Biomarkers for Pulmonary Effects Induced by In vivo Exposure to Cadmium-Doped Silica Nanoparticles

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Abstract

The study evaluated lung damages caused by in vivo exposure to silica nanoparticle doped with cadmium (SiNPs-Cd, 1 mg/rat) in terms of oxidative stress induction, apoptosis, and fibrosis, and assessed the validity of plasma F2-isoprostanes (F2-IsoPs) as marker of pulmonary insult. SiNPs-Cd effect was assessed 24 hr, 7 and 30 days post-intratracheal instillation compared to that caused by CdCl2 (400 µg/rat), or SiNPs (600 µg/rat) characterizing pulmonary superoxide dismutase (SOD1), cyclooxygenase type-2 (COX2) and collagen expression (by immunohistochemistry and TEM), and investigating apoptosis (TUNEL staining). Free and esterified F2-IsoPs were measured in lung and plasma by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis.

Lung: SiNPs-Cd induced enhancement of SOD1 and COX2 immunoreactivity in a time-dependent manner (7<30 days). Total F2-IsoPs also increased 30 days post-exposure (46.7±11 ng/g in SiNP-Cd vs. 32.8±7.8 ng/g in control). Parallely, apoptosis enhanced as following SiNP-Cd>CdCl2>SiNPs. A strong fibrotic response, i.e. interstitial type I collagen over-expression, was also observed starting at 7 days, particularly after SiNPs-Cd.

Plasma: Pronounced elevation of free F2-IsoPs occurred (54.6±2 vs. 28±8 pg/ml in SiNP-Cd and control, respectively) already at day 7 lasting until day 30. In SiNPs-treated animals no changes were observed on oxidative stress parameters. The CdCl2 pulmonary response was milder than that found with SiNPs-Cd.

The results indicate long-lasting tissue injury following SiNPs-Cd pulmonary exposure in rat and a role for plasma F2-IsoPs as a predictive indicator of nanoparticle-induced oxidative insult.

Keywords: In vivo; Cadmium nanoparticles; Nanotoxicology; Silica; Isoprostanes; Superoxide dismutase; Cyclooxygenase type 2; Apoptosis; Collagen; Rat

Introduction

The beneficial applications of nanotechnology in numerous industrial, consumer, and medical uses are extremely promising. However, the outlook for a future improved by nanotechnology is tempered by the fact that relatively little is known about the adverse effects of nanomaterials on human health. This is also aggravated by the large variety of engineered nanoparticles (NPs) increasingly entering the market and under development. With the exponential growing number of representatives, the potential for the respiratory system to be exposed to a seemingly countless number of unique NPs is expected to increase, and essentially most of these NPs has not been sufficiently examined for potential toxicity at this time [1,2].

NPs of different materials (e.g., gold, silica, titanium, carbon nanotubes, and quantum dots) possess exclusive physicochemical properties, thus having their own unique mechanism of toxicity. Therefore, determining the toxicity of nanomaterials is a fundamental question relating to their extremely small size, high surface area and increased surface reactivity (i.e., redox ability) as compared to larger materials [3,4]. However, it appeared that common responses could be detected, and the paradigm of the central role of oxidative stress was developed [4-9]. The uptake of NPs by target cells like macrophages plays a central role in the biological responses such as direct or indirect production of ROS (reactive oxygen species). The activation of pathways, nuclear factors and specific genetic programs depend directly or indirectly on the level of ROS production outside or inside the cell. Oxidative stress could lead to cell death by necrosis or apoptosis or adaptive responses including pro-inflammatory responses, antioxidant enzyme activations, repair processes effects on cell cycle control and proliferation.

Several studies in animals have indicated a range of toxic effects that may be induced by NPs 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 [10-15]. However, there are no epidemiologic data indicating health hazards for the majority of nanomaterials. Some NPs, such as nano sized metals should clearly represent risk factors for lung diseases, as many of these metals in their native form are known to have fibrogenic, inflammogenic or carcinogenic effects in humans.

Concerning 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. Among a number of representative biomarkers already used in clinical practice to evaluate the oxidative stress and inflammation outcomes [15,16],

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F₂-isoprostanes (F₂-IsoPs), specific products of non-enzymatic lipid peroxidation, are recently suggested to represent a more accurate marker of oxidative stress compared with other available methods [17-19]. 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 [20].

The objective of this study was to evaluate firstly the potential lung damage caused by in vivo NPs exposure using a NP model, namely silica nanoparticle (SiNPs) doped with cadmium (SiNPs-Cd), in terms of induction of oxidative stress, apoptosis, pro-inflammatory effects and fibrosis, and, additionally to assess the validity of the plasma F₂-IsoPs as marker of pulmonary insult [21] associated to in vivo NP exposure.

The SiNPs-Cd response in lung tissues of treated rats was assessed by determining: (i) the levels of F₂-IsoPs, which include a series of prostaglandin F₂-like compounds generated by free radical-catalyzed peroxidation of phospholipid-bound arachidonic acid through a cyclooxygenase-independent pathway, (ii) the occurrence of cell death, i.e. apoptosis, and (iii) the expressions of (i) copper–zinc superoxide dismutase (Cu/Zn-SOD1 or SOD1), an antioxidant isozyme involved in oxidative stress pathway, (ii) inducible enzyme cyclooxygenase type 2 (COX2), which synthesizes prostaglandin E₂ (PGE₂) under stimulation by oxidative stressors including cadmium [22], and (iii) type I Collagen. Moreover, F₂-IsoPs levels were parallelly measured in plasma samples. The effect of SiNPs-Cd was assessed at 24 hr, 7 and 30 days post-intratracheal (i.t.) instillation in comparison with that caused by administration of equivalent amount of CdCl₂ and SiNPs.

Although 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 [22,23]. In vitro studies on SiNPs indicated their capacity to induce dose-dependent cytotoxicity and pro-inflammatory changes, and to increase the reactive oxygen species levels. 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 NPs [24]. On the other hand a large body of evidence supports lung toxicity effects after cadmium exposure when inhaled [25]. 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 [26].

Materials and Methods

NP model: Silica nanoparticle (SiNPs) doped with cadmium (SiNPs-Cd)

The method used to produce SiNPs-Cd was described in a previous report [27]. 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 NPs 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 (0.014%) and Mn (0.001%). Other metals were present in quantities less than 1%. Cadmium and silica contents in the NPs were 32.5% and 24.1%, respectively. CdCl₂ was purchased from Sigma Aldrich (Milan, Italy) and SiNPs from Degussa GmbH (Germany).

In vivo study

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 acclimate for at least 2 weeks before treatment, and kept in an artificial 12 hr light:12 hr 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 NP dispersion and avoid formation of agglomerates. No surfactants or solvents were used.

Biochemical and immunohistochemical evaluations were performed 24 hr, 7 and 30 days post-instillation. At each time point, rats were anesthetized by i.p. injection of 35% chloral hydrate (100 µl/100 g b.w.) and divided into two sets (n=3 animals each). Set I: lungs were removed and blood collected in heparinized tubes for the isoprostane analyses, then 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. Set II: lung tissues were used to examine SOD1, COX2, collagen, and apoptosis by immunohistochemistry and TUNEL staining, respectively, after vascular perfusion of fixative [27]. After fixation, the lungs were carefully removed.

Evaluation of oxidative stress, pro-inflammatory effects and fibrosis

F₂-isoprostanes (F₂-IsoPs) in plasma and lung: Free and total (sum of free plus esterified) F₂-IsoPs were determined by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) in plasma and lung samples, respectively, as described by Signorini et al. [28,29]. In previous studies [28-32], 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 (r²=0.999 for free F₂-IsoPs; and r²=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.

SOD1, COX2, collagen in lung:

- Lung sampling and immunocytochemistry for SOD1, COX2, and type I collagen: 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 [33]. From each slice, 2-3 blocks were systematically derived, washed in NaCl 0.9% and post-fixed by immersion for 7 hrs.
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 micrometre thick sections of the samples were cut in the transversal plane and collected on silan-coated slides.

Subsequently, immunocytochemistry was performed using commercial antibodies on rat lung specimens to localize the presence and distribution of the SOD1, COX2 isozymes, and Collagen (Type I) as markers of inflammation-related oxidative stress and fibrosis, respectively.

The reactions were carried out simultaneously on slides of control and treated animals at all stages to avoid possible staining differences due to small changes in the procedure.

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, or the primary goat polyclonal antibody against COX2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:100 in PBS, or the primary rabbit polyclonal antibody against Collagen (Type I) (Chemicon, Temecula, CA, USA) diluted 1:400 in PBS.

Biotinylated anti-rabbit secondary antibody and an avidinbiotinylated 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 slides were dehydrated in ethanol, cleared in xylene, and finally mounted in Eukitt (Kindler, Freiburg, Germany). As negative controls, in some sections the TdT incubation was omitted; no staining was observed in these conditions.

The evaluation of TUNEL-cytochemically positive cells (TUNEL L.I.) was calculated as the percentage (Labelling Index) of a total number (about 500) of cells, for each animal and experimental condition, in a minimum of 10 randomly selected high-power microscopic fields. The slides were observed and scored with a bright-field Zeiss Axioscop Plus microscope. The images were recorded with an Olympus Camedia C-2000 Z digital camera and stored on a PC running Olympus software.

Electron microscopy: Lung fragments (small blocks of about 1 mm³) were fixed for 4 hr by immersion in ice cold 1.5% glutaraldehyde (Polysciences, Inc. Warrington, PA, USA) buffered with 0.07 M cacodylate buffer (pH 7.4), containing 7% sucrose, followed by post-fixation in OsO₄ (Sigma Chemical Co., St. Louis, MO, USA) in 0.1 M cacodylate buffer (pH 7.4) for 2 hr at 4°C, dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin section (about 60 Å thick) were cut from the blocks, mounted on uncoated 200-mesh-copper grids, and doubly stained with saturated uranyl acetate in 50% aceton and Reynolds’s lead citrate solution. The specimens were examined with a Zeiss EM 300 electron microscope operating at 80 kV.

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 SD of the mean.

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

Statistical analysis for TUNEL L.I. evaluation was performed by two-way analysis of variance (ANOVA) followed by the Bonferroni test.

Results

Lung and plasma F₃-Isoprostanes

In lungs (Figure 1A), total F₃-IsopPs 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 F₃-IsopPs were found after 30 days in both groups: F₃-Isopstanone enhancements were 56% and 43% in CdCl₂ and SiNPs-Cd groups, respectively, compared to controls (32.8 ± 7.8 ng/g).

Pronounced increases of free F₃-IsopPs levels were also observed in plasma samples (Figure 1B). Changes in plasma F₃-IsopPs 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). By comparison, the lung and plasma F₃-IsopPs levels were not significantly different between CdCl₂ and SiNPs-Cd groups, at both 7 and 30 days post-exposure.
After treatment with SiNPs (600 µg/rat, i.t.), the lung and plasma F2-IsoPs levels were similar to those observed in the control group at all time points considered (Figures 1A and 1B).

**Pulmonary SOD1 and COX2**

The localization and distribution of SOD1 and COX2, essentially involved in oxidative stress pathway, revealed an extensive spreading in bronchiolar and alveolar cells, as well as in the capillary component, evidencing the pulmonary reaction to the injury, already observable at 7 days after i.t. exposure, and lasting until 30 days (Table 1), with CdCl2 and SiNPs-Cd treatments causing adverse effects of comparable extent. SOD1- and COX2-immunoreactivity significantly enhanced in a time-dependent manner (7<30 days) (Figures 2a-d and 2e-h), after both CdCl2 (Figures 2b and 2f) and SiNPs-Cd (Figures 2c and 2g, respectively) with a more pronounced SOD1 immunoreactivity occurring at day 30 post treatment, especially in the animals given SiNPs-Cd. Administration of SiNPs did not significantly influence any of the two investigated molecules (Table 1; Figures 2d and 2h). The localization and distribution of SOD1, revealed an extensive spreading in bronchiolar and alveolar cells, evidencing the cellular response to the injury, and more intense for SiNPs-Cd observable starting at 7 days after i.t. exposure (Table 1); noticeably, numerous SOD1-immunopositive activated macrophages were detected particularly at 7 days after i.t. exposure (Table 1; Figures 2d and 2f), and SiNPs-Cd showing a significantly more marked effect (Figure 2m; Figures 2i). The immunolabelling changes between control and treated groups were evaluated using two-tailed t test and P value <0.05 was considered statistically significant (*).

| Time Points | SOD1 | COX2 | Collagen |
|-------------|------|------|----------|
|             |      |      |          |
| Control     | 24 hr| ±    | ±        |
|             | 7 days| ±    | *±       |
| 30 days     | ±    | *|±       |
| 24 hr       | ±    | *|±       |
| SiNPs-Cd    | 7 days| ++  | ++       |
| 30 days     | +±  | ++  | ++       |
| 24 hr       | −±  | ++  | ++       |
| CdCl2       | 7 days| +± | ++ |
| 30 days     | ++ | ++  |
| SiNPs       | 7 days| *  | *        |
| 30 days     | ±  | ++  |
| SiNPS-Cd    | * | *   | ++       |
| p Value     | CdCl2| * | * |
| SiNPs       | Ns | Ns  |

Degree of staining intensity: from undetectable (-) to strong (++++)

* p values calculated by Kruskal-Wallis test: (*) <0.05

ns: not statistically significant

**Table 1:** Expression of immunolabelling for SOD1, COX2 and Collagen (Type I) on a semiquantitative evaluation.

Immunopositive activated macrophages were detected particularly evident in collapsed areas, appearing heavily labelled (Figures 2h and 2c).

Regarding COX2-immunolabeling, a marked immunoreactivity was detected both at the capillary and bronchiolar levels (airway epithelium) particularly after SiNPs-Cd and CdCl2 (Figures 2g and 2f), in correlation with the alveolar collapsed status. The marked increase in SOD1 and COX2 immunolabelling, with different expression patterns in bronchiolar, alveolar and vascular epithelium, observed after either CdCl2 or SiNPs-Cd treatment (Table 1), is consistent with (i) the integral and pivotal role played by both SOD1 and the cytokine-inducible enzyme in pulmonary system, particularly in the development and progression of lung injury, and (ii) the known function of the respiratory epithelia as the first line of defence after insult, being also in accordance to previous in vitro and in vivo findings related to cadmium toxicity.

**Type I Collagen**

Immunohistochemistry for Type I collagen evidenced a fibrogenic reaction occurring 7 and 30 days after all types of treatment (Table 1) (Figure 2i). The immunolabelling changes between control and treated rats were characterized by diffuse collagen fibers deposition both in juxta-bronchiolar areas and within the alveolar walls (Figures 2l-2n), with SiNPs-Cd showing a significantly more marked effect (Figure 2m; Table 1). The latter finding was also supported by TEM analysis (Figure 3).

**Apoptosis**

Several apoptotic cells were observed after all types of treatment, with absence of necrotic tissues. The apoptotic phenomenon morphohistochemically characterized by TUNEL-positivity (Figures 4a-4d), and at ultrastructural level characterized by nuclear pyknosis, karyorrhexis, and apoptotic bodies formation (Figures 4e-4g, respectively), increased significantly after all three types of treatment more markedly at the early time point (24 hr) and persisting for 30 days (with tendency to decrease with time) (Figure 5). This apoptotic effects...
was more pronounced for SiNPs-Cd>CdCl$_2$>SiNPs.

**Discussion**

There is growing awareness that ROS accumulation and "oxidative stress" have been implicated in the etiology of a wide array of human diseases and clinical conditions, including lung pathology [20,34]. Several experimental investigations have demonstrated that ROS,
responsible for lipid peroxidation production and DNA damage, have been implicated in cadmium toxicity [35]. Recent data obtained in rats exposed to cadmium indicated Cd-induced lipid peroxidation in several organs [36] and dose-dependent changes in several oxidative stress markers including F2-IsoPs [37]. F2-IsoPs, initially formed in situ on phospholipids (esterified 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 [17,18].

The present study addressed the pulmonary effects of SiNPs-Cd versus CdCl2 and SiNPs linked to oxidative stress, pro-inflammatory response, apoptosis and fibrosis evaluated in rats at different time points post treatment (single i.t. exposure to 1 mg/rat, 400 μg/rat, and 600 μg/rat, respectively) as well as the identification of plasma biological markers of lung insult. The study approach intended to use Cd, for which the bulk of experimental and epidemiological data have abundantly addressed its toxicity till to define the limit value in the human exposure scenario [38], for evaluating the effects of engineered NPs namely cadmium-doped SiNPs. Specifically, Cd, a well-known pneumotoxicant, was applied as a tool to investigate its counterpart-doped nanoparticles in order to identify valuable biomarkers of lung injury. We employed a Cd dosage that is known to induce moderate lung insult evolving into chronic inflammation and fibrosis in rodents [39,40]. In particular, lung injury caused by CdCl2 given i.t. at the dose of 400 microg/rat (245 microg Cd) was shown to represent a good model of human interstitial lung disease [39]. Based on the procedure carried out for the SiNPs-Cd preparation (NP model), SiO2 was dispersed in concentration ratios which produces a sample containing 40% Cd by weight, thus 600 microg was the nominal content of SiO2 in 1 mg of instilled dose of SiNP-Cd sample. The two different type of NPs, namely SiNPs and SiNPs-Cd, contained a comparable amount of silica, i.e. 600 microg.

The findings clearly demonstrated that SiNPs-Cd produce undesirable effects on several lung parameters involved in the above biological pathways. Specifically, both CdCl2 and SiNPs-Cd i.t. instilled caused oxidative stress: elevated levels of F2-IsoPs and pulmonary over-expression of SOD1 and COX-2, extensively spread out in bronchiolar and alveolar cells, as well as in the vascular component, were detected as overt signs of toxic response. Parallely, the apoptotic phenomena enhanced as follows, SiNPs-Cd>CdCl2>SiNPs. A strong stromal fibrotic response was also observed in a delayed manner, starting to manifest at 7 days post i.t., particularly after SiNPs-Cd, as clearly demonstrated by interstitial type I collagen over-expression, mainly detected in the juxta-bronchiolar area and within the alveolar wall.

Furthermore, at plasma level, the F2-IsoPs levels were significantly modified by both SiNPs-Cd and CdCl2.

In SiNPs-Cd-treated rats, 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 CdCl2 instillation. 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, hepatoportal syndrome, acute cholestasis, ischemia/reperfusion injury, and diabetes [18,20,41,42]. Elevated F2-IsoPs levels were reported in individuals exposed to environmental respiratory toxicants such as ozone, cigarette smoke, and allergens [20,42]. 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 [42,43]. Several reports suggest that F2-IsoPs also exert biological action on platelets inducing shape changes thus altering the formation of thromboxane or irreversible aggregation in response to platelet agonist [17,44]. They can also cause vasoconstriction of renal arterioles, stimulation of DNA synthesis and cell proliferation on muscle vascular cells [45] and endothelial cells [46]. F2-IsoPs seem also to mediate the increased production of transforming growth factor-beta1 (TGF-β1) in kidney mesangial and glomerular cells exposed to high ambient glucose such as that produced by streptozotocin-induced diabetes [47].

With regard to our investigation, it is likely that F2-IsoPs level that we determined in our model, may have contributed to the observed lung insult. We could assume a chain mechanism in which the nanomaterial induced production of ROS, with subsequent production of F2-IsoPs, which in turn amplified the responses of cellular adaptation. It is also to be considered that the half-life of F2-IsoPs is of few minutes [48], and that plasma F2-IsoPs levels normalize when the oxidative insult finish [48,49]. Concerning to the present study, the nanomaterial is probably not completely cleared; it is kept into the cells, representing a continuous stimulus to the production of F2-IsoPs. Therefore, in this particular condition, it is very difficult to discriminate the role of the nanomaterial or F2-IsoPs to the final phenotype. 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 [20,50].

In our study, the increased expression of SOD1, critically involved in cellular protection against oxidative stress, was detected in lung tissues after both SiNPs-Cd and CdCl2, with a similar extent, starting at 7 days after treatment and still observable at day 30, mainly localized at bronchiolar and alveolar levels, e.g. in activated macrophages. Based on the notion that Cd tends to induce metallothionein, increase cellular glutathione levels, and activate antioxidant transcription factor Nrf2 [35], we can assume that SOD1 over-expression may represent a possible adaptive cellular defence mechanism in response to Cd-induced pro-oxidative insult. Similarly to SOD1, COX2, highly expressed at day 7 until day 30 in lungs of both SiNPs-Cd- and CdCl2-treated animals, was found to be over-expressed particularly in vascular and bronchiolar epithelia, accompanied by an evident alveolar collapsed status. COX2, critically involved in pathogenesis of lung diseases [51], is usually expressed at minimal levels under normal conditions [52], while is elevated during inflammation catalyzing prostaglandin synthesis (i.e. PGE2). Particularly, COX2, also induced by NF-kB-mediated oxidative stress (Nuclear Factor-KappaB) [53], was shown to up-regulate different pulmonary cell types including endothelial and inflammatory cells [54,55]. Several in vitro and in vivo findings have shown a specific rise in COX2 expressions after exposure.
to Cd [56,57], as well as in different models of oxidative stress in vivo [58,59]. Many studies associate oxidative stress with inflammation, a relationship that has also been described for Cd-induced pulmonary inflammation [60,61].

Moreover, oxidative stress and inflammatory injury to airways are distinctive processes associated with high-level exposure to NPs [8,9,62,63]. In some instances, the primary respiratory effect of inhaled NPs was shown to extend to extra-pulmonary sites (i.e., blood, vascular endothelium, secondary target organs) due to migration of the NPs from lung to the systemic circulation or to secondary organ changes caused by circulating inflammatory factors (e.g., IL-6, IP-10 and TGF-ß1) released from lung following local insult [64,65].

Accordingly, our recent in vivo investigation indicated that not only CdCl₂ but even SiNPs-Cd cause lung damage characterized by morphoarchitectural alterations as well as the occurrence of inflammation, in terms of expression changes of several molecules, e.g., different cytokines/chemokines and metabolic factors (IL-6, IP-10, and TGFß1). These effects were observed acutely (24 h after i.t.) and lasted until the 30th day, with the SiNPs-Cd treatment producing a more marked effect compared to SiNPs and CdCl₂ [27].

In the present study, we also reported a diffuse fibrotic response, characterized by a gradual increased production and deposition of extracellular matrix in a time-dependent manner (starting to be manifest at 7 days post i.t.), underlying the role of interstitial type I collagen overexpression in creating abnormal spatial organization of the alveolar septa at different temporal stages.

Furthermore, we demonstrated an enhanced apoptotic phenomena induced by both CdCl₂ and SiNPs-Cd exposure for which an oxidative stress response was also detected. These findings are in line with previous literature data documenting the inflammation-mediated oxidative damage following NP exposure also remarking that oxidative stress may act as a critical mechanism that links inflammation, excessive oxidative damage following NP exposure also stressing the higher reactivity of SiNPs-Cd (regardless of whether form type is present: original, agglomerate, or with sorbed material at NP surface) compared to SiNPs. Moreover, transcriptional studies indicated that in lung, most of the genes modulated by SiNPs-Cd were different than those modulated by CdCl₂ at 30 days [64]. The overall genomic data showed a complex regulation induced by SiNPs-Cd, only partially overlapping with those of CdCl₂.

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. Such molecular-level biomarkers of NP exposure can provide a mechanistic basis for NP induced changes in biological structure and function. At the present time, few biomarkers have been identified, however. The present finding may give an important contribute in the field of biomarkers of effects in which methods of detecting sub-lethal changes in biological structure and function in response to nanomaterial exposures are still in their infancy.

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