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Carbon nanotubes toxicology and effects on metabolism and immunological modification *in vitro* and *in vivo*

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Abstract

The aim of this research is focused on the biological effects of multi wall carbon nanotubes (MWCNTs) on three different human cell types, laboratory animals *in vivo*, and immunological effects. Large numbers of researchers are directly involved in the handling of nanostructured materials such as MWCNTs and nanoparticles. It is important to assess the potential health risks related to their daily exposure to carbon nanotubes. The administration of sterilized nanosamples has been performed on laboratory animals, in both acute and chronic administration, and the pathological effects on the parenchymal tissues have been investigated. We studied the serum immunological modifications after intraperitoneal administration of the MWCNTs. We did not observe any antigenic reaction; the screening of ANA, anti-ENA, anti-cardiolipin, C-ANCA and P-ANCA was negative. No quantitative modification of immunoglobulins was observed, hence no modification of humoral immunity was documented. We also studied the effects of MWCNTs on the proliferation of three different cell types. MCF-7 showed a significant inhibition of proliferation for all conditions studied, whereas hSMCs demonstrated a reduction of cell growth only for the highest MWCNTs concentrations after 72 h. Also, no growth modification was observed in the Caco-2 cell line. We observed that a low quantity of MWCNTs does not provoke any inflammatory reaction. However, for future medical applications, it is important to realize prosthesis based on MWCNTs, through studying the corresponding implantation effects. Moreover, it has to be emphasized that this investigation does not address, at the moment, the carcinogenicity of MWCNTs, which requires a detailed follow-up investigation on the specific topic. In view of the subsequent and more extensive use of MWCNTs, especially in applications where carbon nanotubes are injected into the human body for drug delivery, as a contrast agent carrying entities for MRI, or as the basic material of a new prosthesis generation, more extended tests and experiments are necessary.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Since their discovery in 1991 by Sumio Iijima (NEC Laboratories) [1], carbon nanotubes have attracted great interest, thanks to their properties of elasticity, high tensile strength and electrical conductivity. The MWCNTs belong to the class of recently discovered technological materials. Nowadays, such innovative materials are interesting not only for their physical and chemical properties but also for their possible pharmacological and prosthetic employment. With so many researchers all over the world involved in the nanotechnology field, it is important to assess human health risks related to the daily exposure to these potentially hazardous materials. The scientific community is most concerned about the toxicity of carbon nanotubes because of their structural resemblance to asbestos. Inhalation of asbestos fibres is known to induce asbestosis (a progressive fibrotic disease of the lung), lung cancer, and malignant mesothelioma of the pleura. The role of asbestos in lung cancer is still under debate and, unfortunately, experiments performed on rats or guinea pigs [2] are not conclusive because their life time is shorter than the time necessary to follow-up an experiment of this kind. Exposure of professional personnel and the general public to nanostructured materials are expected to increase dramatically in the coming years. First of all, this is because carbon nanotubes are considered to be key elements in nanotechnology for their potential applications in biomedicine, nanoelectronics, mechanical engineering and oncology therapy. Thus, it is important to know the health hazards related to the exposure to carbon nanotubes. Significant progress has been reported in incorporating carbon nanotubes in biological applications as new methods of drug delivery [3]. Also, the toxicity of water-soluble MWCNTs has been discussed [2–4]. The aim of this work is to investigate the effects of sterilized MWCNTs *in vivo* on laboratory rats in acute and in chronic intraperitoneal administration. Tissue pathological examination and toxicity tests on laboratory rats were performed. Serum immunity conditions were tested on laboratory rats before and after MWCNTs intraperitoneal administration. We also investigated the effects *in vitro* on three different cellular clone culture administrations. Commercial MWCNT, (Aldrich 659258) were obtained from Sigma-Aldrich, Milan, Italy. The characteristics of the commercial nanotubes as declared by the manufacturer are as follows: the Aldrich 659258 MWCNT sample is largely made of multi-walled nanotubes (at least 90%), with residual amorphous carbon and no metal contaminant >0.1%; the Aldrich 659258 MWCNTs have diameter 110–170 nm, length 5–9 μm, density 1.35 g cm^{-3}, surface area 130 m^2 g^{-1}. Figures 1(a) and (b), show Aldrich 659258 MWCNT TEM and SEM microphotographs we obtained using, respectively, a JEOL JEM 2010 microscope and a ISI ABT-DS 130 S microscope.

2. Materials and methods

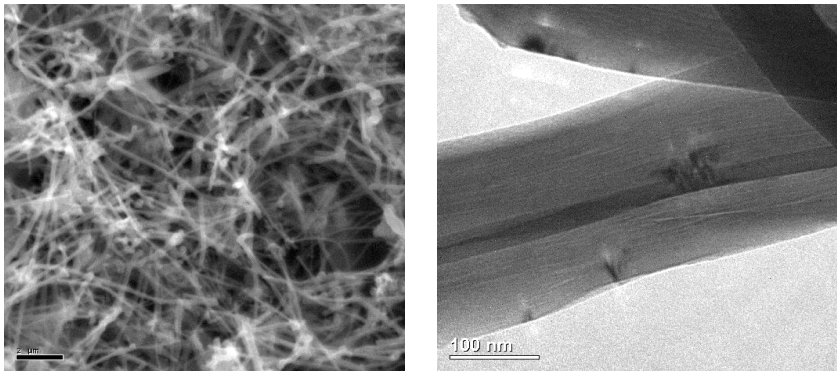
MWCNTs can be readily functionalized by means of chemical groups, with the purpose of making them into efficient carriers for chemicals and drugs [5–8]. Building upon the results of earlier studies of the chemical and physical structure and properties of MWCNTs [9–14], we focused our attention on determining the best techniques to achieve the sterilization of MWCNTs [15–17], without modification of structure and properties, in order to reduce as much as possible the bias effect on the subsequent experiments *in vitro* and *in vivo*.

We realized the sterilization procedure by means of first placing the MWCNT powder in a little polyethylene bag, and then by storing the bag with chemical indicators, showing that a package has been through a sterilization cycle, clearly visible on the outside of each little bag but inside the bigger bag. The packages then were submitted to the sterilization procedure in a steam autoclave at 270 °F (132 °C) for 3 min in a gravity displacement steam sterilizer. This method checks the highest temperature that is reached during sterilization and the length of time that this temperature is maintained. In addition, heat- and steam-sensitive chemical indicators do not reliably document sterility, but they do show that an item has not accidentally bypassed a sterilization process. Microbiological monitoring of steam sterilizers is applied at least once a week with commercial preparations of spores of *Bacillus stearothermophilus* for steam sterilizers (a microorganism having spores that are particularly resistant to moist heat, thus assuring a wide margin of safety). If a sterilizer is working properly and used appropriately, the spores are usually killed. Then we repeated and verified experiments on earlier studied cellular clones [18], and performed the experiment on new cellular lines. In the next stage we extended our experience also *in vivo* on laboratory rats and mice. We tested the effect of MWCNTs with an intraperitoneal administration and also repeated the same injection with the same dose every day for 7 days. We tested the effects of MWCNT implantation and determined the toxicity, survival and modifications of the immunological system after MWCNT administration. Finally, we studied pathological tissue modifications in A and B groups to understand whether MWCNTs are innocuous or not, when introduced inside the body, either as an environmental pollution agent [19] or as a future therapeutical application vehicle. Notice that the MWCNTs we studied have just recently been shown to hamper the barrier function of human airway epithelial cells [20].

2.1. *In vitro tests*

MWCNTs were placed in bidistilled water and dispersed by centrifugation and sonication (30 min), in order to get the homogeneous suspensions which were to be used for the cell count assay.

2.1.1. Cell culture. The human colorectal cancer cell line Caco-2 and the human breast adenocarcinoma cell line MCF-7 were obtained from the European Collection of Cell Cultures (ECACC). Primary human arterial smooth muscle cells (hSMCs) were isolated from a thyroid artery by a collagenase type II digestion and used for experiments from passages 4 to 8. SMCs were identified by positive (>95%) staining with a monoclonal antibody to muscle specific



(a) **(b)**

Figure 1. (a) SEM image of as received MWCNTs; (b) TEM image of as received MWCNTs.

alpha-actin. All cells were seeded into 25 cm^2 flasks (Falcon; Becton Dickinson Laboratoryware; Franklin Lakes NJ, USA) in Dulbecco modified Eagle medium (DMEM) supplemented with 10% foetal calf serum (FCS) and antibiotics (Penicillin 100 IU/ml, Streptomycin 100 μg ml^{-1}, Gentamicin 200 μg ml^{-1}) (standard medium). The cultures were kept at 37 °C in an atmosphere of 5% CO_2 in air. The medium was changed every 3th day. At confluence, the cells were subcultured after removal with 0.05% trypsin–0.01% EDTA. Cell viability was assessed with the Trypan Blue (Sigma Chemical Co., St. Louis MO, USA) dye exclusion method.

2.1.2. Cell proliferation assay. Caco-2 cells, MCF-7 cells and hSMCs were seeded in 6-well culture plates (Falcon, Becton Dickinson Laboratoryware) at a concentration of 1×10^5 cells/well in a standard medium. The following day, the cells were re-feed with a standard medium containing MWCNTs at concentrations ranging from 0.001 to 0.1 mg ml^{-1}. The plates were incubated for 24, and 72 h at 37 °C in an atmosphere of 5% CO_2 in air. The cells were then detached from the wells by trypsinization and centrifuged, and cell pellets were resuspended in PBS. The cell count was performed by a particle count and size analyzer (Beckman Coulter, Inc), and by a Thoma haemocytometer. Two replicate wells were used for each data point, and every experiment was performed three times.

2.2. Statistical analysis

Data were expressed as mean ± standard deviation (SD). Data were statistically analysed with the *analysis of variance* (ANOVA) followed by the Bonferroni post-test. Differences were considered significant at the level of $p < 0.05$. Statistical analysis was performed by using GraphPad Instat software (GraphPad Software, Inc; San Diego, CA, USA).

2.3. In vivo general effects

In our experiments we used male CD1 Swiss mice, weighing 27–32 g, purchased from Charles River (Calco-Lecco, Italy). In order to obtain a stable solution we tried to dissolve MWCNTs in several vehicles. They were insoluble in saline solution, also after sonication for as long as 90 min (the difference with respect to the homogeneous suspension of MWCNTs in bidistilled water used for a cell count assay, see *in vivo* tests above, is probably due to the much larger amount of solution needed for the *in vivo* tests, where the MWCNTs have a higher tendency to aggregate) and the suspension in methylcellulose (MTC 0.5%) exhibited precipitation of the nanoparticles. At last, we obtained a stable suspension using a mixture of Tween 80 (5%), PEG 400 (5%) and methylcellulose (MTC 0.5%). Nanotubes were dissolved in a vehicle volume suitable to administer 1 ml of suspension for 100 g of body weight i.e. 10 ml kg^{-1} body weight. Control animals were treated with the vehicle. MWCNTs were administered intraperitoneally (i.p.) at concentrations of 10, 20, 40 mg kg^{-1} body weight in groups of 3–5 animals each.

The animals were examined by the Irwin test [21]. The Irwin test is used in basic pharmacological screening to assess the effects induced by new drugs. After the treatment the animals were housed in a cage for 20 min and observed for any behavioural change. Then the animals were placed on the table and observed to evaluate the pathologic signs exhibited (tremors, twitches, convulsions, pallor, cyanosis) or the alterations of the physiological functions (righting reflex, pupil size, body tone and muscle tone). The animals were observed during the following four hours to check for immediate death, then housed in cages and monitored over 7 days to note the delayed toxicity. After 7 days the animals were weighed and sacrificed in a chamber saturated with CO_2 in order to carry out the necroscopic examination. Samples of all parenchymatous viscera were collected in order to perform the histological examination. Animal care complied with the principles of laboratory animal care as formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Sciences, National Institute of Health, publication no. 80, 23 revised 1985).

2.4. Repeated administration test

In order to highlight the effects of MWCNTs after repeated administrations, a group of four mice was treated daily

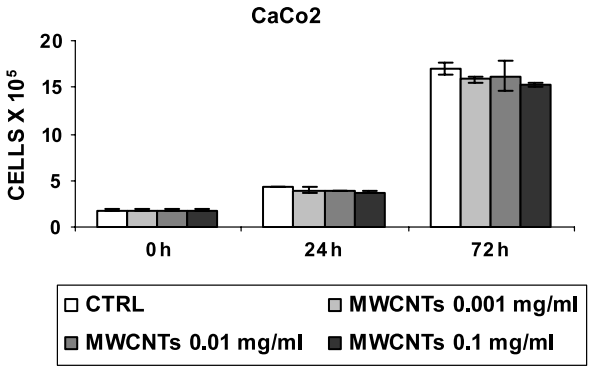


Figure 2. Effects of MWCNTs on the proliferation of the human colorectal cancer cell line Caco-2. The cells were treated with MWCNTs at concentrations from 0.001 to 0.1 mg ml^{-1} for 24 and 72 h. Cell proliferation was determined by a count performed by a particle count and size analyzer. Each column represents the mean value ± SD of three separate experiments performed in duplicate.

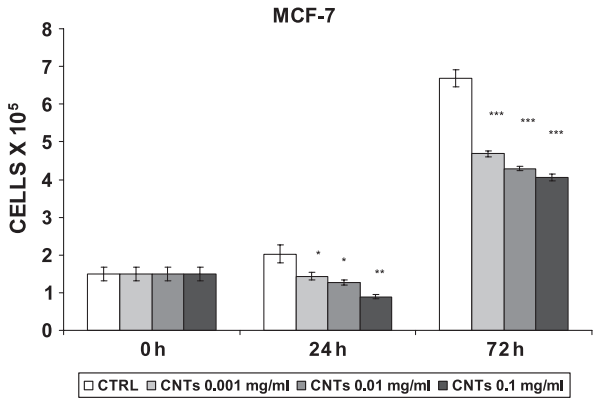


Figure 3. Effects of MWCNTs on the proliferation of the human breast adenocarcinoma cell line MCF-7. The cells were treated with MWCNTs at concentrations from 0.001 to 0.1 mg ml^{-1} for 24 and 72 h. Cell proliferation was determined by a count performed by a particle count and size analyzer. Each column represents the mean value ± SD of three separate experiments performed in duplicate. * $p < 0.05$ versus control (CTRL); ** $p < 0.01$ versus CTRL and $p < 0.05$ versus time 0 count; *** $p < 0.001$ versus CTRL.

intraperitoneally (i.p.) for 7 days with the substance at the dose of 5 mg kg^{-1} suspension with a mixture of Tween 80 (2.5%) and methylcellulose (MTC) 0.5%. The day after the last administration, the animals were weighed and sacrificed in a chamber saturated with CO_2, necroscopy was carried out and tissue samples were collected for the histological examination by microscopic studies.

2.5. MWCNT implantation tests

In order to evaluate the MWCNT implantation effects we treated two 'Sprague Dawley' 300 g male rats. We performed a general anaesthesia with sodium pentobarbital in saline solution at a dose of 53 mg kg^{-1} for intraperitoneal administration through the left iliac fossa at a volume of 1 ml kg^{-1} of body weight.

In the *first experiment* a surgical incision was performed on the back of the rat deep into the muscular fascia. A pocket was created between the muscular fascia and the lumbar muscular tissue with no bleeding. 20 mg of MWCNTs were placed into the pocket and the scar was sutured with absorbable stitches on fascia incision and nonabsorbable scar.

The *second experiment* was performed with the same surgical technique but employing only 5 mg of MWCNTs.

2.6. Immunological assessment

Mice are genetically identical animals employed for laboratory experiments. They have identical genetic assessment, so they react in the same manner to the same stimulation. For this reason we could aggregate the small blood quantities taken from the heart ventricular cavity at the moment of death. The A-group mice were treated only with the control carrier solution, and the B-group mice were treated with MWCNTs diluted in the carrier solution, both of them at 10 mg/pro Kg of body weight concentration. We tested ANA (anti-nuclear antibody), AMA (anti-mitochondrial antibody), ASMA (anti-smooth muscle antibody), ANTIRET (anti-reticolin antibody), APG (anti-gastric wall antibody), ENA (extractable anti-nuclear antigen), and NDNA (native deoxyribonucleic acid).

2.7. Histological examination

In the first stage of our experiment the study material was based on 24 mice and 2 rats. We obtained samples of all viscera, fixed them in Formalin 4% phosphate buffer with pH 7.6, and embedded them in paraffin and then 3 μm sections coloured with Haematoxylin and Eosin staining were prepared 40 times.

3. Results

3.1. Cell proliferation assay

In the human colorectal cancer cell line, Caco-2, treatment with MWCNTs, added to culture medium at concentrations from 0.001 to 0.1 mg ml^{-1} for 24 and 72 h, did not induce any modification of cell growth as compared to the control (not treated cells) (figure 2 and table 1).

In the human breast adenocarcinoma cell line, MCF-7, treatment with MWCNTs, added to culture medium at concentrations from 0.001 to 0.1 mg ml^{-1} for 24 and 72 h, determined a statistically significant reduction of cell proliferation as compared with the control, in a dose-dependent manner (figure 3 and table 2). Moreover, the number of cells treated with MWCNTs for 24 h turned out to be lower than the number of cells counted at time 0, even though only the reduction of the proliferation of the cells exposed to 0.1 mg ml^{-1} was statistically significant ($p < 0.05$) (figure 3 and table 2).

The hSMCs treatment with MWCNTs, added to the culture medium at concentrations from 0.001 to 0.1 mg ml^{-1} for 24 h, resulted in a slight, but insignificant decrease in the cell number as compared with the control (figure 4 and table 3). Treatment with MWCNTs for 72 h gave a statistically significant reduction of cell proliferation at the highest MWCNTs concentrations, but there was no modification of cell growth at a concentration of 0.001 mg ml^{-1} (figure 4).

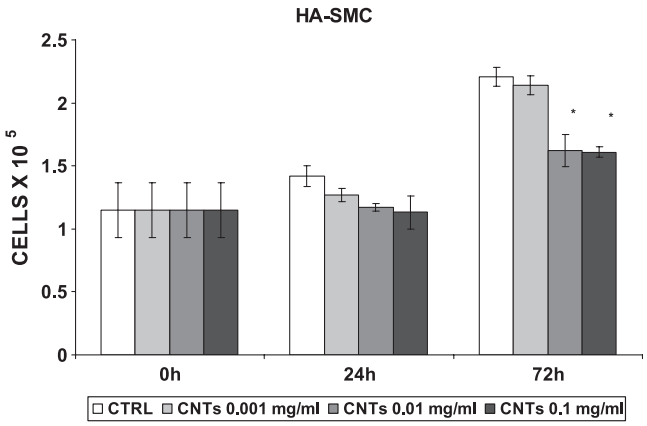


Figure 4. Effects of MWCNTs on the proliferation of the human arterial smooth muscle cells (hSMCs). The cells were treated with MWCNTs at concentrations from 0.001 to 0.1 mg ml^{-1} for 24 and 72 h. Cell proliferation was determined by a count performed by a particle count and size analyzer. Each column represents the mean value ± SD of three separate experiments performed in duplicate. $^{*}p < 0.01$ versus CTRL.

Table 1. Human colorectal cancer cell line Caco-2 matched with MWCNT dilution from 0.001 to 0.1 mg ml^{-1} concentration.

CaCO2			
MWCNT dilution (mg ml^{-1})	0 h	24 h	72 h
0	1.8 ± 0.13	4.34 ± 0.037	16.99 ± 0.61
0.001	1.8 ± 0.13	3.96 ± 0.38	15.87 ± 0.33
0.01	1.8 ± 0.13	3.88 ± 0.027	16.19 ± 1.56
0.1	1.8 ± 0.13	3.75 ± 0.19	15.28 ± 0.14

3.2. General in vivo effects after acute treatment

In the Irwin test no significant neurovegetative or behavioural effects of MWCNTs were registered at all doses tested (10, 20, 40 mg kg^{-1} body weight), except for a slight reduction of spontaneous activity just after the treatment, likely a consequence of peritoneal irritation resulting from the treatment. At the highest dose tested (40 mg kg^{-1}), animals exhibited, 30 min after the treatment, a deep violet colour of the testicles that disappeared in the subsequent hours.

During the seven days post-treatment period the death of one animal, both at 20 and at 40 mg kg^{-1} was registered; the control animal did not exhibit mortality. The total mortality after seven days of monitoring is reported in table 4. The body weight of the animals after the seven day period increased in all groups, although this was more evident in control groups (table 5).

On the seventh day all animals were weighed and then sacrificed. A small body weight increase was observed in the 10 mg kg^{-1} group. The expected body weight increase was not observed at 20 and 40 mg kg^{-1} as shown in table 5.

In the necroscopic examination, viscera appeared normal, in both colour and size, for all tested doses. In mice treated with 40 mg kg^{-1} we registered adhesive peritonitis, involving all viscera, while no pathologic signs either in the pleural or the pericardial cavity were observed. All membranes were

Table 2. Human breast adenocarcinoma cell line MCF-7 matched with MWCNT dilution from 0.001 to 0.1 mg ml^{-1} concentration.

MCF-7			
MWCNT dilution (mg ml^{-1})	0 h	24 h	72 h
0	1.5 ± 0.18	2.02 ± 0.24	6.68 ± 0.23
0.001	1.5 ± 0.18	1.43 ± 0.1	4.68 ± 0.08
0.01	1.5 ± 0.18	1.27 ± 0.06	4.29 ± 0.05
0.1	1.5 ± 0.18	0.89 ± 0.054	4.05 ± 0.09

Table 3. Human arterial smooth muscle cells line hSMC matched with MWCNT dilution from 0.001 to 0.1 mg ml^{-1} concentration.

hSMC			
MWCNT dilution (mg ml^{-1})	0 h	24 h	72 h
0	1.15 ± 0.22	1.42 ± 0.084	2.21 ± 0.075
0.001	1.15 ± 0.22	1.27 ± 0.055	2.14 ± 0.076
0.01	1.15 ± 0.22	1.17 ± 0.029	1.62 ± 0.127
0.1	1.15 ± 0.22	1.13 ± 0.129	1.61 ± 0.044

Table 4. Mortality of animals after single dose administration.

Dose (mg kg^{-1} i.p.)	Animals dead (%)
10	0
20	20
40	33

Table 5. Weight of the animals (mean ± SE) at the start (G_0) and 7 days after the treatment (G_7).

	Body weight (g ± SE)			
	Controls		Treated	
Dose (mg kg^{-1})	G_0	G_7	G_0	G_7
10	30.4 ± 1.2	33.8 ± 1.3	29.2 ± 0.6	31.6 ± 1.0
20	30.7 ± 1.1	33.9 ± 1.4	32.6 ± 2.1	32.8 ± 2.1
40	30.5 ± 0.2	34.0 ± 0.1	27.9 ± 0.9	29.9 ± 1.6

adherent among themselves and nanotube aggregates were observed. At a dose of 10 mg kg^{-1} the animals showed light peritoneal irritation, involving cava viscera, without nanotube aggregates deposition. No pathologic signs either in the pleural or the pericardial cavity were registered. Finally, at a dose of 20 mg kg^{-1} we registered adhesive peritonitis, involving all viscera, but less aggressive than in the previous cases, and without evidence of nanotube aggregates deposition. No pathologic signs either in the pleural or the pericardial cavity were registered. In the animals treated with 40 mg kg^{-1} body weight an adhesive peritonitis in the entire peritoneal cavity was evident. All gut and parenchymatous organs were packed together, and it was possible to observe a black precipitate of MWCNTs. The peritoneum was opaque. No modifications were observed, either in the pleuric or the pericardia cavity. We also prepared blood samples in order to evaluate the immunological parameters.

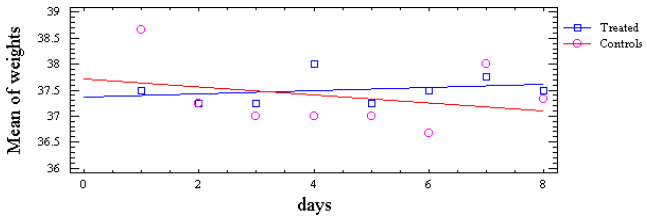


Figure 5. The treated group's body weight has an increasing trend and the control group has a decreasing trend.

3.3. General in vivo effects after repeated administrations

The treatment of animals with MWCNTs at 5 mg kg^{-1} i.p. for 7 days did not induce behavioural effects or mortality in mice. The body weight was unchanged in all animals after 7 days of treatment. At the necroscopical examination, performed after the animals were sacrificed by CO_2 inhalation, widespread intraperitoneal depositions of MWCNTs and several connections between the liver surface and adjacent organs were observed. The liver showed an inhomogeneous structure. Control animals appeared quite normal in the necroscopic examination, except for one mouse whose liver showed whitish dots.

3.4. Statistical analysis

The statistical evaluation was carried out with Statgraphics Plus 4.1 PC software. We studied the comparison of body weight variation regression lines for two mice groups. The treated group received MWCNTs and solvent at the concentration of 5 mg per kg of body weight, administrated by daily intraperitoneal injection for 7 days. The control group received only the solvent in the same way. Even though the results are not significant, it is possible to observe that the treated group body weight has a daily 0.03 g increasing trend and the control body weight group has a daily 0.77 g decreasing trend, see figure 5.

3.5. Implantation test

The first animal died after 180 min. In the necroscopic examination we observed acute lung oedema, a pleural reaction and a pleuric inflammatory liquid in the pleuric cavity. We also observed ischaemic myocardial and brain necrosis. The implantation site was characterized by a local moderate inflammatory reaction. The second animal on the seventh day was weighed and dissected for the necroscopic examination. We observed a normal pleuric cavity, normal heart and pericardia, normal abdomen and peritoneal serosa. The implantation site showed a moderate inflammatory and fibrotic reaction.

3.6. Immunological assessment

Within the time and technique limits the immunological markers: ANA (anti-nuclear antibody), AMA (anti-mitochondrial antibody), ASMA (anti-smooth muscle antibody), Antiret (anti-reticolin antibody), APG (anti-gastric wall antibody),

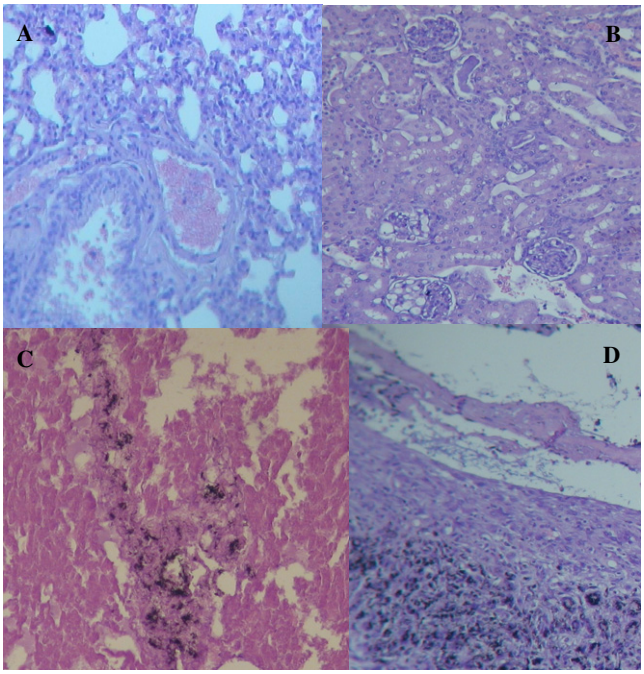


Figure 6. (A) Intense congestion of alveolar septa, emphysematous bullae, alveolar atelectasia, endoalveolar extravasations, flooding with red blood cells, thickening of alveolar septa. (B) Vacuolation of 60% of renal glomeruli. Presence of cylinders inside the tubules. (C) Massive full-thickness intestinal necrosis, muscular necrosis with residual pigmented area (MWCNTs). (D) Granulomatous giant cellular reaction of implantation site under muscular fascia, connected to fibrin deposits in reaction to implantation of 20 mg of MWCNT powder.

ENA (extractable anti-nuclear antigen), NDNA and (native deoxyribonucleic acid), turned out to be negative both in the A-group mice, treated only with the solution, and in the B-group mice, treated with MWCNTs diluted in the carrier solution, at 10 mg/pro kg of body weight concentration. We did not observe any antigenic reaction, as the screening of ANA, anti-ENA, anti-cardiolipin, C-ANCA and P-ANCA was negative (see table 6). No quantitative modification of immunoglobulins was observed, and so no modification of humoral immunity was documented.

3.7. Histological evidence

In the pulmonary parenchyma the administration of MWCNTs determined intense congestion, failure of alveolar septa, emphysematous bullae, intense congestion with alveolar atelectasia, endoalveolar extravasations, alveolar flooding with red blood cells and thickening of alveolar septa (see figure 6(A)). The kidney parenchyma shows alteration of the filtering organs, vacuolation of the filtering component in 60% of renal glomeruli, as well as the presence of MWCNTs inside the tubules (see figure 6(B)). The large intestinal wall exhibits a diffuse and massive full-thickness intestinal necrosis and muscular necrosis with residual pigmented area (owing to the presence of MWCNTs) within the muscular necrosis area (see figure 6(C)). The rat implantation site lesion displays a granulomatous giant cellular reaction, with red blood cells and

Table 6. Immunological markers for both A-group and B-group mice, showing the absence of antigenic reactions.

A-treated		B-control	
ANA	Neg	ANA	Neg
AMA	Neg	AMA	Neg
ASMA	Neg	ASMA	Neg
Antiret	Neg	Antiret	Neg
APG	Neg	APG	Neg
ENA	Neg	ENA	Neg
NDNA	Neg	NDNA	Neg

granulocytes creating a sort of cavity (see figure 6(D)). In the subcutaneous tissue, under the muscular fascia, a pseudo-cystic formation is reported: it is surrounded by a granulomatous giant cellular reaction connected to a necrosis and fibrin deposits, as a consequence of the implantation of 20 mg MWCNT powder. A massive necrosis of the seminiferous tubules of the testicle is observed.

4. Discussion of *in vitro* experiments

In our *in vitro* study, three different cell types were exposed in culture to various concentrations of MWCNTs, in order to estimate their cytotoxic effects. Interestingly, the three cell types gave different responses to the treatment with MWCNTs. The breast adenocarcinoma cells, MCF-7, showed a clear inhibition of the proliferation, when treated with MWCNTs at all concentrations used, for both 24 and 72 h of incubation. In particular, after the first 24 h of treatment, at an MWCNT concentration of 0.1 mg ml^{-1}, there was a significant decrease in the cell number, as compared with the number of cells present in the culture at time 0. This result indicates that even early on, MWCNTs exert a strong cytotoxic action on these cells. After 72 h the cell count assay demonstrated that all cellular samples proliferated, but at the highest MWCNT concentrations the number of cells was significantly lower than the control. These results are consistent with the findings of other authors who demonstrated the toxicity and hazardous effects of carbon nanotubes on a variety of cultured cells, although there exist many different morphologies of MWCNTs, which can also be chemically or functionally modified. For example, Bottini *et al* showed that MWCNTs caused a time-and dose-dependent decrease in the viability of Jurkat T leukaemia cells and an induction of apoptosis in the same cells and in freshly isolated primary human T-lymphocytes [22]. Sato *et al* demonstrated the influence of the length of MWCNTs on the cytotoxicity; they performed their *in vitro* studies on a human acute monocytic leukaemia cell line THP-1 [23]. Monteiro-Riviere *et al* showed that the interaction of MWCNTs with human epidermal keratinocytes led to the internalization of nanotubes in the cytoplasm of the cells, as determined by transmission electron microscopy, and demonstrated both a cytotoxic effect and an increase of the release of IL-8, an irritation marker [24]. Finally, Magrez *et al* [3] showed that MWCNTs induced proliferation inhibition and cell death in three different human lung-tumour cell lines, and that the cytotoxic effects were enhanced by an acid pre-treatment of the particles. In our experiments hSMCs showed a significant inhibition of cell proliferation with respect to control only after 72 h of treatment with MWCNTs at the concentrations of 0.1 and 0.01 mg ml^{-1}; at the lowest concentration tested, there was no inhibition of the cell growth. After 24 h of treatment hSMCs did not show any significant variation in the proliferation pattern in all conditions studied. Our results are in accordance with a study performed on rat aortic smooth muscle cells treated with SWCNTs (single-walled carbon nano tubes) that demonstrated the lack of growth inhibition at the end of the 1st day of cell culture, whereas there was a significant dose-dependent decrease in cell proliferation from day 2.5 to day 3.5 for concentrations from 0 to 0.1 mg ml^{-1} [25]. In the human colorectal cancer cell line Caco-2, the treatment with MWCNTs did not produce any modification of cell growth as compared with the control. The surprising result seems to be consistent with a study in which various kinds of CNTs (SWCNTs and MWCNTs) did not determine any acute toxicity on cell viability and apoptosis in two cell types, i.e. the rat alveolar macrophage cell line NR8383 and the human lung epithelial cell line A549 [26]. Moreover, the MWCNT treatment in both cell lines did not induce the release of inflammatory mediators, but it produced a dose- and time-dependent increase of the intracellular reactive oxygen species (ROS) [26]. In conclusion, our *in vitro* studies showed that the anti-proliferative response to MWCNTs may differ, depending on the cell line and tissue types used. It is necessary that other effects of MWCNTs on various cell types, such as cytokine release and ROS production, be studied, in order to explain the true hazard of these nanostructures.

In order to explore the effect of surface chemical properties on the toxicity, Magrez *et al* [3] performed a set of experiments in which they modified the surface chemistry of the filaments. The surface of MWCNTs has been decorated according to the method reported by Hiura *et al* [27] that involved a chemical modification of the outer layer of the MWCNTs after acid treatment. This procedure results in adding carbonyl (CdO), carboxyl (COOH), and/or hydroxyl (OH) groups onto the nanotube surface. In their experiment, the cells grown in a plain gelatin containing medium served as a reference. When the number of viable cells is compared after the treatment with nanotubes, it becomes evident that the toxicity increases with the chemical surface treatment. This is significant in the case of MWCNTs. The latter observation might be somewhat obscured by the fact that a relatively high toxicity already occurred with unmodified nanotubes. Nevertheless, these results clearly demonstrate that grafting additional, putatively 'toxic', chemical groups on the surface of MWCNT reduces the number of viable cells significantly. These experiments demonstrate that carbon nanotubes generally lead to proliferation inhibition and cell death as showed also by our studies. Although carbon nanotubes are less toxic than carbon nanoparticles, the toxicity of carbon nanotubes increases significantly when carbonyl (CdO), carboxyl (COOH), and/ or hydroxyl (OH) groups are present on their surface. Also for us the exact mechanisms that lead to cell death are still unclear, but nanotubes can induce cell death either after contact with cell membranes or after their internalization, and, in order to demonstrate the real

mechanism, we are going to go on with the experiment. To understand all these mechanisms is mandatory, if we want to use these new devices as an NMR contrast medium, or as anticancer systemic therapy carrier, as well as to create new prosthesis generation.

5. Discussion of *in vivo* experiments

5.1. Single administration experiment

The doses of 5, 10 mg kg^{-1} of body weight are harmless. The dose of 20 mg kg^{-1} of body weight is fatal in 20% of cases in the 1st 24 h and the dose of 40 mg kg^{-1} of body weight is fatal in 33% of the cases in the 1st day.

5.2. Repeated administration experiment

The dose of 5 mg kg^{-1} of body weight is practically innocuous. No lesions are reported either in the abdominal cavity or in pleural or pericardial cavities. Only a small irritation was observed at the injection point.

5.3. Implantation of MWCNTs experiment

The dose of 20 mg kg^{-1} of body weight was fatal within 180 min. It is possible that an excessive quantity of MWCNTs caused the death. In fact 5 mg of MWCNTs neither caused illness, or tissue necrosis, or suffering, but only a granulomatous reaction to a foreign body, which is well tolerated by the subject. For the first rat experiment we presume that the dose of nanotubes was excessive so that:

- It caused an intensive phlogistic reaction that provoked the capillary vessels dilatation and tissue oedema;
- The reabsorbing of the interstitial liquid by the lymphatic circuit caused the removal of the nanotubes from the implantation site and the migration around the body;
- The introduction of MWCNTs from the thoracic duct into the anonym trunk, and afterwards into the cava superior vein and into the heart, occurred. From the heart the MWCNTs were ejected into the pulmonary blood circuit and provoked the necrosis by internalization in the alveolar cells and hence the acute pulmonary oedema;
- Consequently we presume the starting of a respiratory distress and brain and heart ischaemic disease.

For the second case, we think that the low-quantity dose was not enough to provoke a massive phlogistic reaction, and so the MWCNTs did not move from the implantation site. In fact only a low phlogistic reaction was observed by the pathologist in the implantation site, which is well tolerated by the subject.

5.4. Immunological assessment modifications after MWCNT administration

We investigated whether any modification in the immunological assessment occurred, seven days after administration of MWCNT dilution in group A, and seven days after solvent administration in control group B. We did not find any variation in the antibody assessment, that is to say that at the dose administrated MWCNTs are not dangerous for laboratory animals. This result suggests that neither MWCNTs nor the solvent, within time and dosage limits imposed, provoke a phlogistic reaction.

5.5. Histological examination

We believe that the glomeruli damage provoked by MWCNTs causes urine production, tissue oedema and, probably for this reason, a small increase in the body weight of treated mice. The repeated intraperitoneal injection of solvent provokes the reduction in the body weight of control mice, because the latter does not eat its fodder.

6. Conclusions

- It is useful to continue studying MWCNTs, in order to develop a statistically significant number of cases.
- Although SWCNTs are more expensive to produce, it would be interesting to study the corresponding cell culture and animal behaviour comparing the results with the MWCNT case.
- The cytotoxicity of carbon nanotubes increases significantly when carbonyl (CdO), carboxyl (COOH), and/or hydroxyl (OH) groups are present on their surface. Carbon nanotubes can induce cell death, either after contact with cell membranes, or after their internalization. The exact mechanisms that lead to cell death are still unclear. In the present work we did not consider nanotubes functionalized by chemical groups on their surface, but we plan to tackle such a task in future experiments aimed at clarifying the above-mentioned mechanisms of interaction between the cells and nanotube surfaces.
- The higher the MWCNT concentration, the shorter is the growth velocity of the cells in culture. However, in our experience the human colorectal carcinoma cell line Caco-2 is insensitive to the MWCNT's presence. It would be interesting to understand this protective mechanism and further investigation is required, in order to obtain a better interpretation of the data. In truth no other results are available at the moment.
- We did not find any variation in the antibody assessment, as to say that at the dose administrated MWCNTs are not dangerous for laboratory animals. Perhaps the immunological system is not activated or it could be suppressed by the MWCNT's toxicity, we do not know yet. We have to study the limit, if there is one, to understand if we can functionalize the MWCNTs for a therapeutic use.
- We observed that a low quantity of MWCNTs does not provoke any inflammatory reaction, but, for future medical applications, it is important to realize prosthesis based on MWCNTs, in order to study the implantation effects corresponding to such applications.
- Moreover, it has to be emphasized that this study does not address the carcinogenicity of carbon nanotubes, that is, the potential to transform a normal cell into a tumour cell,

which requires a detailed follow-up investigation on that specific topic. In the last five years, the question about potential toxicity of carbon nanotubes has been raised frequently. However our study shows no toxicity at doses of 5, 10 mg kg^{-1} of body weight. No immunological effects were observed at higher doses of 20, 40 mg kg^{-1} of body weight. We imagine, future applications where carbon nanotubes are injected into the human body for drug delivery [28], as contrast agent carrying entities for MRI [29], or as the material to generate a new prosthesis, the toxicity issue must be carefully addressed. Anyway precautions in their manipulation are recommended until complete knowledge becomes available. Notice that recently a toxicological assessment of different types of carbon nanotubes on the human tumour lymphocytic Jurkat cells has been reported [30], indicating that carbon nanotubes might play a role in inflammation, and recommending careful attention to the evaluation of exposure risks.

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Supporting information available: Experimental methods as well as additional time and dose dependence plots of carbon nanotube toxicity on H596 cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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