Toxicology and Applied Pharmacology 342 (2018) 60–68

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Relevance to investigate different stages of pregnancy to highlight toxic effects of nanoparticles: The example of silica

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ARTICLE INFO

Keywords:
Nanoparticles
Toxicity
Pregnancy
Placenta
Fetus
Silica

ABSTRACT

Amorphous silica nanoparticles (SiO_2NPs) have been recognized as safe nanomaterial, hence their use in biomedical applications has been explored. Data, however, suggest potential toxicity of SiO_2 NPs in pregnant individuals. However, no studies relating nanoparticle biokinetic/toxicity to the different gestational stages are currently available. In this respect, we have investigated the possible embryotoxic effects of three-size and two-surface functionalization SiO_2NPs in mice. After intravenous administration of different concentrations at different stages of pregnancy, clinical and histopathological evaluations, performed close to parturition, did not show signs of maternal toxicity, nor effects on placental/fetal development, except for amino-functionalized 25 nm NPs. Biodistribution was studied by ICP-AES 24 h after administration, and demonstrates that all particles distributed to placenta and conceptuses/fetuses, although size, surface charge and gestational stage influenced biodistribution. Our data suggest the need of comprehensive toxicological studies, covering the entire gestation to reliably assess the safety of nanoparticle exposure during pregnancy.

1. Introduction

Due to their high thermal stability, chemical inertness, and ability to be functionalized with different molecules and polymers, silica nanoparticles (SiO_2 NPs) represent a promising material for many biomedical and industrial applications (Bitar et al., 2012). SiO_2 NPs are already widely used as additives to plastic, cosmetics and food, and as filler in different products (Napierska et al., 2010). As a consequence of such a wide use, the possibility to come in contact with these nanoparticles is highly increased. Although toxicity of high doses of silica nanoparticles has been reported both in vivo and in vitro (Lin et al., 2006; Kim et al., 2015; Ye et al., 2010; Yu et al., 2013), studies using purified nanoparticles at realistic doses have demonstrated lack of toxicity in several biological settings (Guarnieri et al., 2014; Ambrosone et al., 2014; Malvindi et al., 2012; Sabella et al., 2014; Liu et al., 2014). On this background, possible adverse effects after exposure to SiO_2 NPs should be evaluated in populations considered more susceptible to putative toxicants, such as pregnant women. Moreover, evaluation of NP toxicity in susceptible population is considered a key point in the recently proposed Intelligent Testing Strategies needed to build up reliable risk assessment models for NPs (Stone et al., 2014; Scott-Fordsmand et al., 2014). Pregnancy may in fact represent a higher risk condition for both the mother, given the increased metabolic demand, and the fetus, due to the ongoing development of key organs and systems (Sachdeva et al., 2009). Recent data suggest that administration of SiO_2 NPs to pregnant mice induces fetal growth restriction, while causing relatively minor effects in dams (Yamashita et al., 2011). It should be considered that pregnancy is a dynamic process, during which the feto-placental unit undergoes profound changes, affecting placental barrier permeability (Prouillac and Lecoeur, 2010; Enders and Blankenship, 1999), which in turn may influence the embryo/fetal susceptibility to xenobiotics.

Therefore, maternal exposure to nanoparticles (NPs) might have different effects depending on the gestational stage. Stage dependent

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https://doi.org/10.1016/j.taap.2018.01.026
Received 13 November 2017; Received in revised form 27 January 2018; Accepted 29 January 2018
Available online 31 January 2018
0041-008X/

feto/placental accumulation of NPs has been observed for gold nanoparticles (Yang et al., 2012). A similar importance might have the physio-chemical characteristics of the NPs, as toxicity of silica NPs is abolished by their functionalization with −COOH and $-NH_2$ groups (Yamashita et al., 2011), and in vitro studies have shown that SiO_2 NPs display different toxicity to embryonic cells depending on their size (Park et al., 2009).

Although placentation and embryonic development differ for many aspects between humans and rodents, some common features, including gestational stage related changes of permeability, make rodents a good model for in vivo toxicological studies (Muoth et al., 2016).

In the present work, we have taken into considerations these open questions by investigating the effect of SiO_2 NPs of three sizes and two surface chemical functionalizations (−COOH and $-NH_2$) administered to pregnant mice at three different gestational stages: 5.5 days post coitum (dpc) when no placenta has been formed, 12.5 dpc, when the placenta has shaped and has low permeability, and 16.5 dpc when it is fully developed and its permeability has strongly increased.

2. Methods

2.1. Materials and reagents

All reagents used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

2.2. Nanoparticle synthesis and characterization

SiO_2 NPs of three different sizes (25, 60, 115 nm) and two different surface functionalization ($-NH_2$ and −COOH) were prepared by following optimized synthesis procedures as previously reported (Guarnieri et al., 2014; Ambrosone et al., 2014; Malvindi et al., 2012) (Figs. 1–3). The nanoparticles were characterized in both aqueous solution and mouse plasma and zeta potential, size and dispersion stability were checked (Figs. 1–3). Transmission electron microscope (TEM) images were recorded by a JEOL JEM 1011 microscope operating at an accelerating voltage of 100 kV. TEM samples were prepared by dropping a dilute solution of nanoparticles in water on carbon-coated copper grids (Formvar/Carbon 300 Mesh Cu). Dynamic light scattering (DLS) and z-potential measurements were performed on a Zetasizer Nano ZS90 (Malvern, USA) equipped with a 4.0 mW HeNe laser operating at 633 nm and an avalanche photodiode detector. Size measurements were made at 25 °C in both aqueous solutions (pH = 7) and in plasma.

2.3. Elemental analysis

Elemental analysis was carried out by inductively coupled plasma atomic emission spectroscopy (ICP-AES) with a Varian Vista AX spectrometer. Tissue samples were weighed accurately with an analytical balance and then dissolved in 0.5 ml of concentrated HCl/HNO_3 3:1 (v/v). The digestion was carried out by subjecting the tissues to various microwave irradiation cycles through the Discover SP-D mineralizator (CEM). The solution was then diluted to 5 ml with ultrapure water and filtered with cellulose acetate filter paper (45 μm), and the resulting solution directly analyzed. The Si concentration measured was normalized to the mg of tissue. At least three biological replicates were analyzed for each tissue.

2.4. In vivo animal studies

Mice used for this study were of the CD1 outbred (Charles River, Calco, Italy), considered a general multipurpose model for toxicological in vivo studies. Animals were kept under standard conditions in the Animal Technology Station of the University of Tor Vergata and humanely treated. All animal procedures were approved by the Institutional Animal Care and Use Committee and the Ministry of Health (authorization number 675/2015). Six to eight weeks old females, weighting between 30 and 35 g, were mated with males of proven fertility and the presence of the plug checked the following day. The day of the plug was considered day 0.5 of pregnancy (0.5 dpc).

For biodistribution studies, 200 μg/mouse of each type of SiO_2 NP was administered to groups of 10 pregnant females at either 5.5 dpc, 12.5 dpc or 16.5 dpc, via intravenous injection in the retro-bulbar plexus in a final volume of 100 μl. Control animals were injected with 100 μl of saline solution (used as the vehicle). As we previously reported (Pietroiusti et al., 2011; Campagnolo et al., 2013), the retro-bulbar injection is technically very easy to perform and represent a valid alternative to the tail vein injection for small laboratory animals. Specifically, after manual restrain, 100 μl of the NP suspension were gently injected in retro-orbital sinus of the right eye, using a 1 ml syringe equipped with a 27 gauge needle. After 24 h from the administration, blood was collected by retro-orbital bleeding of the contralateral eye, and animals were sacrificed for subsequent ICP-AES analysis of maternal tissues, placentas and fetuses, as reported above. The collected blood was used for the determination of the main hematological parameters. Specifically, 20 μl of whole blood was collected in K2-EDTA micro-vacutainers (Boston, Dickinson and Company, USA) and samples were analyzed using the commercially available automated cell counter “Drew3” (BPC BioSed, Italy). The complete blood count (CBC) includes: hemoglobin (HGB), hematocrit (HCT), red blood cell (RBC) count, white blood cell (WBC) count, platelet count (PLT), red cell distribution width (RDW), mean corpuscular volume (MCV), Mean platelet volume (MPV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and Red Cell Distribution Width (RDW), Lymphocytes (LYMF), mid-range cells (MID), Granulocytes (GRAN), platelet distribution width (PDW), Platelet large cell ratio (LPCR), plateletcrit (PCT). Blood from at least three pregnant females was analyzed for each group.

For toxicological studies, groups of 10 pregnant females were administered with the six different types of SiO_2 NPs at 0, 3, 30 or 200 μg/mouse. All animals were sacrificed at 17.5 dpc. At the time of sacrifice control and treated females were euthanized by cervical dislocation, and maternal organs collected for histological and immune-histochemical analysis. Fetuses and placentas were weighted, measured, carefully observed under a stereomicroscope to screen for the presence of structural abnormalities and vascular alteration and then fixed in buffered formalin overnight at 4 °C. Fixed samples were then processed for paraffin embedding following standard protocols.

2.5. Statistical analysis

All experiments were performed at least three times. Data were expressed as mean ± SE and analyzed using the Kruskal-Wallis One Way Analysis of Variance on Ranks. Chi square test was used to compare each other discrete variables, and Student's *t*-test to compare continuous variables. A two-tailed $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Maternal and fetal biodistribution

For biodistribution studies, the six different types of SiO_2 NPs (Figs. 1–3) were intravenously administered to pregnant mouse females at the concentration of 200 μg/mouse. Administration of nanoparticles occurred at three different gestational stages, 5.5, 12.5 and 16.5 dpc, which vary for the presence/absence of a developed placenta and for changes in its permeability. Biodistribution to the feto-placental units was studied by ICP-AES after 24 h from the *i.v.* injection. As shown in Fig. 4, when SiO_2 NPs were administered at 5.5 dpc and 12.5 dpc, only the 25 and 60 nm NPs were detected in the conceptuses. In contrast, at