Histol Histopathol (2011) 26: 357-367

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Comparative pulmonary toxicity assessment of *pristine* and functionalized Multi-Walled Carbon Nanotubes intratracheally instilled in rats: morphohistochemical evaluations

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Summary. Increasing interest in safety evaluation of carbon nanotubes (CNTs) has risen in relation to their wide applications, together with the evidence of their cytotoxic effects.

It has been shown that chemical functionalization extends the applications of CNTs, conferring them new functions that cannot otherwise be acquired by *pristine* CNTs, but also impacts on biological response to CNTs, modifying their toxicological profile.

We assessed the onset of pulmonary toxic effects caused by *pristine* MW-CNTs and functionalized MW-NH_2 or MW-COOH, 16 days after intratracheal instillation (1 mg/kg b.w.); major endpoints tested included (i) histopathology of lung (Haematoxylin/Eosin Staining), (ii) apoptotic/proliferating features examined by TUNEL and PCNA immunostaining, and (iii) presence/distribution of (1) Transforming Growth Factor-beta1 (TGFß1), (2) Interleukin-6 (IL-6) and (3) Collagen (Type I) investigated by immunochemical methods, as markers of lung toxicity, inflammation, and fibrosis, respectively.

Lung histopathology from exposed animals showed dark, particulate-laden macrophages, reflecting carbon nanomaterial engulfing, both at alveolar and bronchiolar levels, after treatment with all the tested CNTs. Alteration of lung architecture was also observed in several areas showing collapsed thick-walled alveoli and the presence of micro-haemorrhagic foci. TUNEL and PCNA, indicative of apoptosis and cell proliferation respectively, showed a significant increase of immunopositive cells at bronchiolar, alveolar and macrophagic levels, as expression of an improved cellular turnover.

Increased immunoreactivity for pulmonary TGFß1 and IL-6 was observed in treated rats, particularly in bronchiolar areas, collapsed alveoli and at stromal level, while evident changes for collagen were not detected.

Taken together these findings demonstrated the general pulmonary toxicity coupled with inflammatory response after *in vivo* exposure to CNTs, without overt signs of fibrosis and granuloma formation, irrespectively of nanotube functionalization

Key words: Nanomaterials, Lung inflammation, *In vivo*, IL-6/ TGFß1, PCNA, TUNEL

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Introduction

Nanotechnology is one of the fastest emerging fields involving development and manipulation of materials ≤100 nm in size. There are numerous potential perspectives for the applications arising from nanotechnology which include their use in a wide range of fields, e.g. energy, medicine and also environmental improvement (Nanoscale Science, 2004; Shvedova et al., 2009). However, much concern has been expressed about the potential of adverse and unanticipated toxic effects on human health and environment, probably due to the unique properties of this novel material. Carbon nanotubes (CNTs), are among the most promising newly engineered nanomaterials. Discovered in 1991 by Iijima (1991), CNTs structurally resemble rolled-up coaxial graphite sheets with one end capped, with a diameter in the order of nanometers (Lam et al., 2004; Shvedova et al., 2009) and exist in different forms, i.e. *pristine*

single-walled CNTs (SW-CNTs), and multi-walled CNTs (MW-CNTs), the latter consisting of several concentric graphene tubes with a diameter up to 100 nm. No other materials have been developed that possess the size (1-20 nm in width, and many microns in length), strength, and surface chemistry properties of CNTs. The high ratio between length and diameter of CNT and their low water solubility make them potentially bio-persistent and may lead to toxic effects similar to those observed with other fibrous particles, such as asbestos fibers (Maynard et al., 2004; Donaldson et al., 2006). Moreover, it should be mentioned that the toxicity of these nanostructured materials can be modified by chemical approaches, such as functionalization, which may impact on the biological response to CNTs at cellular and molecular level suggesting that the toxicological profile may also be modified. Recently developed functionalization schemes have extended the application spectrum of CNTs enabling the implementation of new functions that cannot otherwise be acquired by *pristine* nanotubes. The association between the physical–chemical properties and the target organ dose–effect relationships are an issue awaiting resolution for engineered nanomaterials (Savolainen, 2009). Along with these concerns, and the risk exposure to nanomaterials may pose to workers, issues regarding the most appropriate unifying metric of dose are still unresolved (Maynard, 2007). No single particle characteristic as a hallmark indicator directing fate and pulmonary toxicity has been identified yet (Madl and Pinkerton, 2009).

Numerous epidemiological studies have demonstrated that CNTs may contribute to pulmonary and cardiovascular systemic diseases, depositing in all regions of the respiratory tract, then translocating from alveolar regions to epithelial and interstitial sites or into the blood circulation, where they may migrate to other organs (Nemmar et al., 2001, 2002, 2007, 2009; Oberdörster et al., 2004; Maynard et al., 2006).

Several experimental studies demonstrated that biological and toxicological responses to CNTs may vary by dose, route of dosing, and type of CNTs. Reported research on the pulmonary *in vivo* toxicity of CNTs to date has been conducted by either instillation into the trachea (Lam et al., 2004; Warheit et al., 2004; Muller et al., 2005) or pharyngeal aspiration (Shvedova et al., 2005). All these investigations reported significant pulmonary effects, including inflammation, evidence of oxidative stress, fibrosis, and granuloma formation (Lam et al., 2004, 2006; Warheit et al., 2004; Muller et al., 2005; Shvedova et al., 2005).

Anyway, *in vivo* studies of MW-CNTs are very limited, showing that intratracheal instilled MWCNT in rats caused pulmonary inflammation at 3 and 15 days post-exposure, and induced pulmonary fibrosis at 60 days post-treatment (Muller et al., 2005). Contrastingly, inhalation exposure of mice to MWCNT has been reported to induce no significant lung toxicity, but did cause immunosuppression (Mitchell et al., 2007). Moreover, Shvedova et al. (2004) showed that pharyngeal aspiration of SWCNT elicited pulmonary effects in C57BL/6 mice that was promoted by a robust, acute inflammatory reaction with early onset resulting in progressive interstitial fibrogenic response and the formation of granulomas.

These dissimilar results have been ascribed to differences in MWCNT size and surface area, rodent species used, and differences in observation period (McDonald and Mitchell, 2008). The route of exposure should also be taken into consideration in that it may impact on CNT toxic effects; although intratracheal instillation (*i.t.*) is a more artificial route of dosing bolus material to the lungs and there are differences in distribution, behaviour, clearance and retention of materials when administered by *i.t.* compared to inhalation, the former is widely used to address specific endpoints regarding the respiratory toxicity of nanomaterials (Driscoll et al., 2000; Warheit et al., 2004; Muller et al., 2005; Li et al., 2007; Elgrabli et al., 2008).

Very recent studies reported by Fenoglio et al. (2008) and Muller et al. (2008a) suggest that structural properties may play a major role in the acute lung toxicity of MW-CNTs.

Furthermore, in the last years, numerous *in vitro* studies have been conducted on several cell types, showing different adverse effects (e.g. oxidative stress, inflammatory response, cell death, loss of cellular morphology, gene expression level changes) caused by various CNTs tested at different doses (1-800 µg/ml) and time points (24, 48 and 72 hr) (Monteiro-Riviere et al., 2005; Bottini et al., 2006; Sayes et la., 2006; Davoren et al., 2007).

In addition, our previous *in vitro* investigation (Coccini et al., 2010) highlighted some limitations intrinsical to classical *in vitro* cytotoxicity tests (e.g., Live/Dead-Calcein/PI Test and MTT assay) applied to the study of these CNTs, due to the peculiar physico-chemical characteristics of these new nanomaterials, calling for research approaches complementary to the *in vitro* studies. Indeed, because of investigations in laboratory animals may give essential insights, the present study aimed at assessing the onset of pulmonary toxic effects caused by intratracheal instilled MW-CNTs, *pristine* and differently functionalized with basic (MW-NH_2) or acidic (MW-COOH) groups, 16 days after intratracheal instillation (i.t.) (1mg/kg b.w.), evaluating different markers of general toxicity, lung injury, inflammation, and fibrosis, with the ultimate goal to examine if and how structural properties may modulate pulmonary toxicity. Thus, the major endpoints of this study included: (i) histopathology of lung tissue (through Haematoxylin/Eosin Staining), (ii) characterization of apoptotic/proliferating features by TUNEL and PCNA immunostaining, and (iii) immunohistochemical evaluation of the presence and distribution of (1) Transforming Growth Factor-beta1 ($TGF\beta_1$), (2) Interkeukin-6 (IL-6) and (3) Collagen (Type I), as typical

markers of general lung toxicity, inflammation, and fibrosis, respectively.

Materials and methods

Preparation of functionalized MWCNTs

MWCNTs with different functionalization were prepared as described by Fagnoni et al. (2009). The materials produced included carboxyl (COOH) functionalized MW-COOH and amine-containing nanotubes (functionalized MW-NH_2).

MW-COOH

Five hundred mg of raw MWCNT were refluxed in 25 ml of a mixture of concentrated H_2SO_4/HNO_3 (3/1, v/v) (Figure 1 panel a). The resulting suspension was then diluted with 50 ml of icy water. The excess of acid and water was removed by filtration (Millipore, 0.5 µm). The black solid was washed thoroughly with NaOH 0.1 M and subsequently with HCl 0.1 M to remove oxidation debris. The solid was then washed with distilled water until the eluates were neutral. The resulting MW-COOH were dried under vacuum at room temperature. Approximately 400 mg of MW-COOH were obtained.

MW-CONH($CH_2CH_2O)_2$-$CH_2CH_2NH_2$ (MW-NH_2)

A suspension of 450 mg of MW-COOH in 20 ml of oxalyl chloride was stirred at 62°C for 24 h. The excess of oxalyl chloride was then eliminated in vacuo at 60°C. The resulting solution of MWCNT-COCl and 20 ml of $NH_2(CH_2CH_2O)_2$-$CH_2CH_2NH_2$ in tetrahydrofuran was refluxed for 48 h. After cooling at room temperature, the derivatized CNT were filtered (Millipore, 0.5 µm) and the filter washed several times with methanol. The resulting solid was air-dried at room temperature. Approximately 400 mg of MW-NH_2 were obtained.

Physicochemical characterization of CNTs

Before the *in vivo* exposure by intratracheal instillation (i.t.), exhaustive physicochemical characterization of differently functionalized CNTs was performed by IR spectroscopy, thermogravimetric analysis (TGA), and by Noncontact atomic force microscopy (NC-AFM), as fully reported in our previous study by Coccini et al. (2010).

It has been estimated that one functional chain is anchored each ~16 carbon atoms of CNT surface for the MW-COOH, and each ~100 carbon atoms of CNT surface for the MW-NH_2.

All the three types of CNTs had an outer diameter of 20-30 nm and the wall thickness was 1-2 nm. The particle length was 100-300 nm for both the functionalized CNTs (e.g. MW-COOH and MW-NH_2) and 500-2000 nm for the *pristine* MWCNTs.

Animals and Treatment with Carbon Nanotubes by intratracheal instillation (i.t.)

All experimental procedures involving animals were performed in compliance with the European Council Directive 86/609/EEC on the care and use of laboratory animals.

Adult Sprague–Dawley rats (25 males, 12 weeks old) were purchased from Charles River Italia (Calco, Italy) and allowed to acclimatize for at least 2 weeks before treatment. Throughout the experiment, animals were kept in an artificial 12 h light:12 h dark cycle with humidity at 50±10%. Animals were provided rat chow (4RF21 diet) and tap water ad libitum.

For the treatment, groups of 6 rats were anesthetized with pentobarbital sodium for veterinary use and were intratracheally instilled with MWCNTs, *pristine* or functionalized (see above), dispersed at a dose of 1 mg/kg b.w. in NaCl 0.9%. Just before the i.t exposure, the three different CNT suspensions were prepared by sonication for 15 min with an ultrasonic Sonopuls (Bandelin Electronics, Berlin, Germany) in a short break every two minutes, also vortexing the suspension on ice to further force CNT dispersion, avoiding the tendency to agglomerate and the formation of bundle-like structures. No surfactants or solvents were used. The suspensions of the test materials were immediately used for the treatment.

Sixteen days after the i.t., treated and control rats were deeply anesthetized with an overdose i.p. injection of 35% chloral hydrate (100 microl/100 g b.w.); lung preparation for microscopic evaluations was done by vascular perfusion of fixative. Briefly, 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 H_2O 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.

Tissue sampling

The top and the bottom regions of the right lungs of control and differently treated animals were dissected. Tissue samples were obtained according to a stratified random sampling scheme which is a suggested method for lung tissue in order to compensate for regional differences, which are known to exist in the lung (Weibel, 1979) and to reduce the variability of the sampling means.

From each slice, 2-3 blocks were systematically derived, washed in NaCl 0.9% and post-fixed by immersion for 7 h 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 µM thick sections of the samples were cut in the transversal plane and collected on silan-coated slides.

Histology and Immunocystochemistry

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.

Lung sections of control and treated rats were stained with Haematoxylin/Eosin (H&E) for histological examination.

Immunohistochemistry, providing a rapid and sensitive approach, was performed using commercial antibodies on rat lung specimens, to assess the presence and distribution of (i) Transforming Growth Factor-beta1 (*TGFß$_1$*), (ii) Interkeukin-6 (*IL-6*), (iii) *Collagen* (*Type I*) and (iv) Proliferating Cell Nuclear Antigen (*PCNA-PC10*), as typical markers of general lung toxicity, inflammation, fibrosis, and cell proliferation, respectively.

Lung sections of control and treated rats were incubated overnight at room temperature with: (i) a primary rabbit polyclonal antibody against *TGFß$_1$* (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 or (ii) a primary rabbit polyclonal antibody against *Collagen* (*Type I*) (Chemicon, Temecula, CA, USA) diluted 1:400 or (iii) a primary goat polyclonal antibody against *IL-6* (Santa Cruz Biotechnology) diluted 1:100 or (iv) a primary mouse monoclonal antibody against *PCNA* (American Biotechnology, Plantation, USA) diluted 1:5 in PBS. Biotinylated anti-rabbit, anti-goat and anti-mouse secondary antibodies and an avidin biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) were used to reveal the sites of antigen/antibody interaction. 3,3'-Diamino-benzidine tetrahydrochloride (DAB; Sigma, St. Louis, MO) was used as the chromogen, followed by a light counterstaining with H&E. Then, the sections were dehydrated in ethanol, cleared in xylene, and finally mounted in Eukitt (Kindler, Freiburg, Germany).

In the case of negative controls, some sections were incubated with phosphate-buffer saline instead of the primary antibodies; no immunoreactivity was observed in these conditions.

TUNEL Staining

In addition to morphological criteria, apoptotic cell death was assayed by *in situ* detection of DNA fragmentation using the terminal deoxynucleotidyl-transferase (TUNEL) assay (Oncogene Res. Prod., Boston, MA, USA). The lung sections were incubated for 5 min with 20 µg ml^{-1} proteinase-K solution at room temperature, followed by treatment with 3% H_2O_2 to quench endogenous peroxidase activity. After incubation with the TUNEL solution (90 min with TdT/biotinylated dNTP and 30 min with HRP-conjugate streptavidin) in a humidified chamber at 37°C, the reaction was developed using 0.05% 3-amino-9-ethylcarbazole (AEC) in 0.1 M TRIS buffer (pH 7.6) with 0.2% H_2O_2; in some specimens the reaction was developed using a 0.1% DAB solution. The specimens were lightly counter-stained with H&E.

As a negative control, the TdT incubation was omitted; no staining was observed in these conditions.

Cytochemical assessment

(i) Scoring different specimens, the immunostaining for IL-6, TGFß1 and Collagen (Type I) was evaluated in conventional brightfield microscopy by recording the localization and intensity of labelling according to a semiquantitative scale from absent/undetectable (-) to maximal (++++). Then, to assess the significance of the immunohistochemical results, a Kruskal-Wallis non-parametric analysis of the semiquantitative data was performed. A p value of <0.05 was considered significant.

(ii) The evaluation of PCNA- and TUNEL-cytochemically positive cells (PCNA L.I., TUNEL L.I.) was calculated as the percentage (Labelling Index) of a total number (about 500) of bronchiolar, alveolar and macrophagic cells, for each animal and experimental condition, in different representative microscopic fields. Statistical analyses among the different biological situations was performed by the Student's t-test, and differences between medians were considered significant at $^*P<0.05$.

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.

Results

Cytohistological morphology

After i.t. exposure to all the three different CNTs, lung morphology micrographs, detected by light microscopy, clearly showed a marked uptake of the CNTs into the macrophages, typically localized in different areas, e.g. bronchiolar and alveolar surface, and stromal domain (Fig. 1c-f); noticeably, the presence of such carbonaceous nanomaterial was not detectable in the epithelial cells' cytoplasm.

Histologically, some lung parenchyma regions showed collapsed alveoli with mild interstitial thickening and presence of micro-haemorrhagic foci (Fig. 1d), apparently not accompanied by evident signs of fibrotic reaction. Occasionally, inflammatory shedding of leucocyte clusters characterized the parenchyma, as the consequence of the CNTs spreading before the phagocytic processes by the scavenger cells; nevertheless, the occurrence of true granulomas was

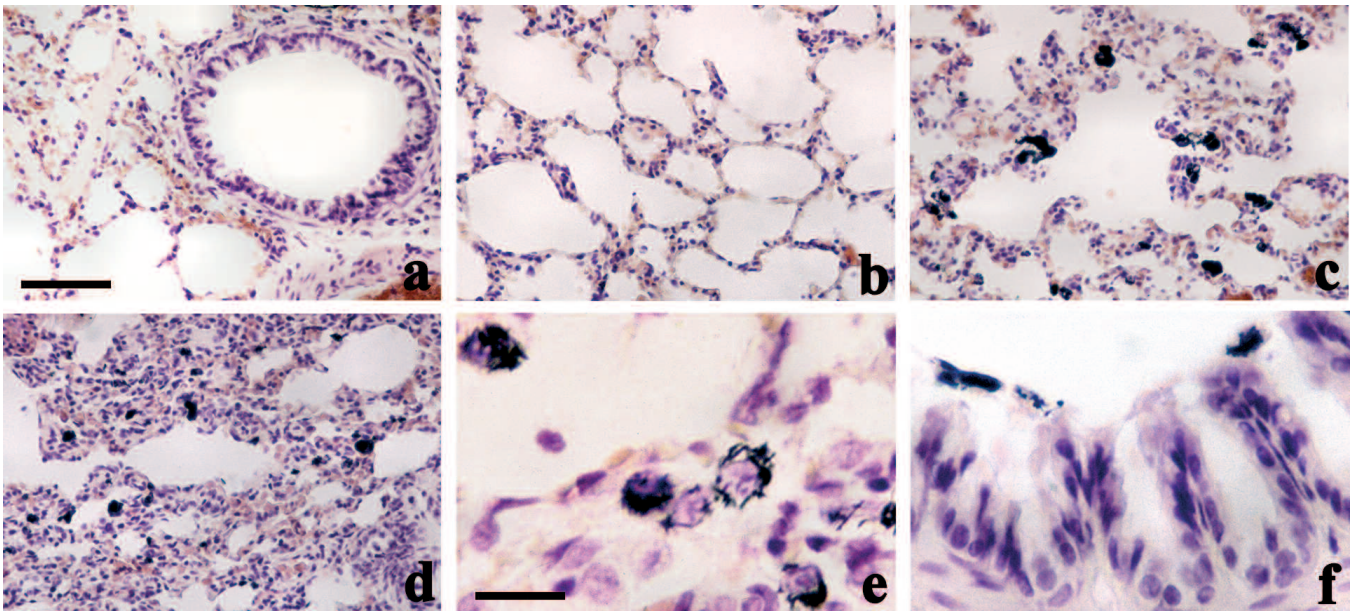

Fig. 1. Representative Haematoxylin and Eosin-stained lung parenchyma specimens from control animals **(a, b)** and rats exposed to 1 mg/kg b.w. MW-COOH **(c)** or MW-NH_2 **(d-f)** by intratracheal instillation. It has to be noted that the normal lung architecture is clearly preserved, both at bronchiolar and alveolar levels, in control animals **(a, b)**, while, on the contrary, after treatment **(c-f)**, dark, particulate-laden macrophages become evident as a consequence of the carbon nanotubes engulfing, at alveolar **(c, d)**, stromal **(e)** and bronchiolar **(f)** levels. In some areas, the thickened-walled alveoli appeared collapsed and the presence of widespread micro-haemorrhagic foci is detectable **(d)**. Scale bar: a-d, 200 µm; e, f, 30 µm.

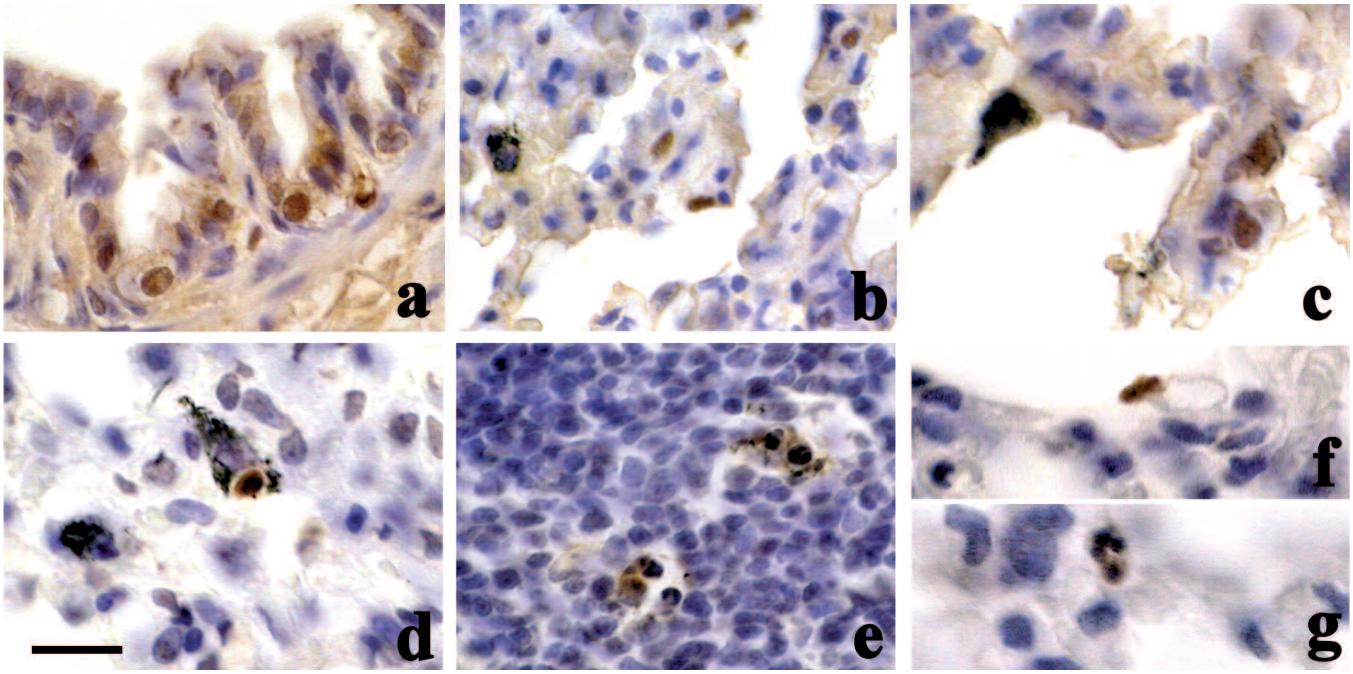

Fig. 2. Cell proliferation **(a-c)** and apoptosis **(d-g)** detected by PCNA immunolabelling and TUNEL staining respectively, after rat i.t. exposure to 1 mg/kg b.w. MW-COOH **(c-e)** and MW-NH_2 (**a-b and f-g**). PCNA positive epithelial cells at bronchiolar (**a**) and alveolar (**b, c**) levels; TUNEL positive CNT-laden macrophages in normal (**d**) and inflammatory (**e**) stromal areas and at alveolar (**f, g**) levels. Scale bar: 30 µm.

never detected.

Cell kinetics (cell death and proliferation) histochemistry

To evaluate cell death as a consequence of CNT injury action in lungs, the histochemical detection of DNA fragmentation with the TUNEL reaction was performed, while the lung cell proliferation response was determined by PCNA expression, being this accessory protein mainly involved during the DNA replication and

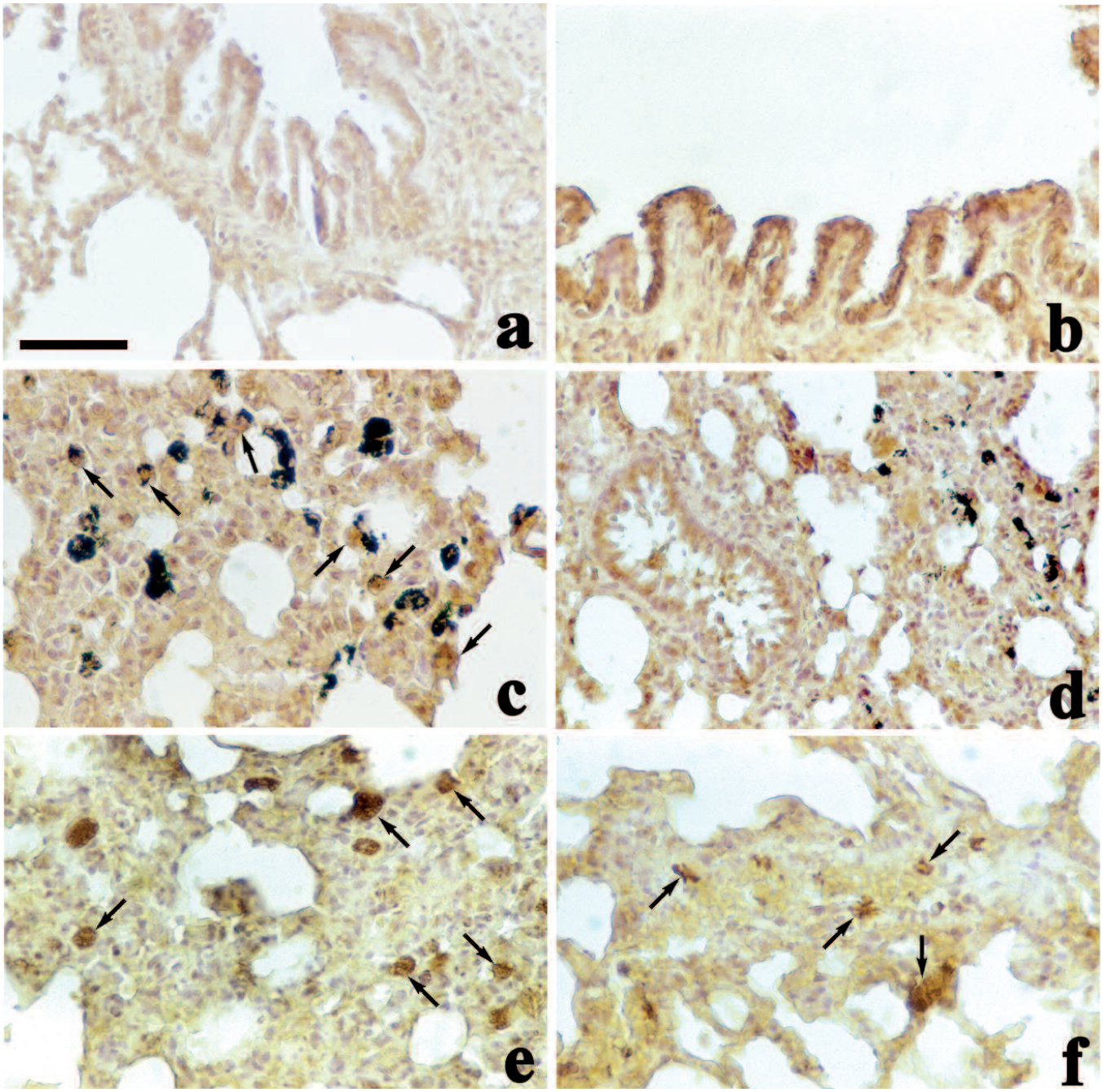

Fig. 3. Immunostaining patterns of IL-6 (**a-d**) and TGF-ß1 (**e-f**) expression in controls (**a**) and rats treated with MW-NH_2 (**b, c, e**) or MW-COOH (**d, f**). Noticeably, low labelling for IL-6 is detected at all lung districts (e.g. bronchiolar, stromal and alveolar levels) in controls (**a**), while, after all CNTs-treatment, bronchiolar areas and collapsed alveoli appeared strongly IL-6-immunoreactive (**b-d**), showing several markedly immunopositive cells (arrows), together with the presence of widespread black particulate. Strong immunoreactivity for TGF-ß1 is observed mainly at stromal level (arrows) of some collapsed alveolar zones (**e, f**) with evident immunopositive cells (arrows). Scale bar: 150 µm.

repair.

Any presence of necrotic tissue degeneration was detected, while, on the contrary, several apoptotic death cells were observed. The canonical apoptotic phenomenon, morphocytochemically characterized by nuclear pyknosis, karyorrhexis, and TUNEL-positivity (Fig. 2), increased significantly after all the treatments, mainly in the alveolar epithelial cells in comparison to the bronchiolar epithelial cells and the macrophages (Fig. 4). Regarding to PCNA expression, an increased number of immunoreactive epithelial and macrophagic cells (Fig. 4) was detected, after i.t. exposure, both at alveolar and bronchiolar levels (Fig. 2), irrespective of nanotube functionalization.

Table 1: Localization and expression of immunolabelling for IL-6, TGF-ß1 and Collagen (Type I) on a semiquantitative evaluation.

	Control	*Pristine* MW	MW-NH_2	MW-COOH	*p* Value
Bronchiolar cells					
IL-6	±	++±	++++	++±	<0.05
TGF-beta1	-	+±	++	++	<0.05
Collagen-I	+	++	+±	+±	NS
Alveolar Cells					
IL-6	±	+±	++	+±	<0.05
TGF-beta1	-	+	+	+	<0.05
Collagen-I	±	+	+±	+±	NS
Stromal cells					
IL-6	±	+±	++±	+±	<0.05
TGF-beta1	±	+++	++++	+++±	<0.05
Collagen-I	+	+±	+±	+±	NS

Degree of staining intensity: from undetectable (-) to strong (++++). *p* values calculated by Kruskal-Wallis test.

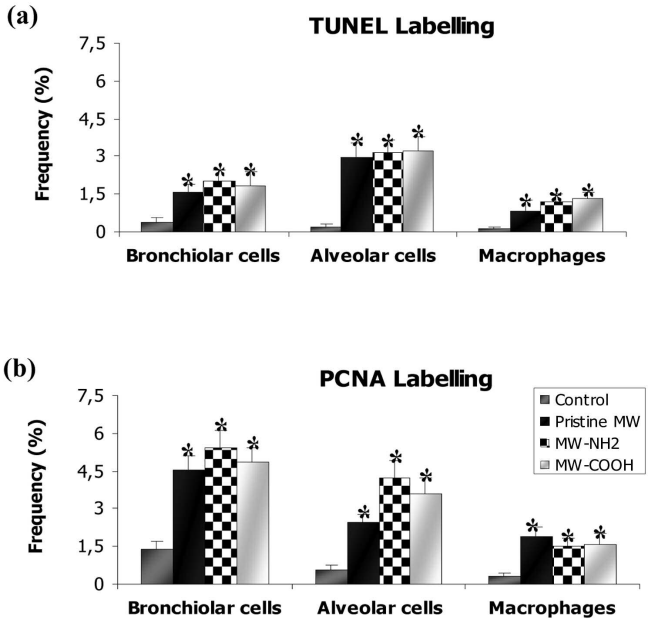

Fig. 4. Histograms showing changes in percentage of TUNEL **(a)** and PCNA **(b)** Labelling Index of bronchiolar, alveolar and macrophagic cells as a consequence of the i.t. exposure to different carbon nanotubes (*pristine* MW-CNT *versus* laboratory-functionalized MW-COOH and MW-NH_2). In all CNT-treated rats, a significant increase (Student's t test) of the above mentioned cell types was clearly observed. Data are expressed as mean ± S.D. (*P<0.05).

Immunohistochemistry

After i.t. exposure to all the three different CNTs, the cellular localization and tissue distribution of IL-6 and TGFß1, both involved in tissue injury and repair pathways, revealed an extensive spreading in the bronchiolar, alveolar and stromal cells, probably evidencing the cellular inflammatory reaction to CNT instillation (Table I). The heaviest IL-6-immuno-positivity was detected in the bronchiolar zone, particularly at the epithelial level; contrarily, the TGFß1 antigen appeared significantly more expressed in the stromal cells (e.g. macrophages, fibroblasts) localized in the collapsed alveolar areas (Fig.3).

As collagen (Type I) concerns, the immunohisto-chemical labelling changes between control and treated rats appeared mild (Table 1) and was only detected at the level of some collapsed thick-walled alveoli.

Discussion

The present study addresses the pulmonary effects of three different MWCNTs (*pristine* versus two diverse lab-made functionalized MWCNTs, i.e. MW-COOH and MW-NH_2) on some cell kinetic (i.e. TUNEL and PCNA) and cytochemical parameters (i.e. IL-6, TGFß1 and collagen) investigated in rats after 16 days from a single intratracheal exposure (1 mg/kg b.w.). We demonstrated the onset of a general pulmonary toxicity coupled with an inflammatory response, clearly deductible by the occurrence of a cellular reaction finalized to obstacle and recover tissue damages, observed both at epithelial and stromal levels, without marked signs of fibrosis and granulomas formation, irrespectively of nanotubes functionalization.

Our experimental results, showing an extensive spreading in the bronchiolar, alveolar and stromal cells of IL-6 and TGFß1, evidencing the cellular inflammatory reaction to CNT instillation, are in agreement with previous literature data reporting toxic pulmonary effects, also including inflammation (characterized by an increase in alveolar cell number and in cytokines, e.g. TNF-alpha and IL-1), and oxidative stress (Lam et al., 2004; Warheit et al., 2004; Muller et al., 2005; Shvedova et al., 2005; Mangum et al. 2006). Different studies demonstrated that several doses of CNT, included also that used presently, (ranging from 0.1 to 0.5 mg or from 0.2 to 5 mg, intratracheally instilled in mouse and rat, respectively), caused dose-dependent toxicity in the lung of both animal species, with effects lasting for at least 3 months (Lam et al.

2004; Warheit et al. 2004; Shvedova et al. 2005).

However, at variance with our data, some authors also reported the onset of multifocal granulomas and the occurrence of fibrosis (Lam et al., 2004; Warheit et al., 2004; Muller et al., 2005; Shvedova et al., 2005; Mangum et al., 2006).

Recent CNT-inhalation studies gave experimental evidence that prolonged exposure (7 or 14 days, 6h/days) to breathable MWCNTs caused in mice neither lung damages like granulomas or fibrosis (for doses varying from 0.3 to 0.5 mg/m^3 corresponding to intra-lung deposition doses of 0.2 to 2.7 mg/kg, respectively) nor inflammation (BAL cellularity studies and IL-6, IL-10 quantification) (Li et al., 2007; Mitchell et al., 2007).

Indeed, Li et al. (2007), comparing the pulmonary pathological lesions induced by MWCNT given by two different routes of exposure, e.g. i.t. versus inhalation (i.e. single i.t. exposure to 50 µg MWCNT with endpoint evaluation after 8, 16, and 24 days *versus* 32 mg/m^3 of MWCNT inhalation exposure during 8, 16 or 24 days, respectively), reported that this type of CNTs induced lung damages and inflammation only after i.t. but not after inhalation, pointing to the fact that the latter exposure method is more physiological than the former one, impacting on the formation and different size of MWCNT agglomerates, resulting in different MWCNT lung distribution and behaviour.

Other experimental studies further contributed to the controversy regarding the *in vivo* toxicity of MWCNT, showing that MWCNT-pharyngeal aspiration exposure in mice resulted in dose- and time-dependent pulmonary inflammation and damage (Shvedova et al., 2005), as well as effects in the central nervous system (Sriram et al., 2007).

It is widely accepted that biological and toxicological response to CNTs may vary other than by dose, route of exposure, observation periods, endpoints evaluated, and rodent species employed. Moreover, CNTs unique characteristics, such as structure, size, high surface area and reactivity, tendency to agglomerate, ability to bind and deliver other molecules, and the type and degree of functionalization, deeply impacting on their toxicological profile, either increasing or reducing their toxicity, may lead to negative health impacts (Sayes et al., 2006; Zhang et al., 2007; Tong et al., 2009). In addition, evidence has also mounted that exposure to the two main different forms of CNTs (e.g.. SWCNTs *versus* MWCNTs) may cause a wide range of adverse effects, due to their different physico-chemical characteristic, resulting in diverse biological activity and behaviour, even though they can both provoke general cytotoxicity, cytokines release and oxidative stress (Muller et al., 2005; Shvedova et al., 2005, 2009; Lam et al., 2006), also playing some role in the pathogenesis of cardiopulmonary disease (Li et al., 2005, 2007; Lam et al., 2006).

The present experimental results obtained *in vivo*, reporting no pulmonary injury differences between *pristine* and lab-made functionalized MWCNTs (MW-COOH and MW-NH_2), seem to match with our previous *in vitro* data, obtained in human A549 pneumocytes by testing the same MWCNTs (at doses ranging from 1 to 100 µg/ml), showing similar toxicity profiles for all three types of MWCNTs when two different cytotoxic dying tests (Live/Dead-Calcein/PI Test and MTT assay) were applied (Coccini et al., 2010).

Moreover, our *in vivo* study showed the widespread presence of dark, particulate-laden macrophages, evident as a consequence of the carbon nanotubes engulfing at alveolar, stromal and also bronchiolar levels in accordance to the poor solubility of the MWCNTs (*pristine*, and lab-made MW-COOH and MW-NH_2) underlined in our previous *in vitro* work (Coccini et al., 2010) in which MWCNT were shown to adhere to each other forming dense micron-sized assemblies completely covering the cell surface, with agglomeration increasing at increasing doses irrespective of kind of functionalization.

Regarding the cell kinetic parameters considered, e.g. TUNEL and PCNA (indicative of apoptosis and cell proliferation, respectively), they could be related with a significant turnover of the cells that directly or indirectly interact with the xenobiotic substances. The higher apoptotic frequency of the epithelial cells, apparently not involved in CNT internalization, compared to the macrophagic component, frequently laden with black particles, suggests a different sensitivity of the two cellular categories, independently of the two types of interaction between fibro-carbonaceous pollutant and biological substrates. The increase in apoptotic frequency that we detected after MWCNT exposure is in accordance with previous *in vitro* and *in vivo* studies showing the occurrence of CNT-induced apoptotic phenomena in different lung cell types, e.g. rat lung epithelial cells, guinea pig and rat alveolar macrophages (Elgrabli et al., 2008; Ravichandran et al., 2009; Wang et al., 2009). Moreover, since failure to eliminate an inciting agent is a classic cause of chronic inflammation (Ackermann, 2007), indeed in our study the onset of MWCNT within the lung and the presence of numerous particulate-laden macrophages, due probably to carbon nanotubes engulfing at alveolar, stromal and bronchiolar levels, as a consequence of failure to eliminate the inciting cause, e.g. the MWCNT, might be the cause of the persistent inflammation observable at 16 days after i.t. exposure.

It is well known that size and morphology (i.e. geometry) of particulate matter have great influence on its toxicity, not only in relation to the deposition, but also in the mode of interaction with the target cells (Lam et al., 2006). The increased number of PCNA-immunopositive cells could be associated with an enhanced cell proliferation aimed at replacing the damaged elements (e.g. epithelial cells) and at increasing the cells involved in the organ defence (e.g. macrophages, fibroblasts). Nevertheless, the role of PCNA in the DNA repair processes must be also taken into consideration in the evaluation of the cell-labelling

index changes after the treatments (Barni et al., 2006), and also by the higher values of PCNA L.I. in comparison with the TUNEL L.I., particularly in the epithelial cells. As the nucleic acids injury concerns, Li et al. (2005) observed a dose-dependent damage to mitochondrial DNA after SWCNT treatment as a consequence of oxidative stress. More recently, Muller et al. (2008b) demonstrated clastogenic and aneugenic effects of MWCNTs in lung pnemocytes both *in vivo* and *in vitro* experiments; in particular, after a single intratracheal exposure in rats (0.5 or 2 mg), a dose-dependent increase in micronuclei formation was found. The genotoxic effects have also been related with the intrinsic action of CNTs to produce oxidants such as ROS and RNS, and these catalytic effects may be implemented in the presence of inorganic or organic substances incorporated by the nanotubes (Muller et al., 2008b). The importance of oxidative stress in pulmonary inflammation is strongly supported by a significant number of studies in which several antioxidants, mainly precursors of glutathione, such as N-acetyl-L-cysteine (Sadowska et al., 2006), or superoxide dismutase (SOD) and SOD mimetics (Salvemini et al., 2002; Kinnula and Crapo, 2004) have been successfully employed as protective anti-inflammatory agents. Contrastingly, some experiments here demonstrated that MWCNTs do not directly generate either oxygen or carbon-centred free radicals, suggesting that metal catalyst contaminates were responsible for the ROS generation (Fenoglio et al., 2006; Shvedova et al., 2009).

Regarding the cytokinetic parameter changes, our results seems to be in line with previous literature data (Magnan et al., 1996; Lam et al., 2006), showing an increase of the two inflammatory cytokines, namely IL-6 and TGFß1, produced by the lung cells and involved in mediating tissue injury and repair during the cytotoxicity of the pulmonary tissue after CNT exposure. Accordingly to the MWCNT-induced histopathological changes connected to cytokines release evidenced by Muller et al. (2005), we showed an extensive, widespread localization of IL-6 and TGFß1, in the bronchiolar, alveolar and stromal cells, evidencing both the cellular inflammatory reaction to CNT instillation and the pleiotropic peptide-mediating tissue repair.

The transforming growth factor TGFß1 has been demonstrated to play a pivotal role in the balance of cell growth, differentiation and apoptosis, also stimulating cell proliferation in different cell types (epithelial cells, fibroblast, myofibroblast, etc.) both *in vitro* and *in vivo* (Chen et al., 2003; Haider et al., 2007), also during the stromal remodelling events derived from the pulmonary parenchyma injury (Parra et al., 2006) in which collagen Type I also appear to be involved. Regarding this latter marker, in the present study, we evidenced that, the immunohistochemical labelling changes in lung specimens between control and CNT-treated rats appeared mild, only detected at the level of some collapsed thick-walled alveoli, possibly demonstrating that tissue repair is not excessive, occurring physiologically without overt signs of fibrosis (e.g. absence of collagen accumulation) and granuloma formation.

Altogether the present results support the evidence that intratracheal instilled MW-CNTs can induce lung toxicity associated with inflammation, irrespectively of nanotubes functionalization.

These effects have been clearly demonstrated after exposing the lab animals to these nanomaterials by i.t., supporting the notion (Donaldson et al., 2009; Park et al., 2009) that *in vivo* studies are essential in the safety evaluation of nanomaterials, together with *in vitro* investigations. Indeed, accordingly to the international guidelines and regulatory bodies suggestions (Stone et al., 2009), we have developed a tiered strategy of tests starting with a first-in vitro-stage aimed at investigating general toxicity endpoints (see Coccini et al., 2010), followed by *in vivo* experiments. Thus, the present study, being the subsequent second essential stage, has given essential insight, contributing to clarify CNT toxicity mechanisms and to understand the different CNT toxicity targets, at different pulmonary cytochemical levels, in order to improve the overall understanding of the possible adverse outcomes resulting from CNT exposure, with the final goal of predicting human health hazards.

Acknowledgements. This research was supported by the Italian Ministries of Health, Education and Research (Grant: PRIN # 2007FA34TE). The authors wish to thank Professors P. Mustarelli and A. Profumo, who kindly provided and characterized the carbon nanotubes, and Dr. V. Bertone for his helpful technical assistance.
Conflicts of interest. The authors have no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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Accepted October 4, 2010