in vitro* studies investigating the cytotoxic, oxidative stress and inflammation potential of nanomaterials are now published, although their value for predicting *in vivo* toxicity still remains to be demonstrated (Sayes et al., 2007; Park et al., 2009). Other toxicity endpoints such as carcinogenicity, immunotoxicity, reproductive and developmental toxicity are scarcely investigated. Evaluation of these types of toxicity endpoints often require long *in vivo* exposure studies, but are nonetheless relevant endpoints to include in risk assessments of nanomaterials. An inventory of nanotechnology-based consumer products currently on the market lists various products that claim use of nanomaterials, including paint, cosmetics, personal care products and food supplements, although the presence of nanoscale entities in these products has not been verified (Woodrow Wilson International Center for Scholars, 2008). Emerging applications of nanomaterials in biomedical and biotechnological fields include biosensors (Zhang et al., 2004), biomarkers (Santra et al., 2001), cancer therapy (Hirsch et al., 2003), DNA delivery systems (Bharali et al., 2005), drug delivery systems (De Jong and Borm, 2008) and enzyme immobilization (Qhobosheane et al., 2001). Hence, human exposure to nanomaterials may involve inhalation, ingestion and dermal routes. Moreover, these particles may be directly injected into the human body for medical

* Corresponding author. National Institute for Public Health and the Environment (RIVM), P.O. Box 1, 3720 BA Bilthoven, The Netherlands. Fax: +31 30 274 4446.
E-mail address: Margriet.Park@rivm.nl (M.V.D.Z. Park).

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purposes. Once systemically available, various types of nanomaterials appear capable of distributing to most organ systems and even may cross biological barriers, such as the blood–brain and blood–testis barriers (Semmler et al., 2004; Kwon et al., 2008). Since the number of applications of nanomaterials is expected to rise even more in the future, long term exposure and potential accumulation of these nanomaterials in the human body may result.

Scarce and inconsistent information exists on the ability of nanomaterials to penetrate across the placental barrier and evoke embryotoxic effects. Sadauskas et al. injected pregnant mice intravenously with either 2 nm or 40 nm gold particles in their 16–18th day of pregnancy. No nanoparticles could be detected in either the placenta or the fetuses (Sadauskas et al., 2007). These findings were supported by Challier et al. who demonstrated impermeability of rat placenta to 4–200 nm radio labeled gold colloid particles in both directions, i.e. mother–fetus and fetus–mother (Challier et al., 1973). However, when rats were exposed intravenously to 5 nm and 30 nm ^{198}Au particles a limited transfer of these particles to the fetus was found, i.e. 0.018 and 0.05% for 5 and 30 nm particles, respectively (Takahashi and Matsuoka, 1981). Additional evidence that small amounts of radiolabeled gold can transfer across the placenta in rats was recently published (Semmler-Behnke et al., 2008). Malformations found in embryos after intravenous exposure of pregnant mice to fullerene C_{60} also indicate transfer of these particles to the conceptus (Tsuchiya et al., 1996). In addition, exposure of zebrafish embryos to fullerene C_{60} nanoparticles resulted in malformations and mortality (Zhu et al., 2007; Usenko et al., 2008). In contrast, Bosman et al. demonstrated that *in vitro* exposure of mouse embryos to polystyrene-based nanoparticles did not affect the development to the blastocyst stage (Bosman et al., 2005). These contradicting results may be attributable to the use of different nanomaterials, but also to the exposure occurring during different stages of embryo development as well as differences in experimental models.

The embryonic stem cell test (EST) was validated as an *in vitro* developmental toxicity test discriminating between chemicals in three classes of embryotoxicity (Genschow et al., 2004b). It investigates the potential of test compounds to inhibit the differentiation of embryonic stem cells (D3 cells) into spontaneously contracting cardiomyocytes and is regarded as a promising alternative method to *in vivo* developmental toxicity studies (Genschow et al., 2004a). To our knowledge this assay has not previously been applied to test nanomaterials. The aim of this study was to investigate the effect of four well characterized silica nanoparticles of different sizes in the embryonic stem cell test.

Materials and methods

Nanomaterials. The nanoparticles used in this study were spherical amorphous silica nanoparticles of four different sizes obtained from Glantreo Ltd., Cork, Ireland. These silica particles were synthesized via the Stöber method without any stabilizer (Stöber et al., 1968). The manufacturer's specifications indicated that the particle solutions contained spherical silica nanoparticles with an average primary particle size of 10, 30, 80 and 400 nm, respectively.

In order to clean the particle suspensions from synthesis residues and solvents that may affect toxicity, the nanoparticles were dialyzed extensively against a very large excess of pure MilliQ water. Sample concentration after dialysis was determined by freeze drying three aliquots of the dialyzed nanoparticle sample for 48 h and weighing the final silica material. Endotoxin concentration was below the detection limit as analyzed by the Limulus Amoebocyte Lysate (Gel-clot) assay. Moreover, nanoparticle stock solutions were void of bacterial and/or fungal infections, as tested by inoculation on Columbia sheep blood agar plates (Oxoid Ltd).

The morphology, mean diameter and aggregation status of the dried silica particles was assessed using transmission electron microscopy (TEM) using methods described previously (Barnes et al., 2008). In brief, particles were deposited in suspension onto carbon film TEM grids and left to dry in air. Mean particle size was determined by measuring more than 100 randomly sampled individual particles.

Dynamic light scattering. Dynamic light scattering (DLS) measurements using a Malvern 3000HS Zetasizer photon correlation spectrophotometer were carried out to determine the hydrodynamic particle size of the silica particles in deionized water shortly after dispersion as described previously (Barnes et al., 2008) and in D3 cell culture medium as a function of time (shortly after dispersion, 24 h and 5 days after dispersion). For these studies, particles were dispersed in D3 cell culture medium at 100 μg/ml, corresponding to the highest particle concentration studied in the embryonic stem cell test.

Cell culture. The D3 murine embryonic stem cell line was purchased from American Type Culture Collection (ATCC, Rockville, USA). Cells were maintained in DMEM (Gibco, cat. nr. 41965, Breda, The Netherlands) supplemented with 20% Foetal Calf Serum (HyClone, Logan, USA), 2 mM L-glutamine (Gibco, Breda, The Netherlands), 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco, Breda, The Netherlands), 1% non-essential amino acids (Gibco, Breda, The Netherlands) and 0.1 mM β-mercaptoethanol (Sigma-Aldrich, Schnelldorf, Germany) in the presence of 1000 U/ml murine Leukemia Inhibiting Factor (mLIF; ESGRO, Chemicon, Billerica, USA) in a humidified atmosphere of 5% CO_2 and 37 °C. The addition of mLIF serves to inhibit spontaneous differentiation of the embryonic stem cells into major embryonic tissues. The D3 embryonic stem cells were routinely cultured in 35 × 10 mm culture dishes (Corning, Sigma-Aldrich, Schnelldorf, Germany) coated with a 0.1% gelatin solution and subcultivated using non-enzymatic cell dissociation buffer (Gibco, Breda, The Netherlands) upon reaching 60–80% confluence. Particle exposure experiments were performed using D3 cells between passages 11 and 25.

Embryonic stem cell test. The embryonic stem cell test (EST) was applied to predict the embryotoxic potential of the four silica nanoparticles. This *in vitro* developmental toxicity test is based on the culture of D3 embryonic stem cells in hanging drops of cell culture medium. Under these conditions, cells will form embryoid bodies and upon plating onto tissue culture plastics, they will differentiate into contracting cardiomyocytes (Scholz and Spielman, 2000). This differentiation process may be impaired by toxic agents.

Stock solutions of silica nanoparticles were diluted serially in distilled water (AD) to yield concentrations ranging from 10 to 1000 μg/ml. These samples were then thoroughly vortexed before dilution 10 times with cell culture medium, immediately before use. The embryonic stem cells were thus exposed to end concentrations ranging from 1 to 100 μg/ml, throughout the entire 10-day test period of the differentiation assay. As a positive control, cells were exposed to 5-fluorouracil at an embryotoxic concentration of 0.045 μg/ml. On day 0 of the assay, embryonic stem cells were harvested and a cell suspension of 15×10^4 cells/ml was prepared. Subsequently, 500 μl cell suspension was added to 1.5 ml freshly prepared cell culture medium containing silica particles, AD or 5-fluorouracil and suspensions were placed on ice. Subsequently, cells in the suspension (3.75×10^4 cells/ml) were allowed to aggregate by preparing so called “hanging drops”. For each exposure concentration, 70 drops of 20 μl cell suspension were placed on the lid of a cell culture dish (Greiner, Sigma-Aldrich, Schnelldorf, Germany) filled with 5 ml phosphate buffered saline (PBS). These hanging drops were then cultured in a humidified atmosphere at 37 °C in 5% CO_2. On day 3, the cell aggregates (embryoid bodies) were transferred with 5 ml cell culture medium containing silica particles, AD or 5-fluorouracil to a bacterial petri dish (Greiner, Sigma-Aldrich, Schnelldorf, Germany) and further

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