

Isoprostanes as Biomarkers for In Vivo Evaluation of Nanoparticle-induced Oxidative Stress: a Study with Silica Nanoparticles Doped with Cadmium

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Abstract-The application of silica/cadmium containing nanomaterials in medicine and technology has attracted much attention in the latest years. However, information on toxicity, exposure and health impact of these nanomaterials is still limited. In this study, in vivo effects of silica nanoparticles (SiNPs) doped with Cd (SiNPs-Cd, 1 mg/rat), soluble CdCl₂ (400 µg/rat), or SiNPs (600 µg/rat) have been investigated by evaluating F₂-isoprostanes (F₂-IsoPs) and superoxide dismutase (SOD1) as markers of oxidative stress 24 hr, 7 and 30 days after intra-tracheal (i.t.) instillation to rats. Free and esterified F₂-IsoPs were evaluated in lung and plasma samples by GC/NICI-MS/MS analysis, and SOD1 expression in pulmonary tissue by immunocytochemistry. The present paper also gives an overview of current scientific knowledge and application of F₂-IsoPs as oxidative stress markers.

Thirty days after exposure, pulmonary total F₂-IsoPs were increased by 56% and 43% in CdCl₂ and SiNPs-Cd groups, respectively, compared to controls (32.8±7.8 ng/g). Parallel elevation of free F₂-IsoPs was observed in plasma samples (by 113 % and 95% in CdCl₂ and SiNPs-Cd groups, respectively), compared to controls (28±8 pg/ml). These effects were already detectable at day 7 and lasted until day 30 post-exposure. Pulmonary SOD1-immunoreactivity was significantly enhanced in a time-dependent manner (7 days <30 days) after both CdCl₂ and SiNPs-Cd treatments with the latter causing more

pronounced effects. SiNPs did not influence SOD1-immunolabeling.

The results indicate the (i) potential of engineered SiNPs-Cd to cause long-lasting oxidative tissue injury following pulmonary exposure in rat and (ii) a possible role for plasma F₂-IsoPs measurement as a predictive indicator of nanoparticle-induced oxidative insult.

Keywords: In Vivo, Cadmium Nanoparticles, Nanotoxicology, Silica, Isoprostanes, Superoxide Dismutase, Rat

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1. Introduction

With the exponentially growing production of engineered nanoproducts, occupational respiratory exposure to nanoparticles is expected to increase, and many aspects related to the small size and biological effects of these agents have raised concerns about safety (Maynard et al., 2006). Toxic processes that may result from the interaction of natural or engineered nanoparticles with living systems are

linked to their high surface-to-volume ratio and enhanced reactivity with the biological system. Nanoparticles can be taken up actively by certain cell types (e.g., macrophages) and initiate formation of reactive oxygen and nitrogen species (ROS/NOS). If over- or chronically produced, these species can lead to increased transcription of proinflammatory mediators via intracellular signalling pathways, including calcium and oxidative stress pathways with consequent tissue changes and DNA, protein and lipid damage (Stone et al., 2007; Simkó et al., 2011). Several studies in animals have indicated a range of toxic effects that may be induced by nanoparticles such as oxidative stress, inflammation, granuloma formation, chronic pulmonary disease, immune system disorders, as well as increased risk of tumor development, and changes that may promote cardiovascular or neurodegenerative diseases (Donaldson et al., 2001; Oberdörster et al., 2005; Borm et al., 2006; Stern and McNeil, 2008; Hubbs et al., 2011; Liou et al., 2012). With respect to oxidative stress, development of specific, reliable and non-invasive testing methods for measuring this endpoint in humans may offer a valuable biomarker and in addition a research tool to elucidate the pathological role of free radicals *in vivo*. A list of representative biomarkers already used in clinical practice to evaluate the oxidative stress and inflammation outcomes is presented in Table 1.

Table 1. Proposed biomarkers of oxidative stress and inflammation

- | | |
|--|--|
| • Blood /plasma antioxidant enzyme activity:
Gpx-1, SOD, MPO (*)
(systemic inflammation) | |
| • Exhaled breath nitric oxide, serum Clara cell protein
(pulmonary inflammation) | |
| • Urine 8-hydroxydeoxyguanosine, plasma 8-hydroxydeoxyguanosine
(oxidative DNA damage) | |
| • Fibrinogen, ICAM-1, IL-6, C reactive protein
(cardiovascular system alterations, pro-thrombotic risk) | |

(*) Gpx1: glutathione peroxidase 1, SOD: copper-zinc superoxide dismutase, MPO: myeloperoxidase.

F₂-isoprostanes (F₂-IsoPs) are specific products of nonenzymatic lipid peroxidation. They include a series of prostaglandin F₂-like compounds which are generated by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid, through a cyclooxygenase-independent pathway (Milatovic et al., 2011). Initially formed *in situ* on phospholipids (esterified F₂-IsoPs), F₂-IsoPs are released into the circulation as free F₂-IsoPs and owing to their relatively low reactivity can be easily measured in biological samples (e.g. plasma, urine) as oxidative stress markers (Milne et al., 2005; Halliweel and Lee, 2010). F₂-IsoPs levels are found to be significantly increased, both in organs and body fluids, in diverse human disease conditions which share increased oxidative stress as a common pathological feature (Janssen,

2008). Recent studies in humans have suggested that F₂-IsoPs may represent a more accurate marker of oxidative stress compared with other available methods (Table 2).

Table 2. Characteristics of F₂-Isoprostanes as biomarkers of oxidative stress related to injury *in vivo*

- | |
|--|
| • specific products of lipid peroxidation |
| • well-known pathophysiological role |
| • rapidly released into the blood compartment after their <i>in situ</i> generation from phospholipids |
| • more stable than other lipid peroxidation products (e.g., lipoperoxides and aldehydes) |
| • production increased in several animal models of oxidant injury |

The main objective of this study was, thus, to evaluate the validity of the circulating F₂-IsoPs as marker of oxidative stress (Milatovic et al., 2011) associated to *in vivo* NPs exposure using a NP model namely silica nanoparticle (SiNPs) doped with cadmium (SiNPs-Cd). The response to SiNPs-Cd in terms of capacity to generate free radicals and induce oxidative stress was assessed in intratracheally (i.t.) treated rats by measuring lung and plasma levels of F₂-IsoPs, as marker of oxidative stress, and immunocytochemical analysis of pulmonary copper-zinc superoxide dismutase (Cu/Zn-SOD1 or SOD1), an antioxidant isozyme also involved in oxidative stress pathway. The effect of SiNPs-Cd was evaluated at 24 hr, 7 and 30 days post-exposure in comparison with that caused by administration of equivalent amounts of CdCl₂ and SiNPs.

Silica/cadmium containing nanomaterials are currently produced on an industrial scale for a variety of technological applications. Information on toxicity, exposure and health impact of these nanomaterials is still limited (Rzagalinski and Strobl, 2009; Vivero-Escoto et al., 2010). Dose-dependent cytotoxicity, increased reactive oxygen species levels and pro-inflammatory changes have been indicated by *in vitro* studies on SiNPs. A limited number of *in vivo* studies have demonstrated largely reversible lung inflammation, granuloma formation and focal emphysema, with no progressive lung fibrosis after respiratory exposure to these nanoparticles (Napieriska et al., 2010). There is also a large body of evidence indicating that cadmium is toxic to the respiratory tract when inhaled (Oberdörster et al., 1994). Although the mechanisms of cadmium toxicity are not yet fully understood, several reports have described pulmonary inflammatory changes and induction of oxidative stress in response to cadmium inhalation exposure (IARC, 1993).

2. Materials and methods

2.1. Nanoparticles

The method used to produce SiNPs-Cd was described in a previous report (Coccini et al., 2012a). The particles presented amorphous and crystalline structure (confirmed by X-ray diffraction analysis), spherical form, primary

particle size range of 20-80 nm and specific surface area of about 200 m²/g. Dynamic light scattering (DLS) determination of the SiNPs-Cd size distribution showed tendency to form aggregates and agglomerates of about 350 nm, and a zeta potential of - 23 mV (in deionized water). Flame-atomic absorption analysis was used to determine metal impurities and the release of cadmium from nanoparticles dispersed in physiological solution. Maximum cadmium release was 15% after 16 hr. Further metal release was negligible in the subsequent 10-day period. Main impurities were Ca (0.3%), Na (0.2%), K (0.2%), Fe (0.04%) and Mn (0.001%). Other metals were present in quantities less than 1 %. Cadmium and silica contents in the nanoparticles were 32.5% and 24.1%, respectively. CdCl₂ was purchased from Sigma Aldrich (Milan, Italy) and SiNPs from Degussa GmbH (Germany).

2. 2. Animal treatment

All experimental procedures were performed in compliance with the European Council Directive 86/609/EEC on the care and use of laboratory animals. Adult 12-weeks old male Sprague-Dawley rats (Charles River Italia, Calco, Italy) were allowed to acclimatize for at least 2 weeks before treatment, and kept in an artificial 12 h light:12 h dark cycle with humidity at 50±10% throughout the experiment. Animals were provided rat chow (4RF21 diet) and tap water *ad libitum*. Rats were anesthetized with pentobarbital sodium and treated with a single i.t. instillation of SiNPs-Cd (1 mg/rat, corresponding to about 250 µg Cd/rat). Separate groups of animals received i.t. an equivalent cadmium dose as CdCl₂ (400 µg Cd/rat), SiNPs (600 µg/rat) or 0.1 ml/rat of saline (control). Treatment groups consisted of 6 animals/group at each time point. SiNPs-Cd suspension was vortexed on ice just before the exposure to force nanoparticle dispersion and avoid formation of agglomerates. No surfactants or solvents were used.

Biochemical and immunohistochemical evaluations were performed 24 hr, 7 and 30 days after treatment. At each time point, rats were anesthetized by i.p. injection of 35% chloral hydrate (100 µl/100 g b.w.) and from a set of animals (n=3) lungs were removed and blood collected in heparinized tubes for the isoprostane analyses. The blood samples were centrifuged at 2400 g for 15 min at 4 °C; the platelet-poor plasma was saved and the buffy coat was removed by aspiration. The remaining set of animals (n=3) was used to examine SOD in lung tissue by immunocytochemistry after vascular perfusion of fixative. The trachea was cannulated, and laparotomy was performed. The pulmonary artery was cannulated via the ventricle, and an outflow cannula was inserted into the left atrium. In quick succession, the tracheal cannula was connected to about 7 cm H₂O pressure source to inflate the lungs with air, and clearing solution (saline with 100 U/ml heparin, 350 mosM sucrose) was perfused via the pulmonary artery. After blood was cleared from the lungs, the perfusate was switched to

fixative consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After fixation, the lungs were carefully removed.

2. 3. Oxidative stress evaluation

2.3.1. F₂-Isoprostanes (F₂-IsoPs) in plasma and lung

Free and total (sum of free plus esterified) F₂-IsoPs were determined by gas chromatography/tandem mass spectrometry (GC/NICI-MS/MS) in plasma and lung samples, respectively, as described by Signorini et al. (2003, 2009). In previous studies (Aldinucci et al., 2007; De Felice et al., 2009; Signorini et al., 2003, 2008, 2009; Soffler et al., 2010), GC/NICI-MS/MS has proved to be a reliable procedure (in term of specificity, repeatability and accuracy) to assess F₂-IsoPs as indicators of free radical-induced lipid peroxidation.

Plasma free, and lung total F₂-IsoPs were expressed as picograms per millilitre or nanograms per gram, respectively. The calibration curve correlations were adequate ($r^2 = 0.994$ for free F₂-IsoPs; and $r^2=0.9987$ for total F₂-IsoPs); accuracy was 97.8% (free F₂-IsoPs), and 98.5% (total F₂-IsoPs); variability coefficient were 2.5, and 2.2%, for free and total F₂-IsoPs, respectively. The minimum detection limit was 5 pg/ml.

2.3.2. SOD1 in lung

2.3.2.1 Lung sampling for immunocytochemistry

The top and the bottom regions of the right lungs were dissected. Tissue samples were obtained according to a stratified random sampling scheme which is a recommended method to reduce the variability and compensate for the existing regional differences in lung tissue (Weibel, 1979). From each slice, 2-3 blocks were systematically derived, washed in NaCl 0.9% and post-fixed by immersion for 7 hr in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), dehydrated through a graded series of ethanol and finally embedded in Paraplast. Eight micrometer thick sections of the samples were cut in the transversal plane and collected on silan-coated slides. To avoid possible staining differences due to small changes in the procedure, the reactions were carried out simultaneously on slides of control and treated animals at all stages.

2.3.2.2. Immunocytochemistry for SOD1

Immunocytochemistry was performed using commercial antibodies on rat lung specimens to localize presence and tissue distribution of the SOD1 isozyme, as a marker of inflammation-related oxidative stress. Lung sections of control and treated rats were incubated overnight at room temperature with the primary rabbit polyclonal antibody against SOD1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in PBS. Biotinylated anti-rabbit secondary antibody and an avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA, USA) were used to reveal the sites of antigen/antibody interaction. The 3,3'-diaminobenzidine tetrahydrochloride peroxidase substrate (Sigma, St. Louis, MO, USA) was used as

chromogen, and Haematoxylin was employed for nuclear counterstaining. Then, the sections were dehydrated in ethanol, cleared in xylene, and finally mounted in Eukitt (Kindler, Freiburg, Germany). As negative controls, some sections were incubated with phosphate-buffer saline in absence of the primary antibody. No immunoreactivity was observed in this condition.

2. 4. Statistical analysis

Isoprostane data were presented as means standard deviation (SD) for normally distributed variables. Differences between groups were evaluated using independent-sample t test (continuous normally distributed data). Two-tailed P values of less than 0.05 were considered significant. In all graphs, error bars represent the standard deviation of the mean.

Differential immunolabeling expression data were not normally distributed; therefore, the Kruskal-Wallis nonparametric test was used. Statistical significance is indicated with a * (P value < 0.05).

3. Results

3.1. Lung and plasma F₂-Isoprostanes

In lungs (Figure 1), total F₂-IsoPs levels were not modified at the earlier time points (24 hr and 7 days) after either treatments (CdCl₂ or SiNPs-Cd), while significantly increased levels of F₂-IsoPs were found after 30 days in both groups: F₂-isoprostane enhancements were 56% and 43% in CdCl₂ and SiNPs-Cd groups, respectively, compared to controls (32.8±7.8 ng/g).

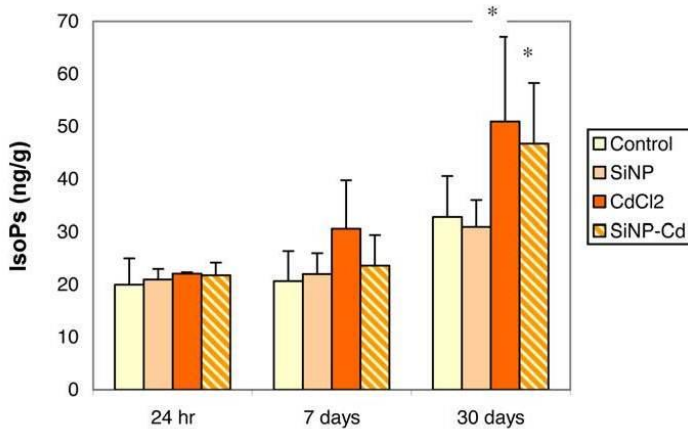


Fig. 1. Total F₂-Isoprostane levels in lungs (mean±SD)

As shown in Figure 2, increased free F₂-IsoPs levels were also observed in plasma samples. Changes in plasma F₂-IsoPs were already observable at 7 days (by 94.9% and 79.5% in CdCl₂ and SiNPs-Cd groups, respectively) and increases were still present at 30 days post-exposure (112.7% and 95.1% in CdCl₂ and SiNPs-Cd groups, respectively, compared to control, 28 ± 8 pg/ml).

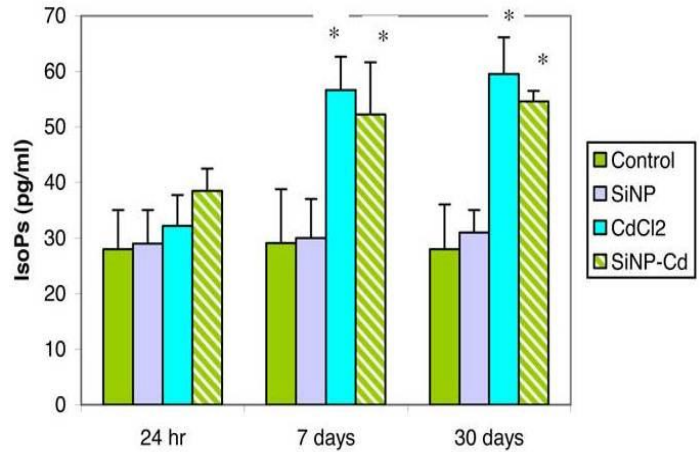


Fig. 2. Free F₂-Isoprostane levels in plasma (mean±SD)

At both 7 and 30 days post exposure, the lung and plasma F₂-IsoPs levels were not significantly different between CdCl₂ and SiNPs-Cd groups.

3.2. Pulmonary SOD1

SOD1-immunoreactivity was remarkably enhanced in a time-dependent manner in lung tissue samples after both CdCl₂ and SiNPs-Cd, with a more pronounced SOD1 immunoreactivity occurring at day 30 post treatment, especially in the animals given SiNPs-Cd (Figure 3). Administration of SiNPs caused no change in SOD1-immunolabeling compared to control rats (Table 3). Noticeably, numerous immunopositive activated macrophages were detected in the bronchiolar and alveolar epithelium of treated animals, particularly evident in collapsed areas, appearing heavily labelled. The localization and distribution of (Cu/Zn-SOD) SOD1, revealed an extensive spreading in bronchiolar and alveolar cells, evidencing the cellular response to injury, which was more intense for SiNPs-Cd observable starting at 7 days after i.t. exposure (Table 3).

Table 3. Expression of immunolabelling for SOD1 on a semiquantitative evaluation.

SOD1				
	24 h	7 days	30 days	p Value
<i>Control</i>	±	±	±	NS
<i>SiNP</i>	±	+	++	
<i>CdCl₂</i>	±	++	++	
<i>SiNP-Cd</i>	±	++	+++	

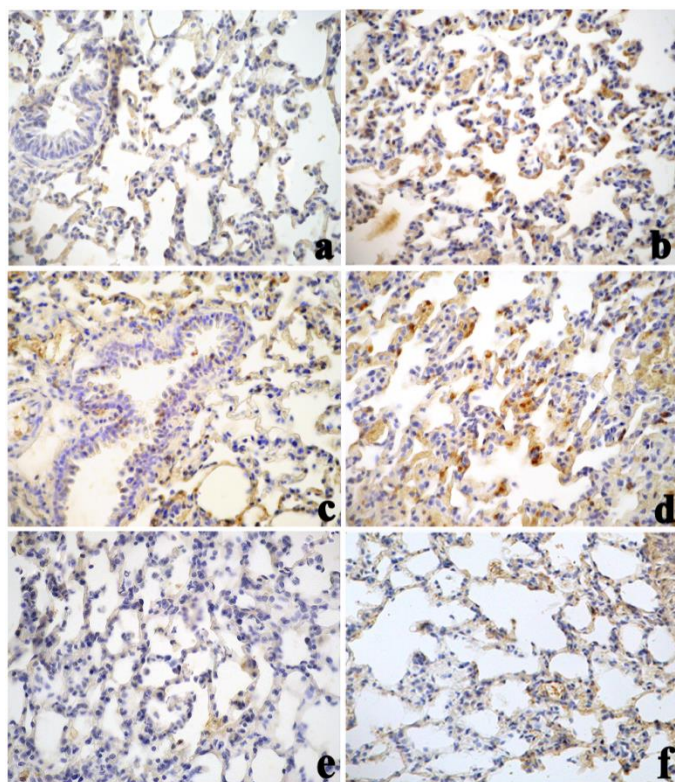


Fig. 3. Immunostaining pattern of SOD 1 expression in lung tissue in controls (a), 30 days after treatment with CdCl₂ (b), 7 and 30 days after treatment with SiNPs-Cd (c and d, respectively) or with SiNPs (e, and f, respectively). Numerous immunopositive activated macrophages were observed in the bronchiolar and alveolar epithelium. *Objective magnification: x 40 (a-d)*

Cadmium exposure has been shown to activate cell defence processes, such as metallothionein synthesis, increase in cellular glutathione levels, and activation of antioxidant transcription factor Nrf2 (Liu et al., 2009). SOD1 over-expression may thus represent an additional adaptive defence mechanism that is activated by cells in response to Cd-induced prooxidative insult.

4. Discussion

Many disease conditions are characterized by ROS accumulation and “oxidative stress” which are thought to contribute to the pathogenesis of a number of human diseases including lung pathology (Thannickal and Fanburg, 2000; Janssen, 2008). ROS producing lipid peroxidation and DNA damage have been implicated in cadmium toxicity (Liu et al., 2009). Recent investigations in rats exposed to cadmium indicated Cd-induced lipid peroxidation in several organs (Galazyn-Sidorczuk et al., 2011) and dose-dependent changes in several oxidative stress markers including F₂-IsoPs (Rogalska et al., 2009).

In our study, oxidative stress was observed in lung tissues and plasma after both CdCl₂ and SiNPs-Cd treatments. The toxic response was reflected by elevated levels of F₂-

IsoPs and pulmonary over-expression of SOD1. In SiNPs-Cd-treated animals, the pattern of changes in isoprostane levels was organ- and time-dependent: in lung, changes were not apparent until 4 weeks after dosing; in plasma, F₂-IsoPs levels were already increased at day 7 and were still enhanced 30 days after treatment. Similarly, elevated plasma F₂-IsoPs levels were found in rats 7 and 30 days after i.t. instillation of CdCl₂. These results indicated comparable oxidative stress response in lung tissues after administration of cadmium as inorganic metal or Cd-nanoparticles. Pulmonary changes were delayed in onset and were preceded by marked increase in F₂-IsoPs levels in plasma suggesting that peripheral plasma may be a sensitive target for Cd-induced lipid peroxidation and that plasma F₂-IsoPs may be early predictive indicators of later pulmonary oxidative insult.

In clinical practice, F₂-IsoPs measurements in plasma or urine have recently been applied as biomarkers to assess the severity of respiratory disorders including acute lung injury, chronic obstructive pulmonary disease, allergic asthma, adult respiratory distress syndrome, pulmonary arterial hypertension, interstitial lung disease, and cystic fibrosis. These biomarkers have also been investigated in non-respiratory disease states such as alcoholic liver disease, hepatorenal syndrome, acute cholestasis, ischemia/reperfusion injury, and diabetes (Basu et al., 2008; Comporti et al., 2008; Janssen, 2008; Halliwell and Lee, 2010). Notably, elevated F₂-IsoPs levels were reported in individuals exposed to environmental respiratory toxicants such as ozone, cigarette smoke, and allergens (Comporti et al., 2008; Janssen, 2008). In addition to their importance as indicator of oxidative damage, F₂-IsoPs can also exert intrinsic biological effects by interacting with tissue receptors involved in constriction of pulmonary vessels and airways (Kang et al., 1993; Comporti et al., 2008). F₂-IsoPs have been shown to exert biological action on platelets inducing shape changes thus altering the formation of thromboxane or irreversible aggregation in response to platelet agonist (Ting and Khasawneh, 2010; Milne et al., 2011). They can also cause vasoconstriction of renal arterioles, stimulation of DNA synthesis and cell proliferation on muscle vascular cells (Fukunaga, 1993) and endothelial cells (Yura, 1999). F₂-IsoPs seems also to mediate the increased production of transforming growth factor-β1 (TGF-β1) in kidney mesangial and glomerular cells exposed to high ambient glucose such as that produced by streptozotocin-induced diabetes (Montero, 2000).

It is possible that the inflammatory effects observed in the animals treated with the nanomaterials could at least in part be mediated by release of F₂-IsoPs secondary to oxidative stress. Presently, little is known about the time-course of lung tissue isoprostane accumulation in disease states and the correlation between changes in isoprostane levels and the onset/progression/regression of specific symptoms (Lakshminrusimha et al., 2006; Janssen, 2008).

Oxidative stress and inflammatory injury to airways are

distinctive processes associated with high-level exposure to nanoparticles (Stone et al., 2007; Li et al., 2008; Simkó et al., 2011; Shvedova et al., 2012). In some instances, the primary respiratory effect of inhaled nanoparticles was shown to extend to extra-pulmonary sites (i.e., blood, vascular endothelium, secondary target organs) due to migration of the nanoparticles from lung to the systemic circulation or to secondary organ changes caused by circulating inflammatory factors (e.g., IL-6, IP-10 and TGF- β 1) released from lung following local insult. Extra-pulmonary organ toxicity (e.g., nephrotoxicity) caused by inhaled nanoparticles have been documented in our previous studies on SiNPs-Cd (Coccini et al., 2011) and in experiments using carbon nanotubes (Reddy et al., 2010).

Additional issues considered in this study were those regarding the toxic potential of Cd-containing NPs compared to Cd salt and the effect of silica nanoparticles absorbed alone. Comparable oxidative stress responses occurred after administration of CdCl₂ or Cd-nanoparticles. In lung and plasma, F₂-IsoPs levels were similarly modulated in the two types of treatment, and the pulmonary expression of SOD1, an enzyme physiologically involved in oxidative stress protection, was also similarly increased in both SiNPs-Cd and CdCl₂ groups. The SOD1 changes were seen at the day 7 and were still observable 30 days post-exposure. On the other hand, toxicogenomic studies on oxidative stress pathways indicated remarkable differences in the gene expression profiles in rat lung and kidney after administration of SiNPs-Cd or equivalent amount of CdCl₂ (Coccini et al., 2011, 2012b).

SiNPs are generally considered to be non-toxic and are largely investigated as potential drug delivery systems (Mamaeva et al., 2012). However, experiments in cell cultures or animal models have indicated dose-dependent cytotoxicity, increased reactive oxygen species, and reversible lung inflammation after exposure to SiNPs (Park and Park, 2009; Napierska et al., 2010; Panas et al., 2012). In the present study, the response to SiNPs differed from that observed with SiNPs-Cd as the latter but not SiNPs were effective in modifying pulmonary SOD1 expression and tissue levels of isoprostanes.

In summary, delayed occurrence of pulmonary oxidative stress was observed after i.t. instillation of SiNPs-Cd in rats. The effects SiNPs-Cd on tissue isoprostanes and pulmonary SOD1 were comparable to the effects caused by CdCl₂. No changes involving these markers were observed in animals treated with SiNPs. These findings would suggest a primary role of the cadmium moiety in the biological response to SiNPs-Cd. However, it seems unlikely that the changes produced by SiNPs-Cd merely reflected the action of cadmium ions released from nanoparticles. Preliminary chemical assays with SiNPs-Cd have indicated limited release of cadmium ions from the nanoparticles dispersed in physiological solution, the maximum metal release being ca. 15% over a 10-day period. Gene modulated by SiNPs-Cd were different than those modulated by CdCl₂ at 30 days. The

overall genomic data showed only partially overlapping with CdCl₂ (Coccini et al., 2011). DLS experiments with the nanoparticles dispersed in physiological solution showed agglomeration and aggregation of SiNPs-Cd to extent greater than that found with SiNPs. The more pronounced tendency of SiNPs-Cd to form aggregates and agglomerates may offer an additional mechanistic explanation of our experimental findings.

The results provided an example on how measurements of F₂-IsoPs levels in plasma may be valuable as a predictive indicator of NP-induced oxidative lung damage. Further studies are needed to establish the validity of F₂-IsoPs measurements in field studies such as biomonitoring exposure to engineered nanomaterials in worksites and human safety assessment of nanomedicines.

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