Research Article

***In Vitro* Toxicity Evaluation of Engineered Cadmium-Coated Silica Nanoparticles on Human Pulmonary Cells**

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Cytotoxicity of cadmium-containing silica nanoparticles Cd-SiO₂NPs (0.05–100 µg/mL) versus SiO₂NPs and CdCl₂ was evaluated by an *in vitro* test battery in A549 by assessing (i) mitochondrial function, (ii) membrane integrity/cell morphology, (iii) cell growth/proliferation, (iv) apoptotic pathway, (v) oxidative stress, after short- (24–48 h) and long-term (10 days) exposure. Both Cd-SiO₂NPs and CdCl₂ produced dose-dependent cytotoxic effects: (i) *MTT-assay*: similar cytotoxicity pattern was observed at both 24 and 48 h, with a more Cd-SiO₂NPs pronounced effect than CdCl₂. Cd-SiO₂NPs induced mortality (about 50%) at 1 µg/mL, CdCl₂ at 25 µg/mL; (ii) *calcein-AM/PI staining*: decrease in cell viability, noticeable at 25 µg/mL, enhanced markedly at 50 and 100 µg/mL, after 24 h. Cd-SiO₂NPs induced higher mortality than CdCl₂ (25% versus 4%, resp., at 25 µg/mL) with further exacerbation after 48h; (iii) *clonogenic assay*: exposure for longer period (10 days) compromised the A549 proliferative capacity at very low dose (0.05 µg/mL); (iv) a progressive activation of *caspase-3 immunolabelling* was detected already at 1 µg/mL; (v) GSH intracellular level was modified by all compounds. In summary, *in vitro* data demonstrated that both Cd-SiO₂NPs and CdCl₂ affected all investigated endpoints, more markedly after Cd-SiO₂NPs, while SiO₂NPs influenced GSH only.

1. Introduction

The rapid development of nanotechnology worldwide is accompanied by massive generation and usage of engineered nanoparticles (ENPs), even though essentially most of these NPs have not been sufficiently examined for potential toxicity at this time [1, 2]. Thus, with the exponential growing production of ENPs, the potential for the respiratory system to be exposed to a seemingly countless number of unique NPs is expected to increase, and many aspects related to the size of these nanomaterials, smaller than cells and cellular organelles, have raised concerns about safety [2–4].

Among ENPs, silica/cadmium containing nanomaterials have attracted much attention in the latest years for their applications in medicine and industrial manufacturing, synthesis, and engineering [5–10]. Though silica nanoparticles

(SiO₂NPs) are generally considered to be nontoxic, experiments using cell cultures or animal models have indicated dose-dependent cytotoxicity, increased reactive oxygen species, and reversible lung inflammation [11–19]. On the other hand, a large body of evidence supports lung toxicity effects after cadmium exposure when inhaled [20, 21], and although its toxicity mechanisms are not yet fully understood, several reports have described pulmonary inflammatory changes and induction of oxidative stress in response to cadmium inhalation exposure [22].

Some NPs, such as metal-based nanomaterials should 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. Evaluation of the NPs biosafety is essentially required by tests examining general toxicity, target organ toxicity, and biocompatibility in

line with regulatory requirements and to identify molecular endpoints and multiple toxicity pathways.

The present study intended to elucidate the toxicological profile of a model nanomaterial namely cadmium-containing silica nanoparticles (Cd-SiO₂NPs) by an *in vitro* testing approach.

For instance, a key concept, developed from the strategy proposed by major institutions [23, 24] and international consensus meetings [25], indicates the use of multi-tiered testing protocols to address toxicological research and health risk assessment for NPs, based on (i) physico-chemical characterization, (ii) *in vitro* models by a battery of cytotoxicity tests, and (iii) *in vivo* experiments driven on the basis of the *in vitro* results. Information generated using *in vivo* studies will also provide a database from which to compare *in vitro* studies for identifying additional evidence that assists in explaining findings from *in vivo* nanomaterial toxicity or health effects. Comparing the *in vitro* and *in vivo* results may also help to assess the concordance/discordance between the alternative methods and the *in vivo* methods, and to test the predictability of the alternative methods for the *in vivo* results [26].

The identification of predictive *in vitro* toxicity assays is in line with the recommended attention that should be given to regulatory acceptance as means of promoting the use of alternative methods to animal testing in human safety assessment [27, 28].

Our recent *in vivo* investigation on Cd-SiO₂NPs indicated long-lasting lung damage, after intratracheal instillation (i.t.) of these nanoparticles in rats, characterized by morphological alterations, the occurrence of inflammation (accompanied by granuloma formation), stromal fibrogenic reaction, and enhancement of apoptotic phenomena followed by a consequent increased cell proliferation [29]. This pulmonary insult was also associated with an oxidative stress response [30].

In this work, a battery of *in vitro* tests have been used to examine the responses of human lung epithelial cells to Cd-SiO₂NPs exposure in terms of metabolic activity (by MTT assay), membrane integrity (by calcein-AM/Propidium Iodide staining), oxidative stress (by GSH content evaluation), apoptosis (by activated caspase-3 evaluation), and growth and cell proliferation (by clonogenic assay) to determine whether the modification of morphological and biochemical parameters evaluated by *in vitro* investigations are in accordance with *in vivo* pulmonary insult caused by these nanoparticles. The effects of Cd-SiO₂NPs were assessed in A549 cell line, which represents a widely used cell model to investigate alveolar cell function [31], after short- (24–48 hours) and long-term (10 days) exposure and compared with those caused by treatments with cadmium chloride (CdCl₂) and SiO₂NPs.

2. Materials and Methods

2.1. Chemicals. All cell culture reagents, chemicals, and cadmium chloride hemi (pentahydrate; CdCl₂) were obtained from Sigma-Aldrich (Milan, Italy). Caspase 3 and Alexa 488-labeled antibodies from Molecular Probes (Life Technologies, Monza, Italy) and the GSH quantification Kit from Oxis

International Inc. (Foster City, Ca, USA). Silica nanosize (SiO₂NP) was purchased from Degussa GmbH (Germany) as HiSil™ T700, average pore size 20 nm, surface area 240 m²/g, and pore specific volume of 0.4 cm³/g.

2.2. Synthesis and Physico-Chemical Characterization of Engineered Cadmium-Coating Nanoparticles (Cd-SiO₂NPs). Synthesis and physico-chemical characterization of Cd-SiNPs are previously described in Coccini et al. [29]. Briefly, Cd-SiO₂NPs were produced by the impregnation of SiO₂NPs with cadmium nitrate dehydrate (Cd(NO₃)₂ · 3.56 × 10⁻² M) in an aqueous solution with silica dispersed in a concentration ratio leading to a sample containing 40% Cd by weight. Powder was later subjected to grinding mills with high energy (200 rpm for 1.5 h, 400 rpm for 1.5 h, 600 rpm for 2 h) to get the most equal distribution of particle size and/or aggregates of particles. The entire synthesis preparation was performed under sterile condition to avoid NPs contamination.

Quantitative analyses by scanning transmission electron microscopy (STEM) showed the aggregation of Cd-SiO₂NPs and the analysis of the elements in High Angle Annular Dark Field (HAADF) mode (energy-dispersive (EDS) spectra) confirmed the presence of Cd, Si, and O. X-ray diffraction demonstrated amorphous and crystalline phases of the sample. Dynamic light scattering (DLS) of the Cd-SiO₂NPs showed tendency to form aggregates and agglomerates of about 350 nm (zeta potential about -23 mV in DMEM). Particles presented spherical form, primary particle size range of 20–80 nm and specific surface area of about 200 m²/g. Metal impurities (Ca (0.3%), Na (0.2%), K (0.2%), Fe (0.04%), and Mn (0.001%)) and the release of cadmium from nanoparticles dispersed in culture medium were determined by flame-atomic absorption analysis. Maximum cadmium release in culture medium (DMEM) was 28% after 16 h, and it was negligible in the subsequent 10-day period.

2.3. Cell Line and Cell Culture. Human lung epithelial cells (A549 cell line purchased from ECACC, Sigma-Aldrich, Milan, Italy) were used for *in vitro* study of the nanoparticle toxicity. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin. Cells were maintained at 37°C in a humidified atmosphere (95% air/5% CO₂).

Stock solutions were prepared by dissolving test materials (Cd-SiO₂NPs, CdCl₂, and SiO₂NPs) in culture medium (DMEM), then cells were exposed to concentrations ranging from 0.05 to 100 µg/mL. Fresh solutions of test materials were prepared shortly before each experiment.

Doses were chosen based on previous experiments in A549 cells showing toxic effects (e.g., apoptosis, necrosis) after cell exposure to concentrations ranging from 5 to 60 µM (corresponding: 0.916 to 10.99 µg/mL) of CdCl₂ [32–35].

2.4. Cytotoxicity Study: Short-Term Exposure (24–48 h)

2.4.1. Mitochondrial Function (MTT Assay) and Membrane Integrity (Calcein-AM/Propidium Iodide Staining). The viability was assessed by two dye-based methods: MTT assay (mitochondrial function) and calcein-AM/Propidium Iodide

(PI) staining (membrane integrity). Cells were seeded in 96-well plates at density of 1×10^4 cells/well in complete medium. After 24 h of cell attachment, the cells were exposed to Cd-SiO₂NPs at final concentration of Cd between 1 to 100 $\mu\text{g/mL}$ for 24 or 48 h at 37°C and compared to equivalent amount of CdCl₂ or SiO₂NPs.

At the end of the incubation period, the mitochondrial function was assessed by 0.5 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) for 3 h at 37°C and was quantified spectrophotometrically at 550 nm in Biorad microplate reader. Data were expressed as a percentage of control (untreated cells).

The membrane integrity was evaluated by the co-incubation of the double staining: 2 μM calcein-AM and 2.5 $\mu\text{g/mL}$ PI for 5 min at 37°C. Cells were examined under a Zeiss Axiovert 25 fluorescence microscope combined with a digital camera (Canon powershot G8). The fluorescence images were taken using 32x objective lens with an excitation wavelength of 400, 495, 570 nm; beamsplitter wavelength of 410, 505, 585 nm; and an emission wavelength of 460, 530, 610 nm. Viability was expressed as percent cells retained calcein compared to the total cells counted (calcein-positive plus PI-positive).

2.4.2. Oxidative Stress Evaluation: Glutathione (GSH) Measurement. The concentration of intracellular GSH was determined by colorimetric assay. Briefly, cells were seeded in six-well plates at density of 5×10^5 cells/well. After the treatments with 2 mL of Cd-SiO₂NPs, CdCl₂ and SiO₂NPs (final concentration ranging from 1 to 100 $\mu\text{g/mL}$ in cell culture medium) for 24 and 48 h exposure, the medium was aspirated and the cells were washed once with phosphate buffer saline (PBS). Then, the cells were scraped and centrifuged at 1100 rpm for 3 min at 25°C, the supernatant was removed by aspiration. The cell pellets were resuspended in ice-cold metaphosphoric acid (MPA) and immediately homogenized (Ultra Turrax, Janke & Kunkel) then centrifuged at 3000 g, 4°C for 10 min. Subsequently, the samples were mixed with 4-Chloro-1-Methyl-7-Trifluoromethyl-Quinolinium Methylsulfate and 30% sodium hydroxide reagents, and then were incubated for 10 min at room temperature (r.t.) in dark. The absorbance was measured spectrophotometrically (Spectrometer Lambda Bio 20, Perkin Elmer) at 400 nm, total glutathione content was determined with a standard curve.

2.4.3. Apoptotic Pathway: Immunofluorescence Detection of Activated Caspase 3. Cells were seeded in coverslips at density of 2×10^5 cells. After 24-h cell attachment, the cells were exposed to increasing concentrations of Cd-SiO₂NPs, CdCl₂, and SiO₂NPs (1–50 $\mu\text{g/mL}$) for 24 h at 37°C. At the end of incubation period, the cells were fixed with 4% paraformaldehyde for 20 min at r.t. and then in 70% ethanol over night at –20°C. After rehydration with PBS, the samples were incubated with blocking solution for 30 min at r.t., and then with polyclonal antibodies recognizing caspase 3 (dilution 1:200 in PBS). After washing, the bound antibodies were revealed with Alexa 488-labeled (dilution 1:100 in PBS) antibody recognizing rabbit-Ig. The slides were counterstained for DNA with 1 $\mu\text{g/mL}$ Propidium Iodide, washed with PBS

and finally mounted with Fluoroshield. Cells were examined under a CX41 Olympus fluorescence microscope, excitation light being provided by EPI LED Cassette (FRAEN, Settimo Milanese (MI), Italy) and combined with digital camera (Infinity2). Digital images were captured using 100x objective lens, measurement conditions were: 470 nm excitation ($T\% = 40$), 505 nm dichroic beamsplitter, 510 nm long pass filter.

2.5. Cytotoxicity Study: Long-Term Exposure (10 Days)

2.5.1. Clonogenic Assay. The procedure for clonogenic assay was adopted from Herzog et al. [36]. A549 cells were seeded in six-well at density of 400 cells/well, each well containing 2 mL of cell culture medium. After attachment (about 14 h, time was shorter than the population doubling time), the cells were washed with 2 mL PBS, and treated with 2 mL of Cd-SiO₂NPs, CdCl₂, and SiO₂NPs (final concentration ranging from 0.05 to 100 $\mu\text{g/mL}$ in cell culture medium) over a time period required to form colonies (about 10 days). A colony being defined as at least 50 clones of one cell. At the end of the treatment, the medium was removed and the colonies were fixed, stained with Hematoxylin and then counted for the evaluation of cell survival after Cd-SiO₂NP, CdCl₂ and SiO₂NP treatments. The colonies were examined under Zeiss Axiovert 25 microscope combined with a digital camera (Canon powershot G8).

Digital photographs were taken from each well using 2.5x objective lens. The number of colonies that arose after treatment (surviving fraction) was expressed in terms of plating efficiency (PE). PE was calculated by dividing the number of colonies formed by the number of cells plated per 100.

2.6. Statistics. Data from acute exposure were obtained from three independent experiments each carried out in six replicates. Data from chronic exposure were obtained from two independent experiments and each experiment was carried out in three replicates. Results are expressed as mean $\pm$ SD. Statistical significance was assessed by one-way ANOVA. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Cytotoxic Activity of Cd-SiO₂NPs Compared to CdCl₂ and SiO₂NPs in A549 Cell Line. *In vitro* cytotoxicity results after short- (24–48 h) and long-term (10 days) exposure of A549 cells to increasing concentrations of Cd-SiO₂NPs, CdCl₂, and SiO₂NPs (from 0.05 to 100 $\mu\text{g/mL}$) are reported and compared. Mitochondrial function, membrane integrity, oxidative stress, apoptosis were considered as endpoints of acute exposure, while the capacity to form colonies was considered as endpoint of chronic exposure.

3.2. Cytotoxicity Results after Short-Term Exposure (24–48 h)

3.2.1. Mitochondrial Function: MTT Assay. Data of mitochondrial function, evaluated by MTT after 24 and 48 h of exposure to increasing concentrations of Cd-SiO₂NPs,

FIGURE 1: Cell viability measured by MTT assay in A549 cells exposed to increasing concentration (1–100 $\mu\text{g/mL}$) of Cd-SiO₂NPs (—□—), CdCl₂ (---△---), and SiO₂NPs (—◆—) after 24 (a) and 48 h (b). Data are mean $\pm$ DS of three separate experiments each carried out in six replicates. Error bars: $\pm$ SD.

CdCl₂, or SiO₂NPs (1–100 $\mu\text{g/mL}$) and expressed as percentage of the viability of control, are presented in Figures 1(a) and 1(b). Both Cd-SiO₂NPs and CdCl₂ produced a dose-dependent cytotoxic effect on A549 cells. The pattern of cytotoxicity was similar at both time points (i.e., 24 and 48 h) for either compound, but Cd-SiO₂NP cytotoxicity was more pronounced compared to CdCl₂. Cd-SiO₂NPs induced mortality (about 40% and 50% after 24 and 48 h exposure resp.) already at the lowest dose (1 $\mu\text{g/mL}$) (Figures 1(a) and 1(b)). The cytotoxic effect of CdCl₂ treatment was detected at 25 $\mu\text{g/mL}$ with about 20% mortality after 24 h (Figure 1(a)) and 45% after 48 h exposure (Figure 1(b)). The maximum effect (about 80% mortality) of the two tested materials reached at the highest dose (100 $\mu\text{g/mL}$) after 48 h exposure (Figure 1(b)).

As graphically represented, in Figures 1(a) and 1(b) (see black line), SiO₂NPs did not show any significant cytotoxic effect after both time points considered (24 and 48 h).

3.2.2. Membrane Integrity: Calcein-AM/PI Staining. Membrane integrity was evaluated by calcein-AM/PI staining after 24 and 48 h exposure to the compounds. Figures 2(a), 2(b), and 2(c) describe a panel of representative and randomly selected microscopic fields of A549 cells treated with increasing concentrations of Cd-SiO₂NPs, CdCl₂, and SiO₂NPs (1–100 $\mu\text{g/mL}$) after 24 h.

Calcein-AM/PI staining indicated cytotoxic effects. A similar dose-dependent cytotoxic effect was observed after both Cd-SiO₂NPs and CdCl₂ treatments (Figures 2(a) and 2(b)). A decrease in cell viability was observed as evidenced by the presence of numerous red coloured cells (indicating damage to the cell membrane), starting at 25 $\mu\text{g/mL}$ dose and becoming markedly evident at the highest concentrations of 50 and 100 $\mu\text{g/mL}$ (Figures 2(a) and 2(b)). Semi-quantitative analysis of selected microscopic fields, after 24 h exposure, in terms of cell counts and expressed as percentage of live cells

(green fluorescence), showed difference in cell death between Cd-SiO₂NPs and CdCl₂ treatments at dose of 25 $\mu\text{g/mL}$. Cd-SiO₂NPs mortality was higher than that caused by CdCl₂ treatment (about 25% versus 4%). In both Cd-SiO₂NP and CdCl₂ groups treated with the lowest dose (1 $\mu\text{g/mL}$) the green fluorescence was uniformly diffused in cell cytoplasm (indicating the maintained membrane integrity), and cell morphology was not altered (Figures 2(a) and 2(b)).

The effects of Cd-SiO₂NPs and CdCl₂ were exacerbated after 48 h exposure. Cell viability was decreased of about 60–100% at doses ranging 25–100 $\mu\text{g/mL}$ (data not shown).

Images obtained from SiO₂NP treatment showed uniformly diffused green fluorescence and normal cell morphology for all treatment concentrations (1–100 $\mu\text{g/mL}$) when compared to control (Figure 2(c)). Semi-quantitative analysis with increasing concentrations of SiO₂NP treatment (1–100 $\mu\text{g/mL}$) showed no effects on A549 cells even at the highest dose investigated of 100 $\mu\text{g/mL}$ and after both 24 (Figure 2(c)) and 48 h (data not shown).

3.2.3. Oxidative Stress: Evaluation of GSH Intracellular. GSH levels were affected by all test materials at both time points considered (24–48 h). The reduction of intracellular GSH levels caused by Cd-SiO₂NP or CdCl₂ treatments was not dose-dependent: the GSH depletion was ranging from 35 to 40% and from 35 to 45% for Cd-SiO₂NPs and CdCl₂, respectively, after 48 h exposure (Figure 3). SiO₂NPs showed dose-dependent depletion of GSH in cells when compared to control with about 55% decrease of GSH intracellular after 48 h exposure at the highest concentration investigated (100 $\mu\text{g/mL}$; Figure 3).

3.2.4. Apoptotic Pathway: Immunofluorescence Analysis of Activated Caspase 3. Figure 4 displays a panel of representative randomly selected microscopic fields of A549

FIGURE 2: Representative images of randomly selected microscopic fields of A549 cells stained with calcein-AM/PI after 24 h exposure to increasing concentration (1–100 μg/mL) of Cd-SiO₂NPs (a), CdCl₂ (b), and SiO₂NPs (c). Dose-dependent cytotoxic effect in both Cd-SiO₂NP and CdCl₂ treatment groups: there was a strong decrease of viability at higher concentrations ranging from 50 to 100 μg/mL (low or no green fluorescence and red fluorescence indicating cell death). SiO₂NPs treated cells showed uniformly diffused green fluorescence at all tested doses similarly to cell controls. Quantitative analysis of the cell loss is shown for each treatment (Green Square Cell Live; Red Square Cell Death). Data are mean ± DS of three separate experiments, error bars: ±SD. (Scale bar: 100 μm).

cells treated with increasing concentrations of Cd-SiO₂NPs, CdCl₂, and SiO₂NPs (1–50 μg/mL). A549 cells exposed to Cd-SiO₂NPs or CdCl₂ showed a progressive activation of caspase 3 as brilliant green intracellular spots (Figures 4(a)–4(d) and Figures 4(e)–4(h), resp.) directly observed with the lowest dose tested of 1 μg/mL. To note, the advanced stage of apoptosis were observed at the highest dose of 50 μg/mL Cd for both Cd-SiO₂NP or CdCl₂ treatments (Figures 4(a)–4(d) and Figures 4(e)–4(h), resp.). In addition, a decrease in cell number along with a marked alteration of the morphology can be easily appreciated. On the contrary, the immunocytochemistry analysis to detect activated caspase 3 after 24 h exposure demonstrated no positivity for cells treated with SiO₂NPs after any tested doses (1–50 μg/mL;

Figures 4(i)–4(l)) and no appreciable morphology alterations compared to control.

3.3. Cytotoxicity Results after Long-Term Exposure (10 Days)

3.3.1. Clonogenic Assay. To determine whether the prolonged exposure (up to 10 days) to increasing concentrations (0.05–100 μg/mL) of Cd-SiO₂NPs, CdCl₂, and SiO₂NPs might have adverse effects, the proliferation ability and colony forming capacity of A549 cells were evaluated. Figure 5 shows representative images of randomly selected microscopic fields of the different treatment groups (Cd-SiO₂NPs, CdCl₂, and SiO₂NPs). The colonies of SiO₂NP groups (Figures 5(a)–5(e)) had roundish colony morphology and similar patterns

FIGURE 3: Glutathione (GSH) measurement in A549 cells unexposed (control) and exposed to increasing concentrations (1–100 $\mu\text{g/mL}$) of Cd-SiO₂NPs, CdCl₂, SiO₂NPs after 48 h. GSH levels were affected by all tested materials: the GSH depletion in A549 cells was not dose-dependent after Cd-SiO₂NP or CdCl₂ treatments, while was dose-dependent after SiO₂NP treatments. Data are mean $\pm$ DS of three separate experiments each carried out in six replicates. * $P < 0.05$ significant difference from control.

to control (data not shown), while, A549 cells treated with doses ranging from 0.05 to 10 $\mu\text{g/mL}$ of Cd-SiO₂NPs and CdCl₂ presented few colonies and a drastic reduced size compared to control (data not shown, Figures 5(f)–5(j), and Figures 5(k)–5(o), resp.). At the higher concentrations of either Cd-SiO₂NPs or CdCl₂ (25 to 100 $\mu\text{g/mL}$) there was a complete inhibition of the colony formation.

Semi-quantitative analysis showed a strong reduction of colony number (about 45%) already at the lowest tested dose (0.05 $\mu\text{g/mL}$) for both Cd-SiO₂NPs and CdCl₂ treatments, until reaching a total inhibition of cell proliferation at the doses from 25 to 100 $\mu\text{g/mL}$ (Figure 6). SiO₂NP treatment did not produce adverse effects, indeed the proliferative activity of A549 cells was not inhibited after prolonged exposure to any SiO₂NP dose tested (0.05–100 $\mu\text{g/mL}$; Figure 6).

4. Discussion

The present *in vitro* investigations indicate that Cd-SiO₂NP treatment produced *in vitro* deleterious effects after short- (24–48 h) and long-term (up to 10 days) exposure with mitochondrial function severely impaired and activation of caspase-3, depletion of GSH, and inhibition of cell proliferation already observable at the lowest concentration doses (i.e., 1 $\mu\text{g/mL}$ for the short-term exposure and 0.05 $\mu\text{g/mL}$ for the prolonged exposure). Similar cytotoxic profile was observed after CdCl₂ treatment. However, the magnitude of effects caused by Cd-SiO₂NPs was more pronounced compared to that produced by CdCl₂.

A549 acutely exposed to Cd-SiO₂NPs showed dose-dependent alterations of mitochondrial function and membrane integrity, as well as activation of caspase-3 already after 24 h exposure. At 48-hr, a further exacerbation of these effects was assessed. CdCl₂ also influenced the same cellular

parameters although the effects were less pronounced than those caused by Cd-SiO₂NPs. On the contrary, SiO₂NPs did not induce cytotoxic effects in this cell model.

Intracellular GSH level changes (decreases) were also observed at both time-points (24 and 48 h) in A549 cells for all tested compounds (Cd-SiO₂NPs, CdCl₂, and SiO₂NPs) suggesting an induction of oxidative stress. Notably, GSH level was the only altered parameter following SiO₂NP exposure.

Clonogenic assay, used to evaluate the effects induced after prolonged exposure (10 days), showed the ability of both Cd-SiO₂NPs and CdCl₂ (at the lowest tested dose of 0.05 $\mu\text{g/mL}$) to drastically inhibit A549 cell proliferation, while, once again, SiO₂NPs were avoided of any effect.

In relation to cadmium toxicity, an extensive database is available on CdCl₂-induced pneumotoxic effects by *in vivo* and *in vitro* models [22]. Mechanistically, Cd, at the cellular level, has been shown to cause oxidative stress by depletion of endogenous antioxidants such as glutathione that is associated with mitochondrial damage and induction of apoptosis [8, 34]. A recent *in vitro* study underlined that low-dose cadmium triggers apoptosis rather than outright necrosis [35]. Indeed CdCl₂ concentrations used in proximal tubule (PT) cell culture model to induce apoptosis ranged from 2 to 10 $\mu\text{g/mL}$ (corresponding to 10–50 μM) [37]. For instance, these values are of similar magnitude as the threshold level of 50 $\mu\text{g/g}$ kidney tissue for the development of signs of kidney dysfunction and PT damage indicated in *in vivo* experimental and human studies of chronic Cd²⁺ [38, 39]. Involvement of caspase-3 has been described in several animal models of chronic Cd²⁺ nephrotoxicity [40, 41].

Apoptosis (5-fold higher than control) was also observed in cultures, of rat lung epithelial cell line, exposed for 48 h to 20 μM CdCl₂ [42], and was preceded by the up-regulation of oxidant stress genes (glutathione S-transferase- α , gamma-glutamylcysteine synthetase, and metallothionein-1), activation of redox sensitive transcription factors (AP-1 and NF- κ B), and changes in various forms of glutathione (reduced, oxidized, and protein-bound); thus, altogether, these features suggesting a key role played by the reactive oxygen species.

Our findings indeed evidenced, after both Cd-SiO₂NPs and CdCl₂, GSH depletion and activation of caspase-3 which is a critical executioner of apoptosis, as it is either partially or totally responsible for the proteolytic cleavage of many key proteins [43].

The observed cytotoxic effects induced by Cd-SiO₂NPs after short- and long-term exposure suggest a crucial role of the cadmium moiety in the biological response to Cd-SiO₂NPs although it seems unlikely that the changes produced by Cd-SiO₂NPs merely reflected the action of cadmium ions released from nanoparticles. Indeed, chemical experiments with Cd-SiO₂NPs have demonstrated limited release of cadmium ions from the nanoparticles dispersed in medium culture, the maximum metal release being ca. 28% over a 10-day period.

In addition, the tendency to form aggregates and agglomerates of these doped NPs [29] may also have contributed

FIGURE 4: Representative images of randomly selected microscopic fields of A549 cells marked with caspase 3 antibody after 24 h incubation with: Cd-SiO₂NPs ((a)–(d)), CdCl₂ ((e)–(h)), SiO₂NPs ((i)–(l)) at the doses indicated in the figure. Activation of caspase 3 is indicated by green spots already at the lowest tested dose of 1 µg/mL in both Cd-SiO₂NP and CdCl₂ treatment groups, while there was no positivity in SiO₂NPs treated cells at all the tested doses similarly to the unexposed cells (control). (Scale bar: 100 µm).

FIGURE 5: Representative images of randomly selected microscopic fields of the colonies formed after 10 consecutive days exposure to increasing concentrations (0.05–100 µg/mL) of Cd-SiO₂NPs, CdCl₂, and SiO₂NPs. SiO₂NP colonies showed similar patterns at the all tested doses to the control (control data not shown). Few colonies with reduced size compared to control were observed at the lower concentrations (0.05–10 µg/mL) of Cd-SiO₂NPs and CdCl₂, while there was a total inhibition of the colonies at the higher tested doses (25–100 µg/mL) for both Cd-SiO₂NPs and CdCl₂. (Scale bar: 600 µm).

in triggering the described Cd-SiO₂NP effects: the DLS data demonstrated an agglomeration and aggregation extent of Cd-SiO₂NPs (about 350 nm) greater than that measured for SiO₂NPs (about 120 nm). Whether those agglomerated particles retain toxic properties of the individual nanoparticles or are capable of subsequently is a critical question [44].

An additional hypothesis may be related to the nano dimension of the material investigated. In our *in vitro* experiments, the nano dimension may have facilitated the

cell concentration and thus toxicity of the administered cadmium. A “Trojan horse”-type mechanism involving silica nanoparticles as effective carriers for the cellular uptake of toxic metals has been described [45]. In the same type of lung cells used in this study, exposure to SiO₂NPs doped with metals such as iron, manganese, cobalt, or titanium was shown to generate higher concentrations of reactive oxygen species and induce more severe oxidative stress compared to equivalent amounts of the respective metal ions [46]. In our

FIGURE 6: Histograms show the number of colonies formed after 10 consecutive days exposure to increasing concentrations of Cd-SiO₂NPs, CdCl₂, SiO₂NPs at the doses indicated in the figure. The formation of A549 colonies appeared to be seriously compromised by both Cd-SiO₂NP and CdCl₂ treatments already at the lowest dose of 0.05 µg/mL, while SiO₂NPs had no effects on their formation. Data are mean ± DS of two separate experiments each carried out in three replicates, expressed as percentage of control colonies. **P* < 0.05 significant difference from control. Error bars: ±SD.

study, incorporation of Cd into SiO₂NPs may have increased the metal dose delivered to target cells although no specific data supporting this process are presently available.

With regard to SiO₂NPs, no cytotoxic effects were observed for all concentrations tested and for all exposure times (acute or chronic), with the only exception for the observed GSH depletion after 48 h treatment. Several recent literature data are consistent with the present findings: SiO₂NPs penetrated A549 cells and did not cause significant toxic effects at the molecular and cellular levels below 100 µg/mL [47]; it induced low cytotoxicity at concentrations up to 200 µg/mL [48], and generated oxidative stress reflected by reduction of GSH levels [16] or oxidant generation [49]. On the other hand, other investigations indicated A549 cell viability decreases after SiO₂NPs exposure down to 100 µg/mL [16], as well as a proinflammatory response triggered by SiO₂NPs without blocking cell proliferation or causing cell death in A549 cells [50].

For comparison, our previous *in vivo* results indicated early and persistent lung damage after i.t. instillation of Cd-SiO₂NPs in terms of enhanced apoptotic phenomena followed by a significant increase of proliferating cells [29], as well as pulmonary inflammation and fibrosis in rats evidenced by a wide-spread immunoreactivity of both cytokines/chemokines and collagen, respectively. The effects were detectable at the earliest time point, 24 h, and persisted until the 30th day post exposure. Similar pattern of toxic insult was also revealed after i.t. instillation of equivalent amount of CdCl₂, although it was less marked than Cd-SiO₂NP treatment. The dose of CdCl₂ per animal was 400 µg (2.1 µmol) ≡ 247 µg Cd. Cd-SiO₂NPs and CdCl₂ also showed the capacity to cause long-lasting oxidative stress by increasing the tissue F₂-isoprostane levels and pulmonary SOD1, COX-2, and iNOS expressions [30].

On the contrary, no changes involving these markers were observed in animals treated with SiO₂NPs.

Altogether, the *in vivo* results showed a higher Cd-SiO₂NPs reactivity (regardless of whether form type is present: original, agglomerate, or with sorbed material at NP surface) than SiO₂NPs and CdCl₂ in the lung tissue.

Both *in vitro* and *in vivo* findings pointed out that Cd-SiO₂NP exposure produces a complex and multicomponent insult leading to an exacerbated toxicity response compared to the toxic pattern caused by CdCl₂ treatment and essentially much more than SiO₂NPs.

5. Conclusions

In vitro experiments in pulmonary cells have provided effective means of screening and ranking the tested materials (Cd-SiO₂NPs > CdCl₂ > SiO₂NPs) using multiple toxicological endpoints (i.e., mitochondrial and membrane alterations, induction of apoptosis, inhibition of growth and proliferation, and intracellular GSH depletion). Coherently, the *in vivo* results have systematically characterized the tissue damage evidenced by lung parenchyma injury and fibrosis, apoptotic phenomena, the occurrence of inflammation, and pulmonary oxidative stress in rats. The *in vivo* targeted tests have complemented and addressed the *in vitro* findings to ensure the adequate evaluation of nanoparticle hazard potential, also in terms of time of appearance and persistence of the toxicological features on living organism.

Highlights

- (i) Cd-SiO₂NPs produced *in vitro* toxic effects after short- and long-term exposure;
- (ii) similar toxic profile was observed after CdCl₂, the effect rank: Cd-SiO₂NPs > CdCl₂;
- (iii) SiO₂NPs influenced oxidative stress pathway only;
- (iv) *In vitro* tests on lung cells provided effective means of ranking the tested materials.

Conflict of Interests

The authors declare that they have no conflict of interests and they alone are responsible for content and writing of the paper.

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