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ENVIRONMENTAL EXPOSURE TO CARBON NANOTUBES RESULTS IN SYSTEMIC DISTRIBUTION VIA THE CIRCULATORY SYSTEM: HEPATOTOXICITY AND CENTRAL NERVOUS SYSTEM INVOLVEMENT

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SUMMARY

Carbon nanotubes (CNTs) are among the most promising nanomaterials and their interesting properties are now exploited in many fields of technology and biomedical applications. However, the rapid growth of CNT employment raises concerns about the potential risks and toxicities to public health, environment and workers associated with the manufacture and use of these new materials. Here we investigate the main routes of entry following environmental exposure to CNTs, their localization and possible role in inducing inflammation and specific pathologies, using a novel rodent model. Following environmental exposure, we observed that CNTs rapidly accumulate in the lungs and brain, later reaching other organs including the liver, where steatosis was found after chronic exposure to CNTs. Since SWCNTs are considered for potential medical applications ad their toxicity is similar to that of MWCNTs, we further examined the effects of chronic intravenous SWCNT administration, which resulted in a chronic inflammatory condition particularly evident in the liver that was associated with a choleostasis-like syndrome. Our data suggest that after environmental exposure, CNTs can rapidly enter and diffuse in the organism via the blood stream, in addition to the lungs, which appear to have a capacity to clear CNTs, the liver may be the major site of CNT induced damage, where long term accumulation results in inflammation, steatosis and a choleostasis-like condition.

INTRODUCTION

NANOTECHNOLOGY AND CARBON NANOTUBES

Materials exhibit unique properties at the nanometer scale: nanotechnology is the art of manipulating materials in nanometer dimensions to take advantage from these unique properties.

Nanotechnologies are currently considered one of the most innovative technologies with a vast variety of applications. Nanostructures, a term which comprehends nanoparticles, nanotubes, and fullerens, with interesting chemical and physical properties are manufactured and used in the industrial field in many numerous ways.

Many nanotech products are now commercially available in a sector that shows explosive growth. Among the nanoparticles, carbon nanotubes (CNTs) are already widely used in industry and their applications are increasing constantly.

CNTs are major building blocks of this new technology: they possess unique electrical, mechanical, and thermal properties, with potential broad applications in the electronics, computer, aerospace and other industries. However, reasonable concerns are being expressed about potential risks to workers, public health, and the environment through manufacture, use, and disposition of these newly developed materials. This requires a careful assessment of potential unexpected biological interactions and toxicities, in a new field known as nanotoxicology [1].

Physical Characteristics and Properties of Carbon Nanotubes

CNTs are cylinders of one or several layer(s) of graphene sheets, exhibiting unique physical, mechanical, conductive, thermal and chemical properties [2,3]. Depending on the number of graphene layers from which a single nanotube is composed, CNTs are classified into single- walled carbon nanotubes (SWCNTs) constructed of a single sheet of graphite, while multi- walled carbon nanotubes (MWCNTs) are larger and consist of multiple concentric graphite cylinders of increasing diameter.

SWCNTs are considered one-dimensional (1-D) nanomaterials [1] and show important optical properties, as strong optical absorption in the near-infrared (NIR) that make theme utilized for photothermal therapy [4,5]. They can be employed for biological imaging, as SWCNTs emit in the biological tissue transparency window [6,7]

In contrast to SWCNTs, MWCNTs exhibit less important optical properties than SWCNTs. Their larger size and ease of manufacture led to use in many industrial sectors, including chemicals, electronics, clothing, as well as food, health care and biotechnology. Both SWCNTs and MWCNTs possess high tensile strengths, are ultra-light weight and have excellent thermal and chemical stability.

The high aspect ratio (ratio between length and diameter), small diameter, long length, and durability in vivo of CNTs recall another particle with similar features: crocidolite asbestos fibers. Crocidolite asbestos fibers are the principle cause of mesothelioma and are also associated with lung inflammation and other cancers. Similar to asbestos

fibers and other nanoparticles, CNTs have been associated with the induction of a chronic inflammatory response [8].

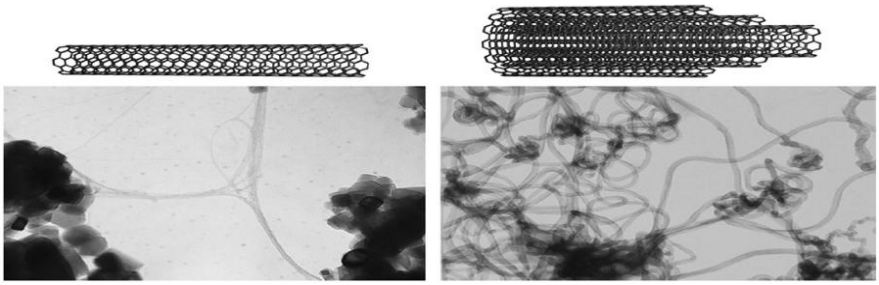

Figure 1. Basic SWCNTs (left) and MWCNTs (right) and typical transmission electron micrographs. (Adapted from Donaldson et al., 2006).

Current Applications of Carbon Nanotubes

CNT applications range from nanocomposites to imaging and treatment of diseases. CNTs are among the strongest and stiffest structures known, and have the strongest tensile strength of any synthetic fiber. As a consequence, the addition of MWCNTs to plastics and composites improves their conductive, mechanical, and flame barrier properties, allowing the construction of spacecraft structures, artificial muscles, combat jackets and land and sea vehicles [9,10].

On the contrary, SWCNTs are less widely employed in industry, however, they are of great interest for their potential medical applications [11]. One of the most interesting applications of SWCNTs is as carrier for drug delivery and treatment, as CNTs are able to enter cells by

themselves. These CNTs are rarely employed as unmodified or pristine from, but rather "functionalized". Pristine CNTs are inherently hydrophobic and aggregation is expected and observed *in vitro* and *in vivo*. As a result, to make CNT soluble in water and biocompatible, the surface chemistry of raw material may be modified covalently or not covalently and this procedure is broadly defined functionalization This "functionalization" consists in the addition of functional groups on the surface of CNTs: the functional group added can be subjected to ordinary synthesis methods to attach virtually any kind of organic compound on the surface.

Functionalization is able to change size and surface charge and alter the solubility and biocompatibility of CNTs. Among the different possible reactions developed to functionalize CNTs, oxidation and functionalization with polyethylene glycol (PEGylation) are the most popular. Although oxidation makes CNTs soluble in water, they aggregate in presence of salts, and so in most of the biological solutions and even if PEGylation makes CNTs and oxidized CNTs stable in solution, chemical reactions could destroy the physical characteristics of CNTs.

CNTs could also be non covalently functionalized with amphiphilic compounds, used to lower the interfacial tension between the solvent and the particulate CNT. These functionalizations are promising for different biomedical applications including imaging: CNTs could also be functionalized by conjugation with fluorescence or radiolabelled probes, peptides and different molecules that allow the attachment of other molecules. Proteins can be also conjugated or absorbed on CNTs for

intracellular delivery. The addition of agents can also protect the particles from being sequestered by the Reticular Endothelial System (RES) largely composed of the liver and spleen, for which the most common functionalizing agent is PEG. Although "properly functionalized" SWCNTs are considered "free of toxicity", there is a phenomenon of "defunctionalization"[12] which suggests that in vivo, particularly in the liver, CNTs can be reverted to a poorly functionalized state. Given the very long life of nanotubes in vivo, this may have implications in the hepatotoxicity and carcinogenic potential of functionalized nanotubes employed in medicine.

Environmental and Occupational Exposures to Carbon Nanotubes

To date, 190 facilities in Italy are working with nanotechnologies, of which 55% are public and 45% private enterprises. In 2011 the number of private organizations using nanotechnology was four times that of 2004. The Italian regions with the highest concentration of public companies active in nanotechnology, are Lombardy and Lazio, followed by Piedmont, Valle d'Aosta and Emilia Romagna (http://www.nanotec.it/). Engineered nanoparticles are completely novel structures that are not found in nature and how these particles interact with biological organisms is to be completely discovered. This is the case of CNTs, whose unusual structure is completely new in terms of evolution and biology. Many of the industries using MWCNTs are in the Lombardy area of Italy, and our data have been obtained using

commercially available MWCNTs used in a local industry. Currently there are no particular precautions employed for those working with CNTs in the industry setting. For these reasons it is critical to investigate the question of their safety for human health.

Fast propagation of nanotechnologies into different industries and consumer products is causing the exponential growth of nanomaterials production and as a consequence, of nanomaterial exposure. People could be exposed to CNTs by coming in contact with the aerosol form of CNTs during production or exposure as a result of environmental diffusion or biomedical use. Toxicity of CNTs is related to properties of the CNT material such as their structure, length and aspects ratio, degree of aggregation as well as to their concentration and dose. These physical characteristics determine the ability of CNTs to enter the environment. In the occupational setting, risk of exposure to nanomaterials could occur for workers at all phases of the material life cycle and depends on how product is being handled and at which phase: exposure could potentially occur during synthesis of the material or in transformation activities by exposure to airborne CNT dust. As previously reported [13], it is important to note that in some factories dust exposure due to lack of respect of industrial hygiene standards is found.

The main exposure routes to CNTs are considered to be inhalation and dermal contact. Ingestion can also occur as a consequence of swallowing inhaled material following mucociliary clearance or as a result of hand-to-mouth contact for individuals with exposure on their hands.

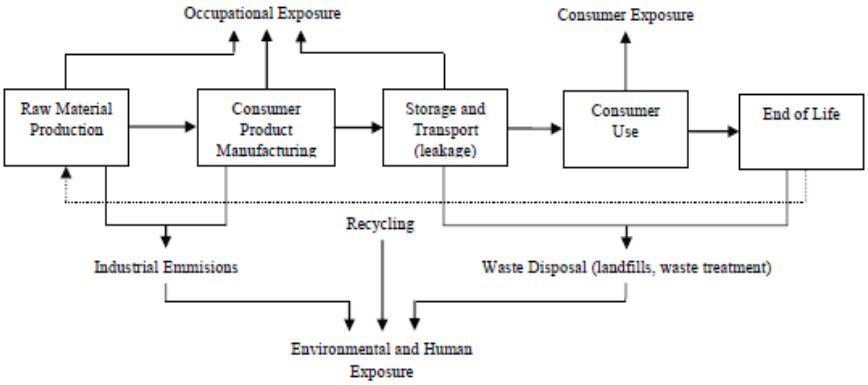

Figure 2. Possible pathways of occupational, environmental and human exposure to nanomaterials (modified from Hristozov D and Malsch I, 2009).

Limited data and guidelines are available for handling CNTs in occupational settings as well as research laboratories. In this contest, the study by Pauluhn and collaborators [14] is of great value for setting guidelines for occupational exposure in workplace environment and deriving occupational exposure limits (OEL). However, there are currently no exposure limits specific to engineered nanomaterials and this requires a careful assessment of potential unexpected biological interactions and toxicities associated to CNTs. Due to the wide use of nanomaterials and CNTs in many industrial fields, nanotoxicology studies have shown that large concentrations of CNTs may be present in occupational environments [13], which deserve particular attention from the standpoint of exposure.

NANOTOXICOLOGY AND TOXICITY OF CARBON NANOTUBES

Toxicity of nanoparticles

With the growing spread of nanotechnology, the necessity to understand the possible interaction of these new materials with the organisms gave birth of a new discipline, called Nanotoxicology. Initially, it was only considered a sub-category of toxicology defined 'to address gaps in knowledge and to specifically address the special problems likely to be caused by nanoparticles' [15]. It was supposed that nanoparticles and nanotubes may be able to induce adverse effects at their site of entry, for example, the lungs, but that some nanomaterials could also translocate from their site of entry to other target organs. At the time it was already known that nanoparticles in food can be adsorbed into the gut and redistribute to other organs more quickly than larger particles [16].

Currently, nanotoxicology has to be considered as 'the other face of the coin' of nanotechnology [17]. The same unique physical and chemical properties that make nanomaterials so promising for many uses may be associated with their potentially adverse or dangerous effects on organisms, cells and tissues. The toxicity of nanomaterials in the context of environmental and occupational diseases is not yet well understood: there is emerging concern that nanosized particles merit a particular assessment of their potential effects on health and environment as the toxicity of nanoparticles could be significantly greater than those of

larger particles. However, specific mechanisms and pathways through which nanomaterials may exert their toxic effects remain largely unknown. Although most people are still unfamiliar with nanotechnology, when CNTs move away from the production settings and the products became commercially available, the debate about the possible toxicities of these unnatural materials and their effects on workers and consumers is growing, that is why it is crucial to investigate benefits and risks associated to nanotechnology and the exposure to nanoparticles.

Methodology to Assess the Potential Toxicity of Carbon Nanotubes

Due to their structures and compositions, CNTs tend to bundle into aggregates [18]. From these structures, they can even form clumps larger than respirable size.

Elucidating the in vivo effects of administered CNT is considered very important in the context of the safety of novel nanomaterials. CNTs are biopersistent and can accumulate in organs, in particular in the lungs and toxicity has been reported in rodent models by pharyngeal aspiration [19,20].intratracheal instillation [13,21,22], inhalation [19] and intravenous [1,23], subcutaneous [24] or intraperitoneal injection [25]. The size and biopersistence of nanotubes has been liken to that of asbestos, and there are extensive concerns regarding the potential health hazards of CNTs, mostly focusing on the lungs [26].

Fibre toxicology and the experience with asbestos

The unique nanometer-scale diameter, the needle-shape and the high aspect ratio make CNTs one of the most attractive structural material, but this fibrous structure has led to concern that they might cause asbestos-like pathologies.

The experience with asbestos and the resemblance between nanomaterials and asbestos has led scientists to assess the potential toxicity and the risks to public health associated to nanoparticles [27]. Several studies have evaluated the responses to CNTs in cell cultures and the lungs of animal models [13,28,29], but the hypothesis that CNTs can behave like asbestos at the mesothelium level has only been partially addressed [30]. The mesothelium is the lining that covers the internal faces of the pleural and peritoneal cavities (parietal mesothelium) and the exterior surfaces of the organs (visceral mesothelium). When cancer occurs in the mesothelium, as is the case of people exposed to asbestos, the cancer is termed mesothelioma [31]. Mesothelioma is almost exclusively found following asbestos exposure and is a unique response to fibre-shaped particles [32].

Fibre toxicology identifies the characteristics of fibres and their most important toxicological features involved in the biopersistence of fibres. Diameter is important, as small diameter allows deposition beyond the ciliated airways. The length is also a key factor in the pathogenicity of fibres: it has been demonstrated that carcinogenesis was linked to fibres

longer than 10 um and long CNTs showed similar ability to asbestos to cause fibrosis and inflammation in the peritoneal cavity [33].

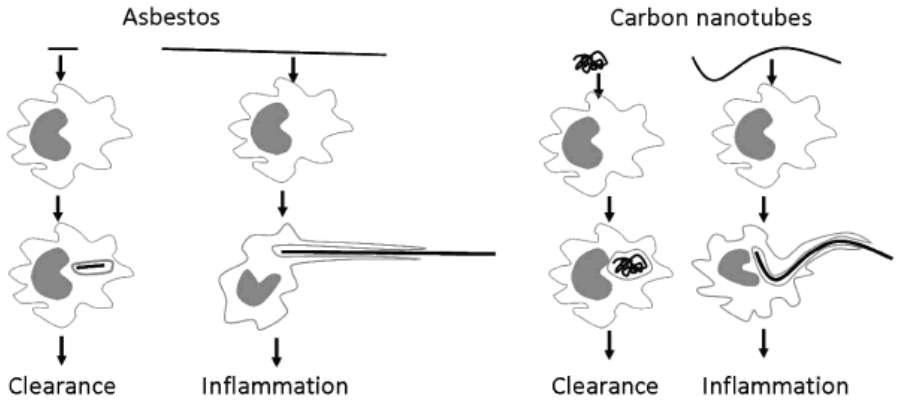

Figure 3. The frustrated phagocytosis paradigm relates to long and short fibres of asbestos (left) and various forms of carbon nanotubes (right). Macrophage can enclose short or tangled CNTs and clear them. However the macrophages are not able to enclose long asbestos or long nanotubes, resulting in incomplete or frustrated phagocytosis, which leads to inflammation (modified from Donaldson et al., 2010).

Biopersistence and length influence the clearance of fibres, but not in the case of long fibres because they cannot be easily phagocyted by macrophages [34]. The failure of macrophage phagocytosis, called incomplete or frustrated phagocytosis, is a pro-inflammatory condition common to asbestos fibres and CNT longer than 15 μm [33]: it is possible for carbon nanotubes to be pathogenic by being thin, long and biopersistent and they can form tangles or ropes still thin enough to be

respirable. Graphene, the basic structural component of CNT is an exceedingly strong material and so is likely to be biopersistent [30].

Pulmonary toxicity of CNTs

The difficult generation of fine dust containing CNTs or aggregates of respirable sizes for inhalation studies [35] and the difficult maintenance of exposure levels of CNT led researchers to assess the effects of CNTs in the lungs by intratracheal instillation (ITI). ITI consists in an administration of a bolus dose of an aqueous suspension containing CNTs directly instilled into the trachea of anaesthetized animals. Instillation studies reported significant pulmonary effects, including inflammation, evidence of oxidative stress, fibrosis [29], granuloma formation [13,28,29,36] and animal death [28]. The mechanism of lethality is associated with the formation of lung granulomas, in a dose-independent manner, and is probably associated with the impact of CNT aggregates that agglomerated the airways of animal models, inducing mortality in 15% of instilled rats within 24 hours post-instillation [28]. However, it is presumed that CNTs with good biodistribution are not toxic enough to cause inflammation and formation of pathological structures in the lung because of the dispersibility. Lam and collaborators [13] showed that a single instillation of SWCNTs in mice induced persistent granulomas and inflammation. Moreover, the severity of lung lesions was dose dependent. Granuloma formation around CNT aggregates was observed in rats and mice [13,29]. In order to clarify if granuloma formation was induced by CNT impurities, Shvedova and

collaborators [29] employed purified SWCNTs administered by ITI, avoiding the generation of oxidant and they found acute inflammatory reaction characterized by a rapid increase in bronchoalveolar lavage (BAL) fluid levels of inflammatory cells [29], inflammatory cytokines, as TNF-α, IL- 6, TGF-β and increased collagen deposition [37]. However, the most important finding was the dose-dependent and progressive development of interstitial fibrosis in lung regions distant from CNT deposition sites. Inflammation and fibrosis were found to be present in lungs after 60 days [36]: after 2 months collagen-rich granulomas were observed in the lung of treated rats. Increased numbers of polymorphonuclear cells and protein exudates (fluid rich in protein and cellular elements that leach out of blood vessels due to inflammation), and high levels of LDH activity in BAL fluid were also observed [36].

In other studies aerosolized MWCNTs did not induce obvious pulmonary toxicity in a 30-day exposure group but induced severe pulmonary toxicity in the 60-day exposure group [38]. The comparison between ITI administration and inhalation of MWCNTs revealed that the main pathological lesions were induced by aggregations, causing proliferation and thickening of alveolar walls. However, the general alveolar structure was still remained [38].

Inhalation of MWCNTs more closely mimics occupational and environmental exposure because CNTs form more dispersed structures when compared to an instilled bolus dose administered in aqueous suspensions. Moreover, inhaled CNTs reach the distal regions of the lung and more accurately represent deposition and pathologic responses to real exposure scenarios. It has been shown that inhaled CNTs caused an

initial inflammatory response followed by granulomas, fibrosis and decreased rates of respiration, as well as activation of a gene that induces lung cancer [19].

Hepatotoxicity of CNTs

Organisms have developed elaborate systems to defend themselves against toxic agents. Most cells in the body are capable to aid in the metabolism of toxic agents of metabolism, however the primary organ for detoxification is the liver and thus is the target organ to investigate the *in vivo* biocompatibility of CNTs.

The oxidative stress and hepatotoxicity biomarkers are usually investigated by evaluation of ROS induction, measurement of LHP, activities of certain liver enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), ALP and histopathological characterization of liver in mice exposed to MWCNTs [24,25]. Serum ALT and AST are important biochemical enzymes indicative of hepatic injury.

AST is a pyridoxal phosphate (PLP)-dependent transaminase enzyme, which catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate. In a subsequent step, oxaloacetate reacts with NADH and the conversion of NADH to NAD+ is proportional to the concentration of AST in serum. ALT catalyzes the transfer of an amino group from alanine to α-ketoglutarate, producing pyruvate and glutamate. Increasing of both ALT and AST reflects damage to the liver.

Several studies reported increased levels of both the enzymes after intravenous injection of pristine or functionalized CNTs [39]. In particular, Jain and collaborators [40] observed that MWCNTs induced severe hepatocyte mitochondrial damage as levels of AST, resident in cytosol as well as in mitochondria, were higher than ALT levels, which is present only in the cytosol.

ROS has been implicated in the toxicity of carbon nanotubes by several authors [19,40,41,42]. Their formation with subsequent cellular damage is considered as the molecular mechanism of carbon nanotube-induced toxicity. Recently, it has clarified that acid oxidation or carboxyl functionalization is crucial to decrease the severity of oxidative stress induced by MWCNTs. In particular, it has been shown that the presence of metals is crucial for the generation of ROS, in fact acid-treated MWCNTs to remove metal contaminant or intensive purification are able to improve CNTs biocompability [40]

Recently, it has been indicated that CNTs were trapped by the reticuloendothelial system and retained mainly in the liver of mice for long time periods after intravenous injection [43,44,45]. Hepatotoxicity involving an inflammatory response, mitochondria destruction and oxidative damage were observed following injection of functionalized MWCNTs, which also can affect several gene pathway expressions in mice, including GPCRs (G protein-coupled receptors), cholesterol biosynthesis, metabolism by cytochrome P450, natural-killer cell-mediated cytotoxicity, TNF-α and NF-κB signaling pathway [46].

AIM

The project that I carried out during my Ph.D course had the purpose to evaluate the biodistribution and the mechanisms of entry of MWCNTs in a simple mouse model that mimics the exposure to CNTs in the environment, since a main route of exposure to CNTs will be through environmental exposure (essentially through aerosol inhalation and the digestive tract contact, as exposure of the digestive tract occurs both by direct trapping in the saliva and by the physiological process of upper airway trapping and respiratory tract mucus removal).

Data from body distribution in rodent model studies to date have been from intravenously administered CNTs [47] and are mainly focused on the systemic distribution of SWCNTs, as this kind of nanomaterials represent the elective choice for drug delivery or therapy. However, also in this context, toxicological data are conflicting.

Toxicity induced by chronic exposure to MWCNTs, instead, is focused on the pulmonary effects. For these reasons, we are interested in clarifying how these particles enter and interact with biological systems and fill the gap regarding the paucity of data regarding possible adverse health outcomes resulting from chronic exposure to MWCNTs.

We investigated the toxicity of an environmental MWCNT exposure, in particular evaluating the biodistribution, modes of entry and effects of CNTs *in vivo* by:

1. Exploitation of a new, simple model of “workplace” exposure to investigate entry mechanisms;
2. Evaluation of the role of CNTs in inducing hepatotoxicity, introduced in the animal model by intravenous injection.

The aspect of novelty of this work is the exploitation of a new and simple in vivo model that mimics a probable workplace exposure scenario. Data from literature are mostly focused on lung toxicity since the main route of exposure is considered to be the respirable tract. Here we focused on the systemic distribution of MWCNTs and the associated toxicities

The main goal of this study is the evaluation of the effects of CNTs exposure through a model that comprehends multiple routes of entry, in particular by the evaluation of hepatic injuries and the possible adverse effects in the central nervous system.

MATERIALS AND METHODS

CNT preparation

Commercially available, industrially employed, Multi-walled Carbon Nanotubes, NANOCYL^{TM} NC7000 (Belgium NANOCYL, Sambreville; average 9.5 nm diameter by 1.5 μm, not functionalized) used in this study were the kind gift of a local industry in Lombardy, the Italian region with the highest concentration of public companies active in nanotechnology. NC7000 were manufactured by CCVD, catalytic carbon vapor deposition process with a purity of 90%.

SWCNTs were obtained from Sigma-Aldrich. The nanotubes cover an average diameter of 1.2-1.5 nm and 2 to 5 μm in length. For the preparation of suspended SWCNTs, the crude SWCNTs were dispersed in Dulbecco's minimal essential medium (DMEM, Sigma Aldrich, Milan, Italy) supplemented with 10% (v/v) of serum of CD1 mice (CD1serum) using a sonication/centrifugation protocol based on that previously described by Yehia et al. [48] but with higher levels of endogenous serum to improve animal compatibility and avoid immune reactions to heterologous sera. Briefly, 10 mg of the crude SWCNT-containing powder was dispersed in an microcentifuge tube containing 10 ml of DMEM/CD1serum, vortexed for ~1 min, and probe sonicated for 15 min at 0°C. Probe sonication was performed using a HD2070 Bandelin Sonopuls sonicator (VWR International, Milan, Italy), a 2 mm diameter probe tip was placed one third of the distance below the surface

of the 1 ml suspension. The resulting suspension was centrifuged for 2 min at 16,000 g and the upper 75% of the supernatant was recovered, placed in a clean tube and centrifuged for 2 min at 16,000 g. Again the upper 75% of the second supernatant was carefully recovered as the final SWCNT dispersion.

Quantification of SWCNT concentrations

To estimate the SWCNTs concentration in the samples used for the *in vivo* experiments we realized a calibration curve by means of FT-Raman measurements [49] on samples of known concentration. The band intensity was calculated as the average over several spectra of the G band peak integrated area from 1573 to 1612 cm^{-1} and the concentration of unknown samples determined using the calibration curve.

Study design and animals

The procedures involving the animals and their care were conformed to the institutional guidelines, in compliance with national and international laws and guidelines for the use of animals in biomedical research. The procedures used were approved by the local animal experimentation ethics committee and by the Health Ministry. Healthy adult male and female CD1 mice (6-8 weeks of age) were used in this study. The animals were housed with steel wire tops and corn-cob beddings,

maintained in a controlled atmosphere with 12-12 dark-light cycle, at a temperature of 22 ± 25 ºC and 50-70% humidity with free access to food and fresh water.

For the murine environmental exposure model, mice were exposed to MWCNTs added directly within the cage litter twice a week, 1.5gr/80 gr litter. Standard rodent diet and water were provided *ad libitum*. The animals extensively agitate the litter, generating an aerosol that contains MWCNTs which cause also the exposure of the respiratory tract as well as the digestive tract, as particulate matter in the lungs trapped by the mucus is expelled into the esophagus along with direct ingestion. During the experimental process, the health status of the animals were monitored by examining for skin alterations, reactivity to stimuli, body weight as well as food and liquid intake. Mice were sacrificed after 1, 2, 3, 4 and 5 weeks after initiating exposure to MWCNTs and the organs (lungs, brain, kidneys, liver and spleen) were removed and either fixed in OCT and immediately stored in liquid nitrogen, or fixed 4% glutaraldehyde and stored at 4°C for subsequent histological staining. The presence and the biopersistence of MWCNTs was assessed by chemical disruption of portions of explanted tissues and analysis by SEM (see below).

For the intravenous injection study, mice were exposed to three doses (0.16 mg/kg; 1.6 mg/kg; 6.4 mg/kg) of SWCNTs injected in the tail vain, either for acute or chronic treatment, the later consisted of injections once every three weeks for nine weeks. One group was chosen as negative control and treated with 200 µl of Dulbecco's minimal essential medium (DMEM) supplemented with 10% (v/v) of CD1 serum (the medium used

to disperse the SWCNTs). Mice were sacrificed either 24 hours, three weeks or four months after the last injection (for chronic exposure) or 24 hours after the first injection (for acute exposure). At sacrifice the internal organs were collected and processed for standard histology and scanning electron microscopy (SEM), blood was collected to produce sera for biochemical analyses.

Optical microscopy

Samples fixed in OCT were used to obtain 5µm sections, stained by crystal violet and basic fuschsin and subsequently observed under a light microscope (Olympus, Tokyo, Japan). Samples fixed in 4% glutaraldehyde were washed in 0.1 M cacodylate buffer pH 7.4 and postfixed with 1% osmic acid in cacodylate buffer, pH 7.4. After serial ethanol dehydration, specimens were saturated with a uranyle acetate solution in 90% alcohol for 30 minutes. Samples were transferred in propylene oxide and Epon-Araldite resin solution 1:1 for 1 hour. Thin (750 nm) sections were obtained with a Reichert Ultracut (Leica, Wien, Austria). For standard histology, the liver, spleen, lungs and kidneys were removed, washed with PBS and 20 mM EDTA to remove excess blood and fixed immediately in 4% formalin. After dehydration in a progressive series of ethanol, samples were clarified in xylene and paraffin embedded. 7µm sections were obtained and stained with haematoxylin & eosin (H&E) for histological examination and observed

under a light microscope (Olympus, Tokyo, Japan). At least 10 slides of each sample were scored for organ histology.

Metabolic analysis

Mice were housed for 24 hours after each injection and every week between the first, the second and the third injection in metabolic cages to collect urine and fecal samples and to analyze the food and water consumption. To analyze the fecal water, feces samples were weighed, placed in a thermostatic oven for an hour, and weighed again.

Serum biochemical analysis

At sacrifice blood samples were immediately collected and transferred into tubes, and allowed to clot for 60 min. After clotting, the sample was centrifuged 60 min at 7,500 g. The samples were stored at -80ºC until ready for analysis. Plasma TNF-α levels were determined by enzyme-linked immuno assay (ELISA, Becton Dickinson, BD - San Jose, California, USA). The absorbance was measured on a microplate reader at 450 nm in a Versamax Microplate reader (MolecularDevice; Genoa, Italy) and the TNF-α concentration in experimental samples was calculated from a standard curve. Aspartate amininotransferase, alanine aminotransferase, alkaline phosphatase, azotemia and creatinine were

performed using Kuadro BPC+BioSed clinical automatic chemistry analyzer (Italy, Genoa).

Flow cytometry analysis

To analyze the $CD45^+$ cells and the lymphocyte subpopulations, the animals exposed to SWCNTs were sacrificed, organs removed and placed in a PBS without Ca^{2+} and Mg^{2+} with 0.53 mM EDTA. A single cell suspension was obtained by mechanical procedure followed by lysis of red blood cells by ammonium chloride solution (ACK, lysing buffer 1X, Life Technologies Europe, Monza, Italy) and finally filtration through 50 µm Nylon filters. Flow cytometric analysis was performed with a CyAn instrument (Dako, Milan, Italy) using Spectraline and Flow-Check FluoroSpheres (Dako) for instrument setting and compensation with standard gating procedures.

Viable cells were selected according to physical parameters (side scatter vs forward scatter), $CD45^+$ were identified by gating $CD45^+CD31^-$ cells using anti CD45 and anti CD31 (marker for endothelial cells) monoclonal antibodies (MAbs). The following MAbs directed against mouse surface markers: anti F4/80 (APC, MB8 clone, BioLegend, Cambridge, UK), anti Gr-1 (FITC RB6-8C5 clone, ImmunoTools, Fiersoythe, Germany), anti NK1.1 (APC PK136 clone, BioLegend) and to CD19 (PE PeCa1 clone, ImmunoTools) were used to identify

macrophages, neutrophil granulocytes, natural killer (NK) and B cells, respectively.

Determination of the presence of nanotube and their localization by Scanning Electron Microscopy

SWCNTs and MWCNTs were observed directly in 750 nm Epon sections of lungs, livers and brain obtained from 5 weeks environmentally exposed mice and injected mice, they were then washed repeatedly in distilled water, dehydrated in an ascending scale of ethanol, critical point dried with CO_2 and sputter- coated with a thin layer of gold in and Emitech 550 sputter coater and observed in backscattered electron (BSE) mode with a scanning electron microscope (Philips XL30 SEM FEG).

Portions of the same samples obtained after 5 weeks of CNT environmental exposure were excised and digested in 5N KOH (Sigma Aldrich) for 2h, then washed repeatedly in dH_20 to remove potassium salt, resuspended in 100 µl of dH_2O and dried on a cover glass for scanning electron analysis. To enhance CNT signals, cover glass were sputter- coated with a thin layer of gold and observed in backscattered electron (BSE) mode with a scanning electron microscope coupled with an energy dispersive x-ray analyzer (Edax Genesis, 2000).

RESULTS

Macroscopic effects of MWCNTs in environmentally exposed mice

All animals environmentally exposed to MWCNTs showed no overt clinical signs and, unlike what reported by several studies on CNT toxicity, all animals survived and appeared healthy at the time of sacrifice. Mice showed no body-weight loss or an altered food and liquid consumption, nor any alteration of reactivity to stimuli, showing a good general health status. MWCNTs did not produce macroscopic lesions (Fig.1), except for the digestive tract, which was dark in exposed mice, suggesting MWCNT accumulation.

Accumulation of MWCNTs in environmentally exposure mice

Although MWCNTs did not induce macroscopic alterations, histopathological effects derived from MWCNTs exposure model were observed on liver, kidneys, lungs and brain explanted from both exposed and control mice. The MWCNT exposed tissue sections showed frequent patches of dark material, likely to be MWCNT aggregates deposited within the lungs, brain, liver and kidneys that were not observed in tissues from control mice (Fig. 2). MWCNTs accumulated primarily as aggregates, in particular the formation of dark material deposits seems to be time-dependent. Temporary, the first organ affected were the lungs, in

line that expected as the major route of exposure is the respiratory tract, where deposits were observed from the very beginning of the exposure period (Fig. 2, I and II weeks of exposure). In the lungs, MWCNTs persisted up to the end of the experiments. Surprisingly, also at the same early time points the brains of exposed mice showed the same brown deposits, but more numerous and less aggregated than those observed in lungs, suggesting that MWCNTs can rapidly reach the brain. This rapid route of entry may be directly through the nasal mucosa. Time-dependent systemic distribution of MWCNTs involved the liver and kidneys, which presented the same brown aggregates observed in the lungs, but only after 4 weeks of MWCNTs administration (Fig. 2). Taken together, these data suggest that MWCNTs in an environmental exposure model are able to quickly reach the distal organs, through the blood stream of exposed mice (see below), where they either directly deposit as aggregates or re-aggregate in liver, lungs, brain and kidneys.

Dark deposits of material in exposed mice are MWCNTs aggregates

The brown deposits were observed only in lungs, liver and brain of MWCNT exposed mice, suggesting that these are due to the presence of MWCNTs in these organs. The presence of MWCNTs in these tissues were confirmed by taking advantage of the chemical resistance of CNTs. Tissues from exposed and control groups were mechanically dispersed and digested with KOH to remove tissue derived material, the resistant

material was collected by centrifugation. This procedure resulted in a visible black material that SEM analysis revealed had the characteristics of aggregates of CNTs that were very similar to the starting material, before and after KOH exposure (Fig. 3D, 3E and 3F). This procedure revealed CNTs from the liver, brain and lung (Fig. 3A, 3B and 3C). Similar structures were found in blood samples taken form MWCNT exposed mice after 3 weeks (data not shown), indicating that CNTs enter into the blood stream. In combination with SEM analysis, Energy-dispersive X-Ray analysis spectrometry was performed on selected areas to detect and visualize the distributions of carbon. Elemental analysis clearly showed co-localization of carbon with the MWCNT aggregates (Fig. 4A and 4B). To confirm these data, we performed SEM analysis directly on a semithin section of 750nm , demonstrating the presence of MWCNT aggregates in liver (Fig. 4C).

Hepatic steatosis in animals environmentally exposed to MWCNTs

When we examined the liver tissues by scanning electron microscopy we observed an accumulation of lipid vesicles in the hepatocytes from animals exposed to MWCNTs (Fig. 4C and 4D). These were not observed in untreated animals, indicating that environmental exposure leads to CNT transport in the blood stream to the liver, where it interferes with liver function and metabolism. While no overt signs of pathology

were observed in the exposed mice, steatosis is indicative of subclinical hepatic stress.

Intravenously injected CNTs accumulate in liver and induce hepatic damage

Environmental exposure to MWCNTs indicates that CNTs are able to enter into the blood stream and targeting diverse organs, in particular the liver, where steatosis is suggestive of hepatic damage. Since previous studies have suggested that injected MWCNTs or SWCNTs showed similar hepatotoxicity profiles in mice [24,25], and SWCNTs have also been proposed for their therapeutic potential, we choose further investigate the systemic toxicity of CNTs, by acute or chronic intravenous injection of SWCNTs.

Macroscopic analysis of organs harvested from mice chronically treated with the highest doses of SWCNTs (6.4 mg/kg dose) and sacrificed three weeks after the third injection indicated that the liver had a macroscopically darker color (Fig.5, panel A). These data suggest that the injected CNTs localized in these tissues. The kidney was affected to a lesser extent (Fig.5, panel A). Similar results were obtained in animals sacrificed 24h and four months after the third administration (data not shown). No differences in the weight of either mice or organs analyzed were observed (Table 1). As, observed in animals environmentally exposed to MWCNTs, histopathology analysis confirmed the accumulation of an amorphous brown material only in CNT treated mice representing SWCNT deposits. These were particularly evident in the

liver and, unlike in the environmentally exposed mice, also in the spleens of all samples analyzed, 24 h (data not shown) and 3 weeks after the last injection. These deposits were found in the lungs at acute time points (animals sacrificed 24 h after the first injection, Fig.6, panel A) but these were cleared by the three-week time point (Fig.6, panel B) . Although the accumulation of what appears to be aggregated SWCNT deposits was observed in livers of treated animals, these organs otherwise showed normal structure with compactly arranged hepatocytes and sinusoids with uniform morphology. No significant alterations were observed in the kidneys. We also performed KOH disgregation on these tissues as above that revealed large aggregates of SWCNTs in the livers of injected mice (Fig. 5A), while these deposits were not found in tissues from control animals. SEM analysis of thin section of the liver showed aggregates, likely to be SWCNTs, in the sinusoids of the liver associated with Kupffer cells (Fig.5B, 5C and 5D).

SWCNTs injection impairs metabolic functions

We examined the effects of CNTs on food and water consumption as well as fecal and urine production using metabolic cages. Following the first administration of SWCNTs there was a trend toward augmented water and food consumption (Fig. 7A and 7B), accompanied by a statistically significant increase in feces production (Fig. 7C). Urine production was not affected (Fig. 7D). Similar effects were reported for all SWCNT doses, and most parameters returned to near baseline at three

weeks from the last administration, in particular for the highest dose. These data indicate that intravenous SWCNTs may interfere with the metabolism and digestive tract function.

SWCNTs can induce chronic hepatic damage

The alterations in food consumption and fecal production suggested potential adverse effects within the digestive tract. We therefore examined several serum parameters reflecting renal and hepatic function. No differences were observed in creatinine levels between treated and control mice (Fig. 8A) while azotemia decreased in mice exposed to acute and chronic doses of SWCNTs (Fig. 8B). Taken together with the lack of effect on urine production, these data suggest that kidney function was not significantly affected by SWCNT administration.

Examination of enzymes reflecting liver damage or malfunction showed a trend towards increased levels of alkaline phosphatase (Fig. 8C) at all doses, and significant increases in the levels of alanine aminotransferase (Fig. 8D) and aspartate aminotransferase (Fig. 8E), particularly for the highest dose. While alanine aminotransferase levels, which increased significantly for the highest dose, returned to baseline after 3 weeks, aspartate aminotransferase remained elevated even after 3 weeks and for all doses. Taken together, these data suggest that chronic exposure to SWCNTs can induce hepatic damage.

SWCNTs introduced into the blood stream are captured by RES system inducing inflammation and choleostasis

To investigate if SWCNT treatment results in inflammation of key organs, we examined the influx of $CD45^+$ cells (a marker for all immune cells) into various organs. In the case of acute treatment, 24 h after the first injection of SWCNTs, we observed an increase of $CD45^+$ cells in the lung at all doses (Table 2), as this is probably the first area affected following intravenous injection. Increases in $CD45^+$ cells were also observed in the liver after 24 hours at the highest dose (6.4 mg/kg). The numbers of $CD45^+$ cells were elevated in both liver and particularly in the spleen even three weeks after the last SWCNT administration (Table 2). Interestingly, analyses of the lymphocyte subpopulations three months following the last SWCNT injection showed that the $CD45^+$ cells remained elevated in the spleen, with increased levels of NK, macrophages, neutrophil granulocyte and B cells (Table 2).

To evaluate the immune response further, we measured levels of TNFα, an indicator of inflammation, necrosis and fibrosis, in the serum. TNFα was elevated in the serum at the highest dose both at 3 weeks and at 3 months after the last dose (Fig. 8F). These data suggest that SWCNTs induce a significant level of inflammation.

DISCUSSION

Nanomaterials and nanoparticles have received considerable attention recently due to their unique properties and applications in diverse biotechnology and life science applications but also in many areas of industry. Despite the rapid progress and early acceptance of nanobiotechnology, the potential for adverse health effects due to prolonged exposure at various concentration levels, in humans and the environment has not yet been established. However, the environmental impact of nanomaterial is expected to increase substantially in the future. In particular, the behavior of nanotubes and nanoparticles inside the cells and cellular responses induced by these nanotubes or particles are still not well understood.

In this study, we focus on the environmentally exposure model that, in our opinion, represents a more realistic scenario of exposure compared to the usual instillation or inhalation procedures explored by several works [13,19,29,41], in particular on the hepatotoxic effects due to chronic exposure and the eventual implications in neurodegenerative diseases.

In vivo studies on CNT pulmonary toxicity are currently discussing about the best protocol to be used to administrate CNTs. It has to be considered that, while inhalation is the most physiological method, it is impossible to quantify the administrated quantity of CNTs, [50]. ITI is the most common administration method, but CNTs tend to create large agglomerate dispersed in suspension in saline that could lead to overestimation of the toxicity induced by CNTs.

Due to the implications that CNTs may adversely affect human health and safety, dosing of animals with an adequate amount of MWCNTs is fundamental for mimicry human exposure levels. The National Institute for Occupational Safety and Health (NIOSH) proposed 7 $\mu g/m^3$ as lowest detectable level for CNTs respirable dust [51]. The dose used in this study, 1.5 g/80 g of litter (the average mouse used weighed 27 g, thus 55 µg of MWCNT/mouse), were selected based on doses reported in previous exposure studies [33]. CNTs were directly dispersed in the litter, where the mice generate a local aerosol containing CNT dust. The dispersed MWCNTs also enter into the digestive tract of the animals: the combination of these two routes of entry, together with skin contact, seems to be a more realistic way in which humans could be in contact with CNTs. Data from this study demonstrated that if MWCNTs in the environmental or occupational settings will be present in a chronic manner, they can penetrate into the organism and rapidly spread in the whole body, without causing any overt clinical signs. We showed that in this model, MWCNTs can form aggregates, ropes and tangles, yet they are still thin enough to be in the respirable size range. The high aspect ratio, ratio of length and width, the nanometer-scale diameter and needle-like shape have draw comparisons with asbestos, which, however, had the lungs as target, resulting in lung cancers, mesothelioma and asbestosis [52], while we find that MWCNTs seem able to reach the distal organs through the blood stream.

It has to be considered that, unlike that previously reported for CNT inhalation or instillation studies [13,19,28,36], we did not found the formation of granulomas or fibrotic tissues: this suggests that the

damages induced by CNTs in inhalation or instillation studies may be quite different from that where CNTs enter through environmental exposure. This represents another feature in common to asbestos, whose exposure causes clinical signs only after many years.

MWCNT aggregates did not affect only the lungs following acute administration, but also the brains of exposed mice. We observed dark deposits of MWCNTs in brain soon after the first administration. These data suggest that either CNTs are able to form aggregates thin enough to penetrate deep into the alveolar region of the lung, then translocate into the blood circulation where they preferentially cross the blood brain barrier, or a more likely scenario of transport from the olfactory nerve into the olfactory bulb and dispersion to other areas of the brain. The ability of nanomaterials to move in the body and reach the central nervous system and heart, spleen, kidney, bone marrow, and liver was previously shown only after intravenous injection. Our data demonstrate that CNTs are able to form stable and numerous deposits in brain even after acute exposition, without being rapidly cleared. Accumulation in fact seems to be dose and time dependent, we found greater MWCNTs after a chronic 5 weeks exposure.

The capacity of CNTs to reach the distal organs may depend on their chemical reactivity, surface characteristics and ability to bind the body proteins. CNTs translocation in distal regions by intravenous administration is expectable, but with this study we are able to demonstrate that MWCNTs can distribute into the body in an environmental exposure model likely reflecting a real exposure scenario.

What we were interesting in was the ability of MWCNTs to accumulate in the liver, mechanism that induced hepatotoxicity, as demonstrated by the SWCNTs injection model, but did not cause obvious damage to the whole organ, neither lesions to the architecture. MWCNT accumulation in the liver due to a respiratory/gastrointestinal route of entry has not been previously reported. Livers appeared to be affected after 3 weeks of exposure and the accumulation of CNTs was continuous until the end of the experiment. Surprisingly, we observed a diffuse steatosis in 5 week treated mice, confirmed by both histological analysis and SEM observation, in spite of clinically healthy status. So, we hypothesize that the chronic exposure to MWCNTs led to development of hepatic injury. However, the absence of macroscopic alterations even in the presence of a diffuse hepatic injury, led us to investigate the specific role of CNTs in the development of hepatotoxicity following a chronic administration induced by intravenous injection, also determining tissue inflammation and differential body distribution. Intravenously administration of CNTs revealed that chronic administration of CNTs impairs metabolic functions, induces chronic hepatic damage and that CNTs are captured by RES system inducing inflammation and choleostasis. Examination of liver enzymes reflected liver damage or malfunction and showed increased levels of alkaline phosphatase and significant increases in the levels of alanine aminotransferase and aspartate aminotransferase. However, there is little effect on renal function, as confirmed by the lack of effect on urine production and the normal level of azotemia.

Taken together, our data suggest that CNTs can rapidly enter and diffuse in the organism after environmental exposure. The data indicate that the

major organs affect may not be the lungs, which appear to have a capacity to clear the CNTs, but rather the liver and brain, where long term accumulation results in inflammation, leading to steatosis and hepatic damage in the liver. The effect of CNTs on the CNS, including inflammation and neurodegenerative diseases, remains to be determined.

FIGURES

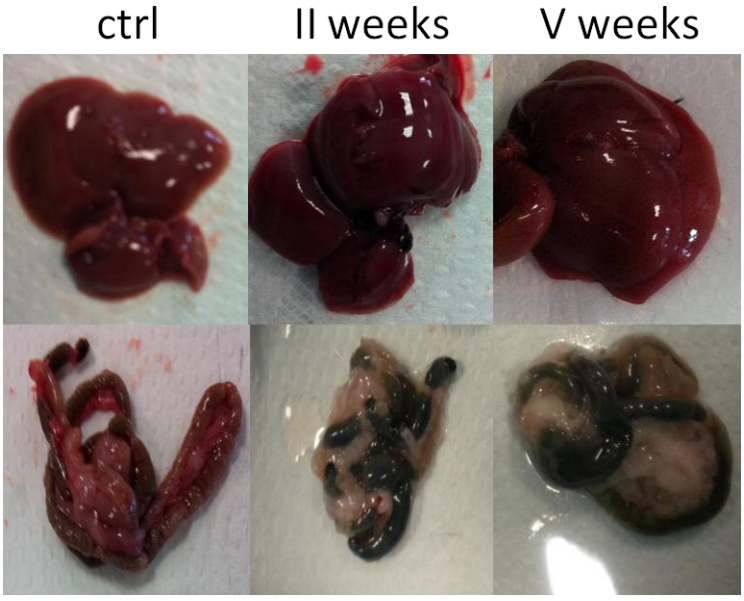

Figure 1: Organs collected after two and five weeks of treatment with MWCNTs. Macroscopic analysis indicated no evidence of lesions except for the digestive tracts which appeared darker, due to the direct ingestion of MWCNTs or ingestion of mucus containing MWCNTs expelled from the lungs.

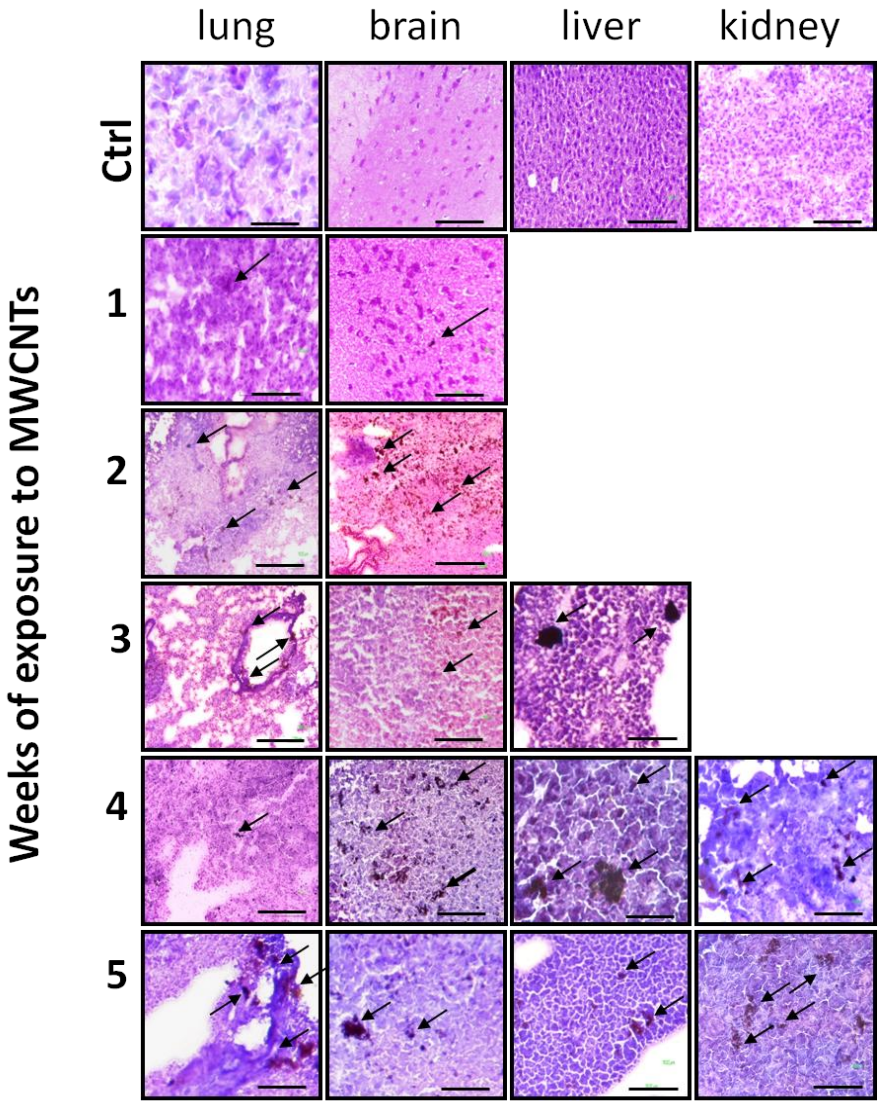

Figure 2: Morphological changes of mouse organs exposed to environmentally administered MWCNTs. Arrows show dark deposits corresponding to MWCNT aggregates. Bars: 100 um.

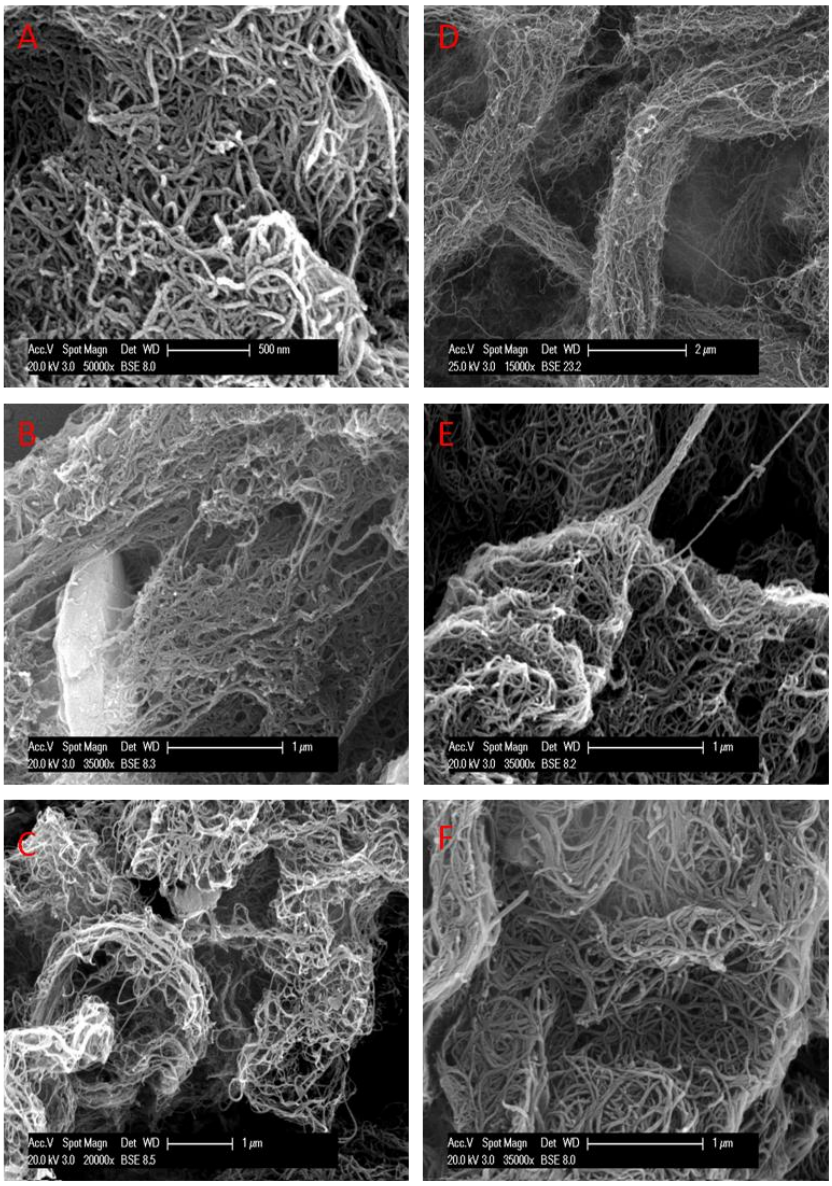

Figure 3: SEM analysis following KOH digestion confirmed the presence of MWCNTs in the liver (A), brain (B) and lungs (C) of 5 weeks exposed animals. SEM characterization of aggregates of MWCNTs crude powder (D), before (E) and after (F) KOH treatment.

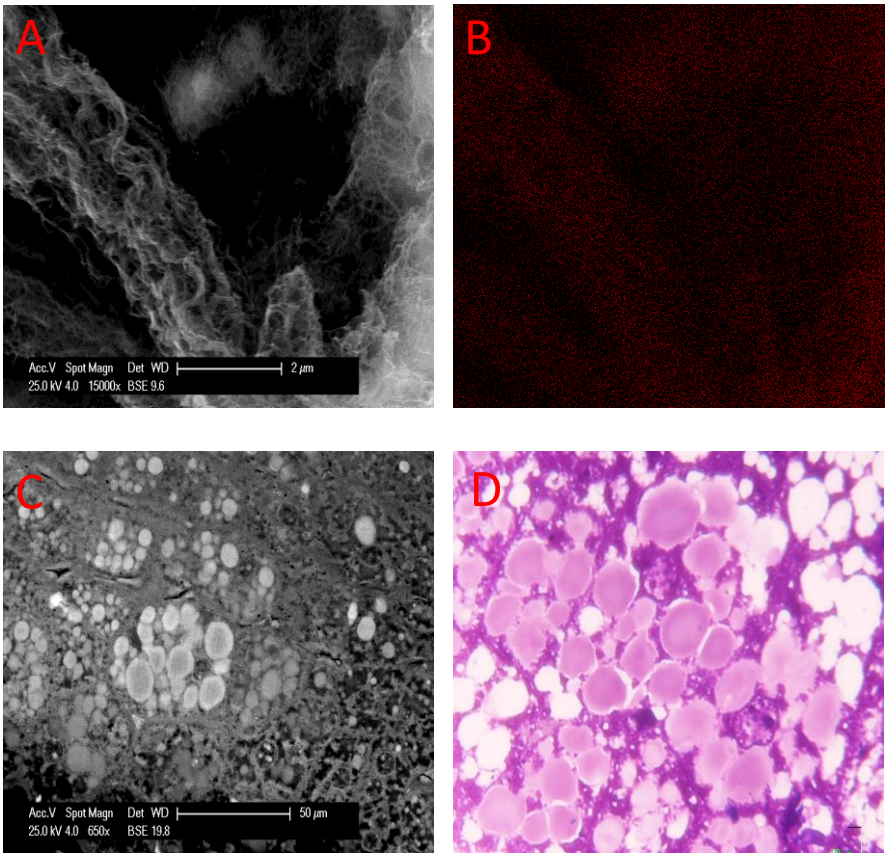

Figure 4.Combination of SEM analysis of MWCNT in liver (A) with elemental analysis (EDAX, B) clearly showed co-localization of carbon with the MWCNT aggregates. SEM analysis confirmed the accumulation of lipid vesicles in the hepatocytes from animals exposed to MWCNTs (C), as revealed by semithin section (D).

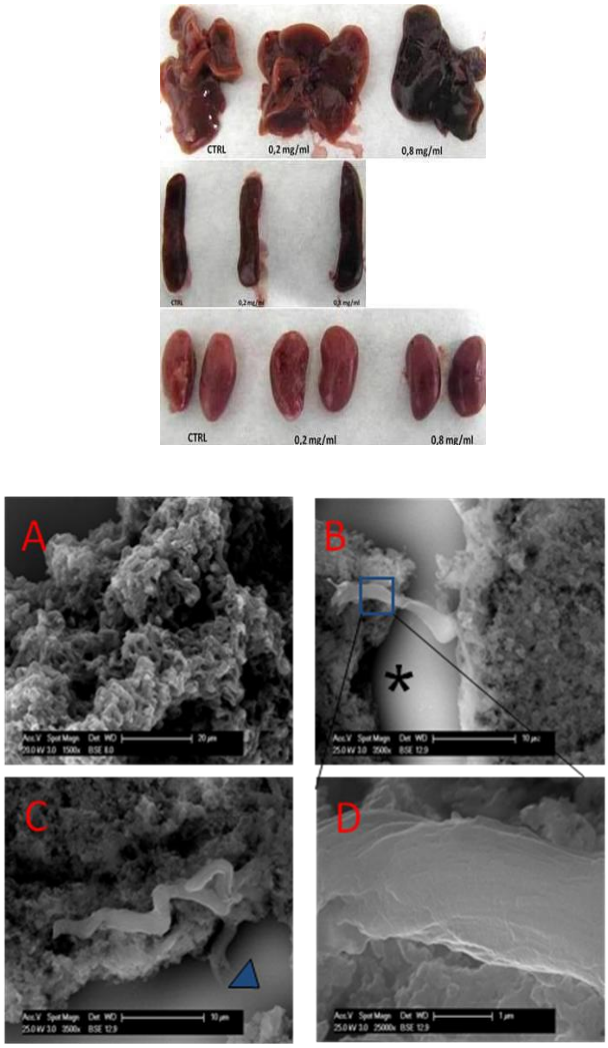

Figure 5. Organs collected 3 weeks after the final administration of SWCNTs. The liver shows macroscopically a darker color, as did the spleen, which was enlarged (Panel A). SEM analysis on liver 6.4 mg/Kg treated, revealed the presence of aggregated SWCNTs associated with portal spaces (B, indicated by *) and sinusoids (C, indicated by ▲).D: Magnification of figure 3B.

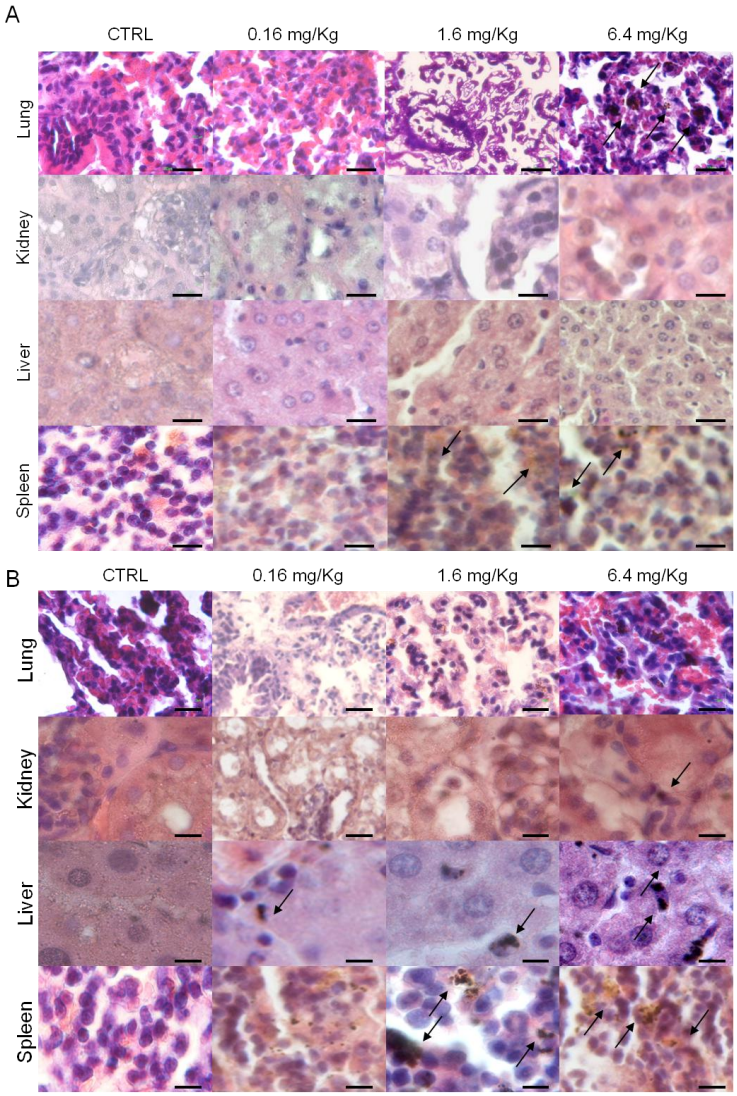

Figure 6: Histopathological analysis of organs in SWCNT treated mice stained with H&E. We observed an accumulation of an amorphous brown material, indicated by arrows, likely representing SWCNT deposits in the liver and spleen in all samples analyzed, 24 h after the first injection (Panel A) and 3 weeks after the last injection (Panel B). Accumulation in lungs occured only after acute treatment with SWCNTs, but they were rapidly cleared (See Supplemental). Bars: 10 um .

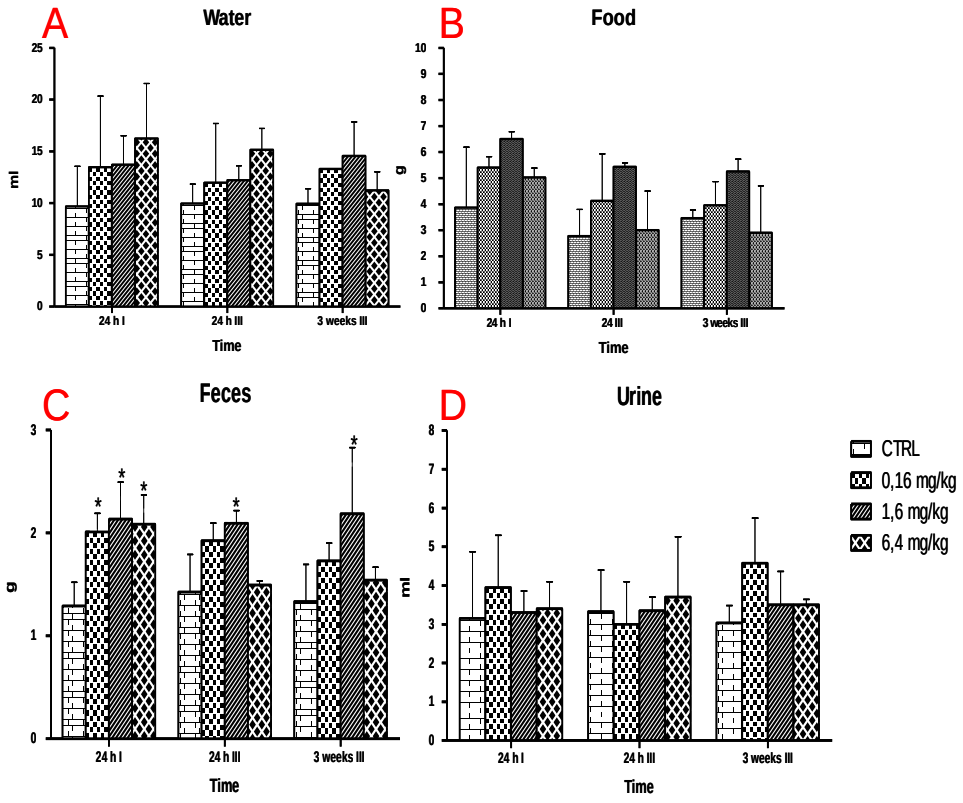

Figure 7. Consumption of food and water, and production of urine and feces as well in samples collected after 24h from the first injection ,24h from the last administration and three weeks after the last injection of SWCNTs. Data are expressed as mean(n > 6) ± SD. Statistically significant *= $p<0.05$, **=0.001 using ANOVA followed by Dunnett's test.

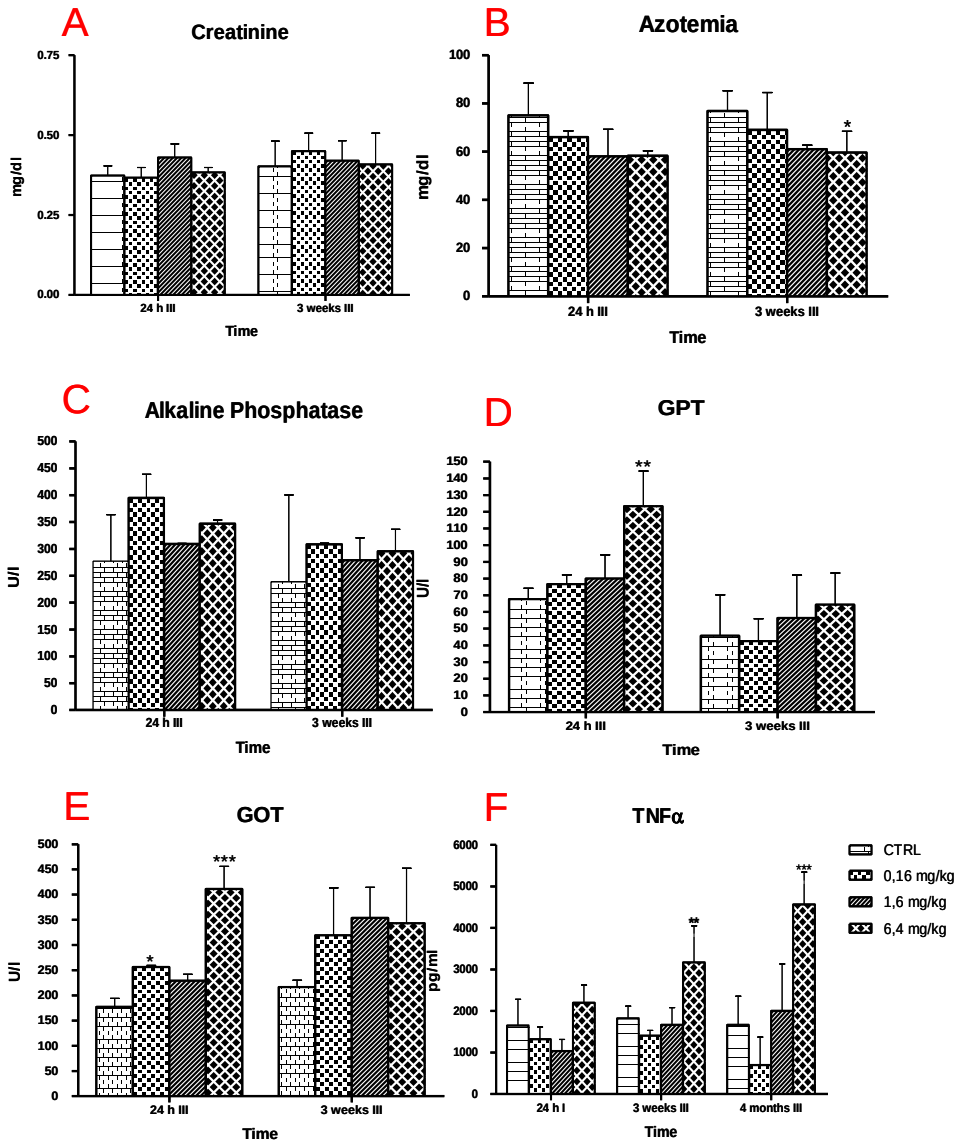

Figure 8.Effect of SWCNTs on the level of creatinine, azotemia,aspartate aminotransferase (GOT), alanine aminotransferase (GPT) alkaline phosphatase and TNFα in the serum of SWCNT treated and control CD1 mice. Each experiment was done in triplicate. Data are expressed as mean (n > 6) ± SD. Statistically significant *=0.5 **=0.1, ***=0.01 Using ANOVA followed by Dunnett's test.

TABLES

	Spleen (g)		Liver (g)	
	24h after I	3weeks after III	24h after I	3weeks after III
Ctrl	0.125±0.031	0.135± 0.017	1.543 ±0.261	1.631 ±0.122
0.16 mg/kg	0.105±0.007	0.130 ±0.042	1.163 ±0.015	1.520 ±0.183
1.6 mg/kg	0.123±0.006	0.255 ±0.230	1.32 ±0.124	1.447± 0.105
6.4 mg/kg	0.110± 0.010	0.159 ±0.016	1.317 ±0.047	1.537± 0.098

Table 1. Macroscopic alterations obseverd in harvested spleen and liver (see Figure 5) do not corrisponde to a significant alteration in organ weights, even for the highest dose administered

	24h after I		3weeks after III		3months after III*				
	Liver	Lung	Spleen	Liver	Spleen				
					CD45+	BL	MP	N	NK
Ctrl	30	35	57	2.05	47	14	3.35	1.5	0.7
0.16 mg/kg	32	85	90	4.03	72	26	3.34	2	1.45
1.6 mg/kg	27	89	93	6.08	74	21	13	2.5	2.38
6.4 mg/kg	80	89	95	9	80	25	15	2.8	3

Table 2. Flow cytometic analysis for CD45+ cells (a marker for all immune cells) in different organs harvested 24 h after the first injection, 3 weeks after the last injection or 3 months after the last injection. Each experiment was done in triplicate. Data are expressed as mean of percent of total CD45+ cells vs. controls.

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