Current Topics in Toxicology
Vol. 8, 2012

Review

Cyto-genotoxicity of engineered nanomaterials: Implications for occupational health

Delia Cavallo*, Cinzia Lucia Ursini and Sergio Iavicoli

INAIL-Research Area, Department of Occupational Medicine, Via Fontana Candida 1, 00040 Monteporzio Catone, Rome, Italy

ABSTRACT

The growing production and use of engineered nanomaterials (NMs) in many applications and the inadequacy of information about the associated health risks mean that it is essential to boost our knowledge of their potential biological effects (at the molecular-cellular and organ-system levels). The production, spread and use of engineered NMs is relatively recent and exposure assessment is complex, so no epidemiological studies or information on their toxicity, particularly on exposed workers, are available yet. Studies to date have been mainly *in vitro* or on animals - mostly mice. Some have highlighted the potential cytotoxic and genotoxic-oxidative effects of NMs. Most have used high concentrations of NMs and mainly found cytotoxicity. The studies available on the exposure to low concentrations of engineered NMs have detected genotoxic, oxidative and inflammatory effects that may have implications in carcinogenesis; however, there is still much uncertainty, and the results are contrasting. This review examines important cyto-genotoxicity studies on NMs such as multi- and single-walled carbon nanotubes, fullerenes, metal/metal oxide nanoparticles and quantum dots, which are representative of NMs already on the market or about to enter it, and are included in the priority list of manufactured NMs issued by the Organization for Economic Co-operation and Development (OECD). The focus is particularly on studies using experimental conditions similar to occupational exposures, with implications for the health and safety of workers employing, handling and producing NMs.

KEYWORDS: engineered nanomaterials, cyto-genotoxic effects, occupational exposure

INTRODUCTION

Nanomaterials (NMs) are widely employed for many purposes: in drug delivery, polymer composites, paints, cosmetics, electronics, biomedicine, optical devices, and energy. In view of this widespread use, the potential hazard for health is one of the main public concerns. Exposure to NMs is mainly by inhalation so the respiratory system is the most important target organ, though dermal penetration and ingestion may also occur.

The production, dissemination and use of engineered NMs is fairly recent and assessment of exposure is complex, so no epidemiological studies are available yet and there is very little information on their toxic effects on exposed populations. However, considering the increasing production and employment of engineered NMs in workplaces (experts have predicted that as many as ten million people could be working in processes involving nanotechnology by the year 2014 [1]), the potential exposure risk for these growing numbers of workers and the paucity of data on the health risks associated with these compounds, it is essential to implement what

*Corresponding author
d.cavallo@inail.it

knowledge there already is on the potential biological effects.

In the only study of workers exposed to polyacrylate spray paint containing nanoparticles (NPs) in a poorly ventilated workplace, Song *et al.* reported clinical symptoms such as pleural effusion, progressive pulmonary fibrosis and pleural damage, and death [2]. NPs were found in pulmonary tissue, bronchoalveolar lavage fluid and chest effusion of affected workers and in the raw material used at work. The same authors, examining the chemical composition of NPs found in pulmonary tissues and pleural membranes from affected workers, reported finding silica NPs, suggesting their involvement in pulmonary and inflammatory disease [3, 4]. Song's study, although it was done in extremely poor industrial conditions, highlights the need for further assessments of the potential risks and hazards associated with human exposure to engineered NMs.

Studies so far have been mainly *in vitro* or on animals (mostly rodents) and the effects of NMs on organs and systems are sometimes extrapolated from results obtained at the cellular level. Potential cytotoxic and genotoxic-oxidative effects have been seen at cellular level, and respiratory, dermal, immunologic, neurotoxic and cardiovascular effects at the organ-apparatus level.

In the nano size range, the properties of materials differ substantially from the "bulk" material, though so far they do not require separate registration; as specified in Regulation (EC) no. 1907/2006 on the Registration, Evaluation, Authorisation and Restrictions of Chemicals (REACH), registration is only required if production exceeds one ton/year [5]. The European Commission (DG Environment) has asked the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) to evaluate the current risk assessment methods laid down in the Technical Guidance Documents, in order to determine their appropriateness for NMs and to make detailed proposals for improvements where possible and appropriate [6]. The SCENIHR stated that not all NP formulations have been found to involve a more pronounced hazard than the bulk formulations of the same substance. This suggests that the hazard characterization of NMs and their classification and labeling should be done on a case-by-case basis.

Most of the investigations on the effects of NMs at cellular level used high concentrations and mainly found cytotoxic effects. Studies of the exposure to low concentrations of engineered NMs have reported genotoxic, oxidative and inflammatory effects that may be involved in carcinogenesis [7]. However, there is still great uncertainty, and results remain contrasting.

Most of these studies have used carbon nanotubes and metal oxide NPs which may cause DNA damage, directly or indirectly, by inducing oxidative stress. The genotoxic effects of NMs depend on their size, large surface area and physico-chemical properties (such as metal contaminants and surface charges) which determine their reactivity and aggregation state. These properties give NMs unexpected genotoxic properties which make it complicated to study their effects and mechanisms of action [8]. Depending on their size and state of aggregation, NMs are able to penetrate the cell by passive diffusion or receptor-mediated or protein-mediated endocytosis; they then enter the nucleus through the nuclear membrane (if sufficiently small) and through nuclear pore complexes or after dissolution of the nuclear membrane during cell division (if larger or aggregated). Once inside the nucleus, they can damage the genetic material directly through interactions with DNA and histone proteins or indirectly through inhibition of the nuclear proteins involved in DNA replication and transcription.

Genotoxic damage can also be induced indirectly through interactions with other cell proteins like those involved in cell division, through the induction of oxygen free radicals, inflammatory processes or by altering the functional status of proteins involved in DNA damage repair.

There is experimental evidence that engineered NPs can penetrate the systemic circulation and reach organs and systems. The main routes for NP uptake are assumed to be lungs, nasal mucosa, skin and gastro-intestinal apparatus with subsequent accumulation in many tissues such as kidneys, muscles, spleen and thigh bone [9]. At the organ and system level, *in vivo* studies have looked into the effects on the respiratory, cardiovascular, nervous, dermal and immunological systems of rodents.

Several studies have reported that engineered NMs, particularly carbon nanotubes (CNTs) and metal NPs, may induce oxidative stress and pulmonary inflammatory processes. Most studies have focused on CNTs and their adverse effects on the respiratory system seem to be related to the toxicity on different cell populations, capacity to induce fibrosis, asbestos-like activity, bio-accumulation and the potentially low levels of bio-degradation of NMs. Some similarities have been observed between the pathogenic properties of multi-walled carbon nanotubes (MWCNTs) and the properties of asbestos fibers in terms of inflammatory response and oxidative stress.

Studies of the potential effects of engineered NPs on the cardiovascular system have been mainly conducted *in vivo* on rodents exposed to CNTs. These can have effects on atheroma development, arterial thrombosis and blood platelet aggregation; the critical aspects of some of these studies, though, are the doses, routes of administration and small number of animals employed. Other studies have examined the potential effects of CNTs on the systemic inflammation which is thought to be one of the main predisposing factors for atherosclerosis; both MWCNTs and single-walled carbon nanotubes (SWCNTs) - but particularly the former - are able to activate systemic inflammation parameters such as granulocytes, IL-6, CXCL 1, IL-5, CCL11, CCL22 and neutrophil activation biomarkers.

In vivo studies of the effects of NPs on the central nervous system (CNS) mostly involve metal NPs and have found neurotoxic effects mainly induced by oxidative stress. There is scientific evidence that inhaled NPs are able to shift from the uptake sites to the CNS through trans-synaptic transport, or can be captured through the nerve endings of the nasal (olfactory and trigeminal nerves) or tracheo-bronchial mucosa (vagus nerve afferences). Furthermore, inhaled NPs penetrate respiratory barriers and, through the circulation, can reach the CNS by crossing the blood-brain barrier (BBB) if it is malfunctioning on account of specific pathological factors.

Dermal exposure to NPs may cause local effects on the skin or serve as a route of uptake into the systemic circulation. Further investigations on the different types of NMs are needed as their diffusion and dermal effects may vary according to their size and chemical composition. To date, most knowledge in this field comes from the pharmaceutical industry which has studied the effects of titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs used in sunscreen formulations. Very little information is available on other types of NP.

The data on the immunological effects of NPs suggest that, once inside the systemic circulation, the particles interact with proteins circulating or deposited on the cell surface, triggering an autoimmune response. NPs may also interfere with opsonization and, as a consequence, with the clearance of extraneous material (e.g. microorganisms) normally eliminated by this process; finally, they may also activate the complement, which can be either harmful or beneficial depending on the circumstances.

In this review we look at the most important *in vitro* and *in vivo* studies on cytotoxic and genotoxic effects of engineered NMs such as single- and multi-walled CNTs (SWCNTs and MWCNTs), fullerenes, metal/metal oxide NPs and quantum dots (QDs), representative of NMs already on the market or about to enter it, which are included in the priority list of manufactured NMs issued by the Organization for Economic Co-operation and Development (OECD) [10]. The focus is particularly on studies using experimental conditions similar to occupational exposures, with implications for health and safety of workers employing, handling and producing NMs.

1. Cytotoxic effects

Several studies are already available on the cytotoxic effects of NMs, indicating that they may produce a wide range of cytotoxic effects (Table 1) depending on their size, surface area and physico-chemical properties (metal contaminants or surface charges) which determine their aggregation state, penetrability and reactivity; this makes it complicated to observe their effects and modes of action. Several *in vivo* studies are currently available on the toxic effects of NMs and are listed in Table 2.

1.1. Carbon-based NMs

Carbon-based NMs consist of different structures that include tubes (CNTs), spheres (fullerenes),

Table 1. *In vitro* studies on nanomaterial cytotoxicity.

NMs	Cytotoxic effects
SWCNTs	Cell viability reduction in different cell types and NF*k*B activation in keratinocytes [13]. Decreased cellular adhesion and proliferation, induction of apoptosis and altered cell cycle regulation in human kidney cells [14]. Dose and time-dependent cytotoxicity as increase of oxidative stress, nuclear and mitochondrial changes in human keratinocytes [16]. Very low or no acute cytotoxicity in A549 cells [15, 44].
Functionalized ***SWCNTs***	Functionalized SWCNT are less toxic than pristine [19, 20]. Acid-functionalized SWCNTs are more toxic than pristine [21-24]. Concentration-time dependent cytotoxicity for both pristine and functionalized SWCNTs [25].
MWCNTs	Cell penetration and cell viability reduction in keratinocytes [35]. Cell viability reduction in macrophages and A549 cells [38, 39, 41-43]. Inhibition of cell proliferation in A549 cells [40] and MCF7 cell line but no in Caco-2 cells [37]. Cell membrane damage [46-48, 43]. No cell viability reduction [44, 45].
Functionalized ***MWCNTs***	Oxidized MWCNTs induce higher apoptosis than pristine in lymphocytes [51]. COOH-MWCNTs induce dose-dependent cell viability decrease, DNA damage and apoptosis in human dermal fibroblasts [50]
Fullerenes	
C60 fullerene	No cytotoxicity for macrophage cell lines [38, 57, 58]. Apoptosis induction in human fibroblasts, liver carcinoma and astrocyte cells [59] caused by membrane lipid peroxidation.
Functionalized *Fullerenes*	Hydroxil-functionalized C60 is less toxic than pristine in liver carcinoma cells and fibroblasts [60]. Aminoacid-functionalized fullerene induce dose-dependent cytotoxicity in human epidermal keratinocytes (HEK) [62].
Metal NPs	
Ag	Disruption of membrane integrity. Cytotoxicity in different cells [77-79]. Moderate-low cytotoxicity in A549 and THP1 cells [76].
Au	Inversely size-dependent cytotoxicity [71, 73]. PEG coniugated Au-NPs are not cytotoxic [72]. Ability to penetrate intact and damaged skin [74] and BBB [75].
Co	No cytotoxicity of Co-NPs [76]
Al	Dose-dependent toxicity in spermatogonic stem cells of rats [77]. Low cytotoxicity in A549 and THP1 cells [76].
Cu	Cytotoxic effects in A549 and THP1 cells [76].
Metal oxide NPs	
TiO_2	Membrane disruption, apoptosis induction [83]. No membrane disruption and no cell death [84]. No cytotoxicity but DNA damage [85].
ZnO	Inflammation. Inversely size-dependent cellular toxicity and immune response [81]. Cytotoxicity at 50 μg/ml in human nasal cells [80].
Fe_3O_4	No cytotoxicity [85].
CuO	More cytotoxic than ZnO, $CuZnFe_2O_4$ and MWCNTs [85].
Quantum Dots	
CdSe	Release of Cd ions inducing cell death and mithocondrial damage [101]. Activation of p53 and chromatinic condensation.
ZnS-CdSe	Decreased cytotoxicity due to ZnS [98].
Coated-CdSe ZnS QDs	Increased toxicity for MUA-coated QDs [102, 103]. Reduced toxicity for PEG-coated QDs [104].

Table 2. *In vivo* studies on nanomaterial toxicity.

NMs	Toxic effects
SWCNTs *(intratracheal instillation)*	Pulmonary inflammation, lung fibrosis, granuloma and development of mesothelioma in mice [26, 28]. Cardiovascular effects in rats [33]. DNA damage and inflammation in mice [119]. Non-dose dependent granuloma and transient lung inflammation in rats [27].
SWCNTs *(inhalation)*	No pulmonary toxicity in rats [29].
SWCNTs *(i.v. injection)*	No acute toxicity in rabbit but liver accumulation [32].
SWCNTs *(oral gavage)*	Inflammatory response and oxidative DNA damage in liver and lung of rats [118]. No MN induction in mice [120].
Functionalized **SWCNTs**	Inhaled acid-functionalized SWCTs induce in mice increased pulmonary toxicity in respect to pristine [34]. Oxidized SWCNTs induce in mice early miscarriages and fetal malformations in higher percentage in respect to pristine at doses $\geq$100 ng/mouse [24]. No-low toxicity of PEG-functionalized SWCNTs after intravenously injection in mice [30, 31].
MWCNTs (*intratracheal instillation*)	Transient pulmonary inflammation at 0.2 mg/rat, small granulomatous lesions at 1 mg/rat [29]. Acute pulmonary toxicity in rats exposed to 2 mg/rat [52]. Oxidative stress in rats [132]. No oxidative and inflammatory effects [131].
MWCNTs *(inhalation)*	Transient pulmonary inflammation and fibrosis after low exposures (10 and 20 μg/mouse) [54]. Lower pulmonary inflammation response compared to intratracheal instillation in rats [29]. Different inflammatory responses after short or long MWCNTs in mice [133]. No oxidative and inflammatory effects [130].
MWCNTs *(intraperitoneal injection)*	Inflammation, fibrosis, granuloma [53] and mesothelioma development depending on the exposure duration and fiber length in mice [129].
Functionalized **MWCNTs**	Intratracheally instilled acid-functionalized MWCNTs [46] and Taurine-MWCNTs [55] induce lower acute pulmonary toxic effects than pristine in mice. Intraperitoneal injection of COOH-functionalized MWCTs induce oxidative stress and hepatotoxicity in mice [56].
Fullerenes	Oxidative effects after oral exposure to C60 in rats [118]. Kidney accumulation and nephropathy after intraperitoneal and intravenous administration of C60 in rats [63]. Pulmonary inflammation [69] and DNA damage induction [138] in mice after intratracheal instillation. Lipid peroxidation in brain [66] and oxidative stress and malformations in Zebra fish embryo [67, 68]. No significant pulmonary toxicity after intratracheal instillation [64] and inhalation [65] of C60 in rats. No genotoxicity in mice [141, 119].
Metal NPs	Small granulomatous lesions and chronic alveolar dose-dependent inflammation in rats after inhalation of AgNPs [89, 90]. Disruption of BBB integrity after exposure to AlNPs by intravenous instillation in mice [91] and after intraperitoneal injection in rats [92]. Alterations of BBB induced by Al, Ag and Cu NPs in rats with Ag and Cu NPs showing the most toxic effects [93].
Ag *Al* *Cu*	
Metal oxide NPs	Pulmonary effects after intratracheal instillation in rats [94, 96] and after instillation in mice [95]. Lung carcinoma induction in rodents after TiO_2 inhalation or tracheal instillation [161, 162]. Genotoxic and oxidative effects in mice exposed to TiO_2 [163].
TiO_2	
TiO_2, ZnO, CuO	Significant malformations on Xenophus Laevis development by TiO_2, ZnO, CuO NPs [97].
Quantum Dots	Hepatoxicity due to oxidative stress in mice exposed to CdSe QDs [108]. Transient lung inflammation in rats after inhalation of CdS/Cd(OH)2 QDs [109]. DNA damage in BAL fluid of mice after instillation of charged CdTe QDs [119]. DNA damage, MN induction and DNA adducts in mice after orally administration of MAA surface modified CDSe QDs [169].

particles (nanoparticulate carbon black) and fibers (graphite nanofibers). CNTs are composed of graphene sheets rolled up into a tubular structure comprising a single layer (SWCNTs) or multiple layers (MWCNTs). CNT widths range from a few to tens of nanometers, and their lengths from less than a micrometer to a few millimeters. Their similarity in shape to asbestos suggests they may have asbestos-like toxicity.

Fullerenes consist of more than 60 carbon atoms linked by hexagonal or pentagonal rings in a caged ball-shaped structure. The fullerene with 60 carbon atoms, C60, with a diameter of 0.71 nm, was described by Kroto *et al.* in 1985 [11], after fullerenes with more than 60 carbon atoms (C70, C76, C78 and C82) and metal encapsulated fullerenes had been discovered.

Single-walled carbon nanotubes (SWCNTs)

***In vitro* studies**

SWCNTs are widely used in industrial and medical applications such as in automobiles, biosensors, and carriers for drugs and genes. They have a strong tendency to aggregate in microscopic bundles and their surface can be modified with functional groups to change their *in vivo* and *in vitro* behavior [12]. Studies in the last decade have given contradictory results; some show toxic effects while others found either no effect or very low toxicity after SWCNT exposure.

The toxicity of SWCNTs was evaluated on human keratinocytes, uterine cervix carcinoma cells HeLa, human alveolar (A549) and lung cancer (H1299) cells in a study by Manna *et al.* (2005) [13] who observed oxidative stress and dose-dependent reduction of cell viability in all the cells tested, and activation of NF-*k*B by SWCNT particles in keratinocytes. Cui *et al.* (2005) [14] treated embryonic human kidney cells with SWCNTs and observed a dose- and time- dependent reduction of the cells' adhesion capacity, reduced cell proliferation, increased induction of apoptosis and altered regulation of the cell cycle. Davoren *et al.* (2007) found very low acute toxicity of SWCNTs [15] on A549 cells in terms of cell viability reduction, confirmed by the lack of intracellular localization of SWCNTs after 24h exposure, even if the numbers of surfactant storing lamellar bodies were increased in exposed cells.

Several hypotheses have been put forward to explain the cytotoxicity observed with SWCNTs. One relates to the mode of production, as the synthesis of SWCNTs requires the use of metal catalysts, which can be toxic themselves. Shvedova *et al.* (2003) [16] reported dose- and time-dependent cytotoxicity in human epidermal keratinocytes exposed to SWCNTs. With higher concentrations and longer incubation times, increased oxidative stress, reduced glutathione levels, and nuclear and mitochondrial changes were found. The addition of a metal chelator reduced cytotoxicity, suggesting that residual iron catalyst in solution may play a role in the cytotoxicity.

Particle aggregation might also influence NP cytotoxicity. Wick *et al.* (2007) [17] investigated the cytotoxicity of SWCNT at various degree of agglomeration on the mesothelioma cell line MSTO-211H, to determine how agglomeration influenced SWCNT cytotoxicity. Only the well-dispersed SWCNT bundles had no adverse cellular effects, indicating that SWCNT agglomeration leads to cytotoxicity. However, an earlier study by Tian *et al.* (2006) [18] on fibroblasts, testing raw SWCNTs and SWCNTs purified with HCl (which removes metal catalysts and modifies the aggregation state) found lower cytotoxicity with the raw, unpurified SWCNTs. They proposed that the unpurified SWCNTs were less toxic as a result of their aggregation into larger - hence less toxic - particles. This, however, contradicts Wick *et al.* (2007) [17], who suggested that the agglomerated SWCNTs were cytotoxic because of their stiffness and larger size, making the NTs emulate the effects of asbestos fibers. Although the conflicting results may reflect the use of two different cell lines (fibroblasts as opposed to mesothelioma cell line MSTO-211H) the effect of SWCNT aggregation is still questionable.

Some studies found that functionalized SWCNTs had less cytotoxic effects than purified nanotubes. Shi Kam *et al.* (2004) [19] showed that SWCNTs functionalized with carboxylic groups, biotin and fluorescein were not toxic to HL60 cells (human leukemia cell line) after one hour's exposure. Sayes *et al.* (2006a) [20] ran *in vitro* cytotoxicity screens of three functionalized SWCNT samples (SWCNT-phenyl-SO_3H, SWCNT-phenyl-SO_3Na, SWCNT-phenyl-(COOH)2) on cultured human

fibroblasts. As the degree of sidewall functionalization increased, the SWCNT sample became less cytotoxic. Saxena *et al.* (2007) found SWCNTs were more toxic after treatment with strong oxidative acids [21]. In a study of the cytotoxicity of carboxylic acid functionalized-SWCNTs on differentiated and non-differentiated Caco-2 cells derived from a human intestinal adenocarcinoma, cytotoxic effects were more evident on the differentiated cell cultures [22].

The toxicity of SWCNTs before and after functionalization on the RAW264.7 mouse macrophage cell line was compared by Dong *et al.* (2012) [23] who reported greater toxicity for acid-functionalized SWCNTs (AF-SWCNTs) than pristine SWCNTs; this might be due to greater bioavailability of AF-SWCNTs and to the surface functional groups. The same study also found that AF-SWCNTs altered the expression of genes related to ribosomes, mitochondria, inflammatory response, cell cycle/apoptosis, and the proteasome pathway.

Oxidized SWCNTs were also more toxic than pristine SWCNTs in a study examining their embryotoxicity, using the embryonic stem cell test (EST), a validated *in vitro* assay that predicts the embryotoxicity of soluble chemical compounds [24]. In another recent study on HUVEC both SWCNTs and COOH-functionalized SWCNTs induced concentration- and time-dependent toxic effects [25].

In vivo toxicity studies

Intratracheal instillation of purified SWCNT induced dose-dependent epithelioid granulomas and interstitial inflammation in mice [26] but non-dose-dependent granulomas and transient inflammation in rats [27]. Shvedova *et al.* (2005) [28] found that metal-reduced SWCNTs could enter mouse lung tissues and induce acute inflammation, progressive granulomas and fibrosis. In a more recent study the inhalation of 0.03 or 0.13 mg/m^3 of well-dispersed SWCNTs for four weeks did not cause pulmonary toxicity in rats [29]. Schipper *et al.* (2008) also detected no toxicity after injection of PEG functionalized SWCNT (100 μM) into the bloodstream of mice [30] and Yang *et al.* (2008) observed only low toxicity after intravenous injection of 40 μg-1 mg of SWCNT in mice [31]. Both these studies reported long-term accumulation of SWCNTs in spleen and liver. Liver accumulation with no acute toxicity was also reported by Cherukuri *et al.* (2006) [32] in rabbits intravenously injected with 75 μg of SWCNTs.

Legramante *et al.* (2009) [33] found adverse cardiovascular effects in rats after instillation of relatively low doses of SWCNTs (1 μg/g body weight). Tong *et al.* (2009) [34] studied mice exposed by inhalation to low doses (10 or 40 μg per mouse) of pristine and acid-functionalized SWCNTs, and only the high-exposure group had a pulmonary inflammatory response and an increase of pulmonary toxicity with acid functionalization.

Low doses (from 10 ng to 30 μg/mouse) of pristine and oxidized SWCNTs were used by Pietroiusti *et al.* (2011) [24] to study the effects of SWCNTs on embryonic development. Doses of 100 ng/mouse or more affected embryonic development with, in particular, a higher percentage of early miscarriages and fetal malformations in females exposed to oxidized SWCNTs than in those exposed to pristine NMs. Extensive vascular lesions and increased production of ROS were detected in placentas of malformed fetuses. The study identified the oxidized SWCNTs as more toxic and oxidative stress as responsible for their effects.

Multi-walled carbon nanotubes (MWCNTs)

In vitro studies

Studies on MWCNT toxicity have increased in recent years, particularly since 2008, and have demonstrated that in the toxicity evaluation the differences in diameter and shape must be addressed. Studies on MWCNT cytotoxicity report discordant results probably due to different chemico-physical characteristics such as size, shape, surface charge, which can all influence the dispersivity and agglomeration, with different effects on the cells. Monteiro-Riviere *et al.* (2005) [35] reported that in human epidermal keratinocytes (HEK) exposed to 0.1-0.4 mg/mL of MWCNTs there was a slight dose- and time-dependent decrease in cell viability coupled with an increase in release of cytokine IL-8 at the higher MWCNT concentrations. The study also showed a time- and concentration-dependent increase of cells containing

MWCNTs. Therefore the authors suggest that the cytotoxicity is due to MWCNT attachment to the cell membrane or MWCNT internalization. Sato *et al.* (2005) [36] also found MWCNT aggregates in THP-1 cytoplasmic cells. Chiaretti *et al.* (2008) [37] observed that MWCNTs inhibited proliferation in the human mammary adenocarcinoma cell line MCF-7 and human smooth muscle cells (hSMC), but not in Caco-2 cells**.**

MWCNTs' effects have been examined in several respiratory *in vitro* models [38-44]. A dose-dependent decrease in cell viability was evident in alveolar macrophages exposed to >95% purified MWCNTs, in the study by Jia *et al.* (2005) [38]. Muller *et al.* (2005) [39] exposed macrophages to MWCNTs and found induction of dose-dependent cytotoxicity and overproduction of TNF-α. Magrez *et al.* (2006) [40] reported that carbon-based NMs (CBNMs) caused inhibition of cell proliferation and cell death in human lung tumor cells. MWCNTs induced DNA damage and cytotoxic effects in the murine macrophage cell line RAW 264.7 [41]. A concentration- and time-dependent decrease of viability was found by Tabet *et al.* (2009) [42] in A549 cells exposed to different concentrations (0.1-100 μg/mL) of industrial MWCNTs dispersed in dipalmitoyl lecithin, ethanol and PBS. In our recent study [43] A549 cell viability also decreased after 24h exposure to 5-100 μg/mL of commercial MWCNTs, starting from the lowest concentration. However, Pulskamp *et al.* (2007) [44] found no acute cytotoxicity after exposure of these same cells and rat alveolar macrophages NR8383 to 5-100 μg/mL of commercial SWCNTs and MWCNTs. Flahaut *et al.* (2006) [45] also detected no loss of viability in HUVEC exposed to 0.5-0.9 μg/mL of MWCNTs.

Some studies indicate that MWCNTs induce cell membrane damage after prolonged exposure (24-48h) to concentrations between 4.5 and 200 μg/mL [46-48]. Di Giorgio *et al.* (2011) [49] exposed mouse macrophage cells (RAW264.7) to 50 μg/mL of MWCNT or SWCNT for 24, 48 and 72h; scanning electron microscopy (SEM) indicated no cell surface modifications for MWCNTs and a reduction in the number of microvilli for SWCNTs. In addition after 72h exposure to 50 and 100 μg/mL of SWCNTs and MWCNTs there was significant cytotoxicity (reduced cell proliferation and apoptosis) and a higher percentage of necrotic cells after 24h exposure to 50 μg/mL of MWCNTs. In our study on A549 cells we showed that MWCNTs induced cell membrane damage already after 2 and 4h of exposure to 40 and 100 μg/mL and we detected clear changes on the cell surface, such as reduced number of microvilli, holes and tears after 4h exposure to 5-100 μg/mL MWCNTs, examined by SEM [43].

There are very few studies on functionalized MWCNTs. Patlolla *et al.* (2010) [50], in human dermal fibroblasts exposed to 40, 200 and 400 μg/mL of COOH-functionalized MWCNTs, found a dose-dependent decrease in cell viability, DNA damage and apoptosis starting from 40 μg/mL after 48h. Bottini *et al.* (2006) [51] exposed human isolated lymphocyte T cells for 24-120h to 40 μg/mL and 400 μg/mL of pristine and oxidized MWCNTs and found greater induction of apoptosis with oxidized MWCNTs after 76h exposure to 400 μg/mL; this indicates that hydrophobic pristine MWCNTs cause less toxic effects than tubes coated with hydroxyl or carboxyl groups.

***In vivo* toxicity studies**

Induction of acute pulmonary toxicity (granuloma formation) in rats after intra-tracheal administration of MWCNTs was reported by Muller *et al.* (2008a) [52]. Poland *et al.* (2008) [53] showed that abdominal instillation in mice of long MWCNTs (50 μg/mouse) resulted in asbestos-like, length-related, inflammation of the abdominal walls seven days after exposure. Another study in mice exposed by aspiration to 50-nm diameter MWCNTs at doses of 10-40 μg/mouse, approximating estimated human occupational exposures, found pulmonary inflammation and fibrosis; however, at 10 and 20 μg/mouse this returned to control levels by 56 days post-exposure [54]. The recent study by Morimoto *et al.* (2012) [29], using well-dispersed MWCNTs to expose rats by intra-tracheal instillation (0.2 or 1 mg/rat), found transient pulmonary inflammation in the low-dose group and small granulomatous lesions in the high-dose group. In the same study a group of rats was exposed to the same MWCNTs by inhalation (0.37 mg/m^3 for four weeks) and there were fewer pulmonary inflammatory responses with smaller amounts of MWCNTs in the lungs than after intra-tracheal instillation. In the study

by Kim *et al.* (2010) [46] tracheal instillation of 10 or 100 µg/mouse of pristine and acid-functionalized MWCNTs induced dose-dependent granulomatous inflammation with more severe acute toxic effects for pristine ones tubes. Wang *et al.* (2010a) [55] too, using water-soluble taurine-MWCNTs and pristine MWCNTs (0.25-1 mg/kg) in mice exposed by intra-tracheal instillation, found the pristine MWCNTs caused more acute toxic pulmonary effects; however, pulmonary inflammation was recoverable with both MWCNTs. Hepatotoxicity induced by activation of the mechanisms of oxidative stress was reported by Patlolla *et al.* (2011) [56] in mice after intraperitoneal injection of COOH-functionalized MWCNTs.

Fullerenes

***In vitro* studies**

Fullerenes are believed to be less toxic than CNTs. A number of studies demonstrated that the cytotoxic response to fullerenes depended on the cell type. No cytotoxicity was seen in macrophages while in some other cell types there was a dose-dependent cytotoxic effect. Jia *et al.* (2005) [38] applied non-treated C60 fullerenes, SWCNTs and MWCNTs to guinea pig macrophages and reported there was less cytotoxicity with C60 than CNTs. C60 also caused no cytotoxicity in murine macrophages, as reported by Fiorito *et al.* (2006) [57]. Porter *et al.* (2006) [58] studied the effects of C60 in human monocytes/macrophages and found no significant cytotoxicity though at the subcellular level the fullerenes tended to accumulate in the cell (in lysosomes, cytoplasm, along the nuclear membrane and inside the nucleus). In human dermal fibroblasts, human liver carcinoma cells and normal human astrocytes, Sayes *et al.* (2005) [59] found that C60-induced cell apoptosis was due to the peroxidation of membrane lipids by oxygen radicals. The addition of an antioxidant, L-ascorbic acid, prevented the oxidative damage and the fullerene-induced toxicity. In an another study Sayes *et al.* (2004) [60] treated human liver carcinoma cells and dermal fibroblasts with unground C60 fullerenes and four different fullerene derivatives with surface functional groups, added to enhance solubility; only the highest concentration (2400 ppb) of unground C60 showed cytotoxicity, the increase of functional groups reducing cytotoxicity. Yamawaki *et al.* (2006) [61], using HUVEC exposed to 1-100 µg/mL of water- soluble hydroxyl-functionalized C60 [C60 $(OH)_{24}$], observed cytotoxicity only at the highest concentration. Rouse *et al.* (2006) [62] exposed human epidermal keratinocytes (HEK) to amino acid-functionalized fullerenes for 24 and 48h and found a dose-dependent decrease in cell viability.

***In vivo* toxicity studies**

Animal experiments on C60 toxicity have been done mainly in rats and fish. Chen *et al.* (1998) [63] administered C60 orally, intraperitoneally and intravenously to rats and observed no lethal damage after oral administration; the LD50 was 600 mg/kg after intraperitoneal injection. The fullerene injected intraperitoneally and intravenously accumulated in the kidney, inducing nephropathy. Sayes *et al.* (2007) [64] reported no significant pulmonary toxicity after intra-tracheal administration of C60 to rats, and Baker *et al.* (2008) [65] found no lung lesions after inhalation of C60 aggregate aerosol.

Studies on fish reported significant lipid peroxidation in brain [66] and oxidative stress and malformations in Zebra fish embryos [67, 68]. In mice exposed to C60 fullerene by instillation, there was a pulmonary inflammatory response [69].

1.2. Metal and metal oxide nanoparticles

***In vitro* studies**

Gold (Au) has long been used in medicine and therapy, and nano-sized gold particles, with their unique properties, are increasingly being employed in many applications such as cancer therapy, imaging and medical diagnostics [70]. Some studies indicate inversely size-dependent cytotoxicity of Au NPs. Pan *et al.* (2009) [71] found that very small Au NPs (1.4 nm diameter) caused cell death by oxidative stress and mitochondrial damage while larger particles (15 nm diameter) were less cytotoxic. Gu *et al.* (2009) [72] used Au NPs conjugated with PEG (3.7 nm diameter) to expose HeLa cells; they found no cytotoxic effects although the particles penetrated the cell nucleus. Dose-dependent induction of apoptosis and up-regulation of pro-inflammatory genes was reported in a study using Au NPs of different sizes (from 2-4 to 20-40 nm) on murine macrophages [73].

A recent study demonstrated that Au NPs could penetrate human intact and damaged skin in an *in vitro* diffusion system [74].

The potential effects on permeability and pro-inflammatory response of the BBB induced by various-sized Au NPs (3-60 nm) were investigated in primary rat brain microvessel endothelial cells (rBMEC). Smaller NPs (3-7 nm) seemed more able than larger particles to accumulate in the cells, inducing moderate cytotoxicity [75].

A quantitative analysis of the cytotoxicity of 24 types of NPs with the same diameter was done on human A549 and THP-1 tumor cell lines [76]. Copper- and zinc-based NPs showed the highest toxicity. Titanium, aluminium, cerium, silver, nickel and zircon oxide NPs showed moderate to low toxicity, and no toxicity was found with tungsten carbide and Co.

Braydich-Stolle *et al.* (2005) [77] found dose-dependent toxicity for silver (Ag), molybdenum trioxide (MoO_3) and aluminium (Al) NPs on spermatogonic stem cells, with Ag NPs the most toxic and MoO_3 NPs showing the least toxicity. Cytotoxic effects of Ag NPs were also seen on osteoblasts and osteoclasts [78]. In human mesenchymal (hMSC) cells 10 μg/mL of Ag NPs induced cytotoxic effects and significant IL-6, IL-8 and VEGF release [79].

Zinc oxide (ZnO) NPs are used in commercial products applied topically for skin care. The toxic effects of different concentrations (0.01-50 μg/mL) were evaluated by Hackenberg *et al.* (2011b) [80] in human nasal mucosa cells: significant cytotoxicity was observed only at the highest concentrations [80]. The recent study by Feltis *et al.* (2012) [81] to evaluate immune cell function and cytotoxicity of ZnO NPs in human macrophages and monocytes found that smaller particles induced a greater cellular response.

Titanium dioxide (TiO_2) NPs, widely used in consumer products such as sunscreen formulations, paints, and pharmaceutical preparations, shows cytotoxic effects in different kinds of cells [82]. Apoptosis was induced in human liver HepG2 cells even by very low concentrations of TiO_2 NPs [83]. Koeneman *et al.* (2010) [84] showed that TiO_2 NPs penetrated the epithelial lining of an intestinal model by transcytosis without disrupting epithelial integrity, and altering apical microvillar organization and increasing intra-cellular free calcium.

Several studies have analyzed the cytotoxicity of metal oxide NPs in comparison with CNTs. Several metal oxide NPs (CuO, TiO_2, ZnO, $CuZnFe_2O_4$, Fe_3O_4, Fe_2O_3) were compared with CNPs and MWCNTs by Karlsson *et al.* (2008) [85] in human A549 cells; CuO NPs were the most cytotoxic, with CuO>ZnO>$CuZnFe_2O$>MWCNTs, while the others caused little or no toxicity, though some of them induced DNA damage (TiO_2, $CuZnFe_2O_4$).

Another study evaluating the cytotoxicity of metal oxide NPs (Al_2O_3 and TiO_2) and MWCNTs on A549 cells found CNTs were more toxic than metal oxide NPs [48]. Sohaebuddin *et al.* (2010) [86] compared the cytotoxicity of TiO_2 and SiO_2 NPs and differently-sized MWCNTs on 3T3 fibroblasts, RAW264.7 macrophages and human bronchial epithelial cells (hT). RAW264.7 macrophages were the most susceptible and 3T3 fibroblasts more resistant to NM toxicity. SiO_2 showed the highest cytotoxicity. MWCNTs with the largest diameter (> 50 nm) were more toxic than smaller ones.

In vivo toxicity studies

There are fundamental differences in NP transfer routes to blood and body organs when NPs are administered into the respiratory tract or intravenously [87]. Whereas dose rates associated with direct intravenous injection are obviously very high, both dose and entry rate of NPs from lung deposits into the blood compartment (arterial) are low, and this must be taken into consideration in studies *in vivo*.

Rinderknecht *et al.* (2009) [88] administered differently-sized gold NPs (5, 50, 200 nm) with different surface modifications (citrate, albumin, polyethylene glycol - PEG) by intra-tracheal microspray or intravenous injection to rats. All three factors modified the biodistribution to extrapulmonary organs: particle size, surface modification and the portal of entry. In particular, 5-nm albumin-coated gold NPs, injected intravenously, were retained preferentially in the liver, whereas after intra-tracheal administration they were retained in the bone marrow. There was

also a minimal translocation from the lung to the blood over 24h; blood concentrations were only between 3 and 20 ng/mL despite a high dose of 50 μg to the lung, which highlights the need to consider realistic low doses when designing *in vitro* studies with cells from extrapulmonary target organs.

In two studies on Sprague-Dawley rats exposed by inhalation (6 h/day for 90 days) to Ag NPs at similar concentrations (0.7×10^6, 1.4×10^6 and 2.9×10^6 particles/cm^3) small granulomatous lesions and chronic alveolar dose-dependent inflammation were observed [89, 90]. The effects of Al NPs (8 to 12 nm) on the BBB and brain's vascular system were examined in mice after intravenous instillation [91] and rats after intraperitoneal injection [92]. In both studies the Al NPs reduced tight junction protein expression and caused marked fragmentation of occludin, with disruption of the BBB. These results suggest that Al NP neurotoxicity is associated with the ability to influence permeability and alter BBB integrity.

The influence of 50-60 nm Cu, Al and Ag NPs on the BBB was investigated in Sprague-Dawley rats [93] after intravenous instillation, intraperitoneal injection and cortical perfusion. BBB integrity was markedly altered by NM exposure, intravenous instillation and cortical perfusion causing the most acute adverse effects; Cu and Ag NPs were the most toxic.

Most *in vivo* studies on metal oxide NPs toxicity have focused on TiO_2 in rats and have used intratracheal instillation of high doses of NPs, so the toxic effects cannot be directly extrapolated to humans under realistic lower exposures. Warheit *et al.* (2007) [94] found that intratracheal instillation of 1 to 5 mg/kg of different types of TiO_2 NP in rats induced pulmonary effects due to the chemical composition and crystalline structure of the particles. In another study, adult male ICR mice were given a single intra-tracheal dose of 0.1 or 0.5 mg TiO_2 NPs (19-21 nm) and pulmonary emphysema, macrophage accumulation, extensive disruption of alveolar septa, type II pneumocyte hyperplasia, and epithelial cell apoptosis were found [95]. In rats given 1.5 and 5 mg/kg of TiO_2 NPs of different sizes and aggregation states by intra-tracheal instillation a size- and time-dependent inflammatory response was observed [96]. In a recent study of the teratogenic potential of commercial CuO, TiO_2 and ZnO NPs on *Xenopus laevis* development, the NPs did not cause mortality at concentrations up to 500 mg/L but malformation rates were significant and the gut seemed to be the main target organ [97].

1.3. Quantum dots (QDs)

QDs consist of a nucleus containing metal elements (some, such as Cd, Te, Se and Pb, are highly toxic), a protective coating layer (cap/shell) mainly made of ZnS, and functional coating groups (carboxylic group, amine group and PEG) which make them sufficiently hydrophilic, enhance their biocompatibility and bioactivity and make them more stable by reducing their potential toxicity [98]. These unique properties are put to extensive use in many biomedical applications, particularly biomedical imaging and electronics, but have been suggested for use in computer memories, visual displays, solar cells and lasers [99] and a replacement for organic dyes on account of their superior quantum yield and resistance to photo-bleaching [100].

In vitro studies

Studies on ZnS-coated CdSe QDs indicate that the ZnS shell can reduce cytotoxicity. Uncoated QDs release cadmium ions and cadmium induces cell death through oxidative stress and mitochondrial damage [101]. Several authors report different effects of different surface coatings on QD toxicity. Shiohara *et al.* (2004) [102] studied the cytotoxicity of three Cd/Se/ZnS QDs coated with mercapto-undecanoic acid (MUA) at different spectral emissions (green, yellow and red) in three cell lines and found that MUA coating increased the QD toxicity. MUA-coated QDs (100 μg/mL) also showed toxicity in the murine T cell lymphoma cell line EL-4 (Hoshino *et al.* 2004) [103].

The cytotoxicity and inflammatory potential of CdSe ZnS QDs with three different surface coatings (PEG, PEG-amines, or carboxylic acids) were assessed in primary neonatal human epidermal keratinocytes (HEKs) by Ryman-Rasmusse *et al.* (2007) [104]. They found QDs coated with carboxylic acid and PEG-amine caused cytotoxicity and PEG-coated QDs caused less. Another study using the same kind of coated QDs in human mammary epithelial cells (MCF10 and MCF7)

found no uptake for PEG and PEG-amines but extensive internalization of carboxylic acid- coated QDs which, however, did not induce cytotoxicity after 72h exposure at 0.8 nM [105]. Lovric *et al.* (2005) [106] reported that mercaptopropionic acid and beta-mercaptoethylamine coating reduced the cytotoxicity of CdTeQDs in rat pheochromocytoma cell cultures. They also found that the cytotoxicity was size-dependent, with different subcellular distribution: small QD cations deposited in the nucleus, and larger ones in the cytoplasmic matrix.

In a recent study of the effects of a series of different surface-coated (organic, carboxylated [COOH], amino [NH_2] PEG) QDs on J774.A1 macrophages cells, organic-coated QDs were the most toxic, although core material also had a significant impact on QD toxicity [107].

In vivo toxicity study

Although several studies have looked at the accumulation of QDs in organs, the biological effects of QD exposure and accumulation have only rarely been addressed. Moreover, little is known about the molecular mechanisms responsible for QD-mediated biological events and cytotoxicity. The effects of acute and chronic exposure to CdSe QDs in adult mice were evaluated by Liu *et al.* (2011) [108] who found that the liver was the main site of QD accumulation, leading to significant hepatotoxicity in terms of morphological alteration of hepatic lobules due to oxidative stress. The inhalation toxicity of water-soluble core-shell CdS/$Cd(OH)_2$ QD was evaluated in male Wistar rats head-nose exposed for 6 h/day on 5 days to the technically maximum concentration (0.52 mg Cd/m^3) [109]. These QDs caused local neutrophil inflammation in the lungs, which partially regressed after the three-week recovery period.

2. Genotoxic and oxidative effects

The main genotoxic-oxidative effects reported in the current literature for NMs are illustrated in Table 2 (*in vivo* studies) and Table 3 (*in vitro* studies).

2.1. Carbon-based NMs

Investigations of the genotoxic effects of CNTs are extremely important in view of the similarities to asbestos which is known to damage DNA and induce carcinogenesis on account of its long bio-persistence, local generation of free radicals and subsequent prolonged inflammatory response. Studies of the genotoxic and oxidative effects of SWCNTs or MWCNTs have given contradictory results so far, probably because of the differences in their characteristics (purity, size, shape, presence of metal contaminants, functionalization), the dispersion medium, presence of surface charges and exposure-related conditions, which are not always described in detail. Fibrous NMs may induce genotoxicity directly through the interaction with DNA (SWCNTs have been observed in the nucleus) or the mitotic fuse and indirectly through the induction of oxidative stress and inflammatory responses [41].

Single-walled carbon nanotubes (SWCNTs)

In vitro studies

Most of the *in vitro* studies conducted so far on CNTs have focused on the SWCNTs and highlighted the induction of oxidative stress and DNA damage in different cell types. In particular, there are reports of generation of free radicals, accumulation of peroxidation products and reduced antioxidant activity in human keratinocytes [16], induction of ROS in rat pulmonary cells [110], ROS generation and DNA damage in human mesothelial cells [111] and DNA damage in human bronchial cells (BEAS-2B) [112]. Lindberg *et al.* (2009) [112] examined the effects of exposure to commercial CNTs (SWCNTs >50%, other CNTs about 40%) in BEAS-2B cells for 24-72h using the Comet assay and micronucleus (MN) test. Dose- and time-dependent increase of DNA damage by comet assay, and MN induction, only after 48h exposure, were observed. Pacurari *et al.* (2008) [111], studying human mesothelial cells exposed to SWCNTs containing metal contaminants, also found DNA damage and ROS generation by Comet assays.

The genotoxicity and oxidative damage may be related to the fibrous nature of the SWCNTs used and to the presence of metals. Pulskamp *et al.* (2007) [44] found that metal traces associated with commercial SWCNTs were responsible for the biological effects: there was a dose-time dependent increase of intracellular ROS and decrease of the mitochondrial membrane potential with commercial SWCNTs, but purified SWCNTs had no effect.

Table 3. *In vitro* studies on genotoxic-oxidative effects of NMs.

NMs	Genotoxic-oxidative effects
SWCNTs	Oxidative stress [16, 110]. DNA damage by comet assay [115, 111, 49]. DNA damage by comet assay and MN induction after 24h [116] and after 48h exposure [112]. Aneuploidy induction [117]. No DNA breakage by comet assay but ROS induction [113].
Functionalized ***SWCNTs***	Altered expression of genes related to ribosome, mitochondria, inflammatory response, cell cycle/apoptosis and proteasome pathway by acid-functionalized SWCNTs [23].
MWCNTs	Direct but no oxidative DNA damage by comet assay [85, 43]. ROS generation [44, 123, 124]. Oxidative stress [125]. Oxidative DNA damage by comet assay [41]. Direct DNA damage induced by low concentrations (1-3 μg/ml) of MWCNTs [49]. MN induction [121]. Point mutations [67]. Lack of DNA damage induction [127]. No induction of chromosome aberrations [122]. No alteration of mRNA expression of oxidative response genes [42].
Functionalized ***MWCNTs***	COOH-MWCNTs induce DNA damage in human dermal fibroblasts [50].
Fullerenes	Oxidative stress, induction of DNA damage, mutagenicity and induction of chromosome aberrations and micronuclei [59, 137, 138]. No DNA damage induction [139, 140]. No cyto-genotoxicity [134, 135].
Metal NPs	
Ag, Co, Co-Cr	DNA breakages, oxidative stress, increase of MN frequency and chromosome aberrations [142-144].
Au	Oxidative DNA damage at concentrations >50 μg/ml [145-147]. No oxidative DNA damage at ≤0.2 μg/ml [148].
Metal oxide NPs	
TiO_2	SCE induction, increased MN frequency, DNA damage, increase of HPRT gene mutations [157-159]. ROS and oxidative DNA damage enhanced by UV radiations [153, 154]. Anatase TiO_2 induces oxidative DNA bases at greater extent [155] and is more genotoxic [156] than rutile form. No double strand DNA breakage [160].
ZnO	Chromosomal aberrations [151], DNA damage by comet assay [149, 152]. ROS production in presence of UV radiations.
Quantum Dots	
CdTe	Penetration into the cell nucleus through membrane and induction of breakages in DNA chain [165], DNA damage and ROS generation [166] by CdTe QDs. Activation of p53 and chromatinic condensation.
Cd Se	Decreased cyto-genotoxicity due to coating with ZnS [7]. CdSe-ZnS QD-induced DNA damage mediated by photogenerated or surface-oxide-generated ROS [167].

Several studies using pure SWCNTs have reported discordant results relating to DNA damage and oxidative effects [113-115]. Jacobsen *et al.* (2008) [113], in a study using the Fpg Comet assay to investigate the direct-oxidative DNA damage in murine lung epithelial cells exposed to highly pure SWCNTs, found oxidative stress induction but no DNA breakages. However, the Comet assay detected DNA damage in Chinese hamster lung fibroblasts (V79) exposed to pure SWCNTs, as

reported by Kisin *et al.* (2007) [115]. A recent study employing the Comet assay and MN test to evaluate the genotoxicity of SWCNTs in human cells of the oral cavity exposed to 50-150 µg/mL for 24h found genotoxic effects at all concentrations. This study also showed a significant increase of ROS production at all doses [116]. Another effect of SWCNTs was the induction of aneuploidy by their interaction with the mitotic spindle apparatus, demonstrated in primary Human Small Airway Epithelial (SAEC) and immortalized BEAS-2B airway lung epithelial cells after 24h exposure to 24-96 µg/cm^2 of SWCNTs [117].

***In vivo* toxicity studies**

In vivo studies on rodents indicate that SWCNTs may induce oxidative stress and the inflammatory response [118, 119]. Folkman *et al.* (2009) [118] exposed rats by gavage to 0.064 and 0.64 mg/kg body mass of highly purified SWCNTs and found oxidative DNA damage in some organs (liver and lung). Jacobsen *et al.* (2009) [119] exposed mice by intra-tracheal instillation of 54 µg/mouse of SWCNTs and the Comet assay detected significant DNA damage. A recent study of the genotoxic potential of SWCNTs using a battery of *in vitro* and *in vivo* assays showed that mice given 60 and 200 mg/kg of high pure and well-dispersed SWCNTs by gavage for two days presented no genotoxicity, assessed by the bone marrow MN test [120].

Multi-walled carbon nanotubes (MWCNTs)

***In vitro* studies**

The genotoxic potential of purified MWCNTs was reported by Muller *et al.* (2008b) [121], who used two complementary approaches based on the MN test *ex vivo* (on type II pneumocytes after intra-tracheal exposure) and *in vitro* (on rat lung epithelial cells). This study indicated that MNs may be induced by both clastogenic and aneugenic events. In addition, MWCNTs may induce point mutations that might be responsible for their carcinogenicity [67]. However, Wirnitzer *et al.* (2009) [122], found no genotoxic effects (induction of chromosome aberrations) for agglomerates of MWCNTs (baytubes) in V79 cells. Karlsson *et al.* (2008) [84] found that significant DNA damage was induced even at low concentrations (2, 40 and 80 µg/mL) of commercial MWCNTs using the Comet assay in human lung epithelial cells (A549) exposed for 4h. They found no oxidative DNA damage after exposure to 40 and 80 µg/mL and no increase in intracellular ROS generation. Tabet's study (2009) also found no oxidative stress, examined by mRNA expression of the different genes implied in an oxidative response [42]. ROS generation was detected in telomerase-immortalized human bronchial epithelial cells (hT) by Sohaebuddin *et al.* (2010) [85]. This study found no ROS generation after 2h exposure to MWCNTs with two different diameters (20-30 nm and >50 nm), whereas other authors [123, 124] found induction of ROS after MWCNT exposure in A549 cells.

MWCNT exposure in human embryonic kidney cells led to concentration-dependent cytotoxicity, cell membrane damage, increased lipid peroxidation and reduced intracellular glutathione levels, indicating that oxidative stress induced cytotoxicity [125]. Migliore *et al.* (2010) [41], using the Fpg Comet assay in a murine macrophage cell line (RAW264.7), found oxidative DNA damage after 24h exposure to 1 and 10 µg/mL of MWCNTs but not at the highest concentration (100 µg/mL). Pulskamp *et al.* (2007) [44] found that metal traces associated with commercial MWCNTs, as for SWCNTs, caused ROS induction and lowered the mitochondrial membrane potential, while purified MWCNTs had no such effects.

These differences might be partially explained by the experimental protocols used, illustrating the difficulties of assessing the health effects of CNT exposure, because of the reactivity of these NMs, that depends on several characteristics such as dimensions, presence of metals, agglomeration and structural defects.

Significant induction of DNA damage by CNTs including MWCNTs was found with the Comet assay using different cell types, concentrations and exposure times [43, 112, 126]. In our recent study on the cyto-genotoxicity of MWCNTs [43], direct DNA damage was detected on A549 cells after short exposure (2h) to low concentrations of MWCNTs. DNA damage was also seen in mouse macrophages exposed to low MWCNT concentrations (1 and 3 µg/mL) for 24h in the study by Di Giorgio (2011) [49]. In another recent study on A549 cells exposed for 24h to 7.5 and

30 µg/mL MWCNTs no DNA damage induction was detected [127].

***In vivo* toxicity studies**

Inflammation, fibrosis and pulmonary granuloma were reported in mice exposed to MWCNTs in a pilot study by Poland *et al.* (2008) [53] and long-term studies indicate that MWCNTs might promote the development of mesothelioma [128, 129]. However, other studies produced no evidence of oxidative or inflammatory effects on rodents exposed to MWCNTs [130, 131]. Reddy *et al.* (2011b) found induction of oxidative stress with reduced total anti-oxidant capacity in rats after intra-tracheal instillation of MWCNTs [132]. Different inflammatory responses after exposure to short or long MWCNTs by pharyngeal aspiration were reported in mice [133], with short MWCNTs having greater potential to induce polymorphonuclear cells whereas long MWCNTs increased IL-6 production.

Fullerenes

***In vitro* studies**

Fullerenes appear to have antioxidant properties without significant cyto-genotoxic effects [134, 135]; however, some studies reported induction of oxidative stress, DNA breakages, increased MNs, mutagenicity and chromosome aberrations [136, 7]. In the Comet assay colloidal dispersions of C60 fullerenes in water had genotoxic effects on human lymphocytes [137]. In addition, Totsuka *et al.* (2009) [138] demonstrated that exposure to C60 fullerenes induced the formation of MNs in A549 lung cells. Other *in vitro* studies have found no DNA damage after exposure to C60 fullerenes [139, 140].

The different findings on the genotoxic effects of fullerenes are probably due to factors such as exposure time, preparation and treatments of fullerenes including the presence of ligands and cell types; today, since chemico-physical characterization is available only in very few studies, it is difficult to compare data.

***In vivo* toxicity studies**

In vivo studies, mainly on rats and mice, show that exposure to fullerenes can have oxidative and inflammatory effects. Folkmann *et al.* (2009) [118] reported that the oral exposure to low doses of C60 induced the formation of high levels of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) in rat liver and lungs. In another study, on mice, instillation of C60 seemed to induce inflammatory responses in lungs [69]. Jacobsen *et al.* (2009) [119], however, found no significant DNA damage in mice exposed to C60 fullerene by intratracheal instillation. Another study not showing any genotoxicity of C60 fullerene was by Sinohara *et al.* (2009) [141], who used the bone marrow MN test on mice exposed up to 88 mg/kg of C60. DNA damage, measured by the Comet assay, was found by Totsuka *et al.* (2009) [138] in lungs of mice exposed to 0.2 mg/body of C60 fullerene for 3h.

2.2. Metal and metal oxide nanoparticles

***In vitro* studies**

Transition metal ions (cadmium, chromium, cobalt, copper, iron, nickel, titanium and zinc) released by specific NPs may induce the production of hydroxyl radical ($^{\cdot}OH$), which is one of the main species causing DNA damage. Fe(II) too may cause H_2O_2 production from molecular O_2. Metal nanoparticles like silver, cobalt and cobalt-chromium appear to have genotoxic effects, inducing, for instance, DNA breakages, oxidative stress, increased MN frequency and chromosome aberrations [142-144].

The potential of gold NPs to induce DNA damage is still not clear given the conflicting results described in literature. Three studies on different cell lines showed that gold NPs could cause oxidative DNA damage at concentrations higher than 50 µg/mL [145-147] and a role of NP size in the genotoxic potential was evidenced by Kang *et al.* (2009) [146]. Another study [148] of the genotoxicity of three gold NP reference materials (10, 30 and 60 nm) developed by the National Institute of Standards and Technology (NIST) in HepG2 cells and calf thymus DNA did not find oxidative DNA damage at concentrations up to 0.2 µg/mL or any free radical production confirming the above studies.

Metal oxide NPs (TiO_2, ZnO, SiO_2, Fe_xO_x) cross the cell membrane and concentrate in the perinuclear region, indirectly causing genotoxic damage by promoting oxidative stress [149, 150] and inflammatory response; they may also enter

the nucleus (TiO_2 and SiO_2) where they aggregate with the nuclear proteins involved in DNA replication and transcription, inhibiting them and, as a consequence, inducing DNA damage. An *in vitro* study by Karlsson *et al.* (2008) [84] compared the genotoxic effects of metal oxide NPs (CuO, TiO_2, ZnO, $CuZnFe_2O_4$, Fe_3O_4, Fe_2O_3) with those of carbon NPs and MWCNTs on A549 cells using the Comet assay and 2',7'-dichlorofluorescein diacetate (DCFH-DA) to measure ROS concentrations. All particles except iron oxides caused DNA damage after 4h exposure; CuO particles were the most powerful, followed by TiO_2 particles. CuO particles also caused the greatest oxidative damage and were the only ones that raised intracellular ROS levels.

Nano-sized ZnO and TiO_2 are widely used in industrial products such as cosmetics and pharmaceuticals, medical materials, paints and for the decomposition of organic environmental pollutants on account of their capability to generate ROS when exposed to UV radiation. ZnO NPs had genotoxic effects, causing chromosomal aberrations in Chinese hamster ovary (CHO) cells [151] and DNA damage, shown by Comet assay, in human nasal mucosa cells [152] and in a human epidermal cell line (A431) even at a very low concentration (0.8 μg/mL) [149].

TiO_2 exists in two crystalline forms, anatase and rutile, with the anatase form having greater photocatalytic activity. Nano-sized TiO_2 induce ROS, DNA damage and oxidative DNA damage *in vitro* with enhancement on exposure to UV light or simulated sunlight [153, 154]. The anatase TiO_2 NPs seem to induce oxidative DNA bases to a greater extent [155] and are more genotoxic [156] than the rutile form, probably reflecting the higher photocatalytic activity. Moreover, TiO_2 NPs induce sister chromatid exchange (SCE), and increase MN frequency and DNA damage [157], and hypoxanthine-guanine phosphoribosyl-transferase (HPRT) gene mutations [158]. However, the cellular response in terms of MN induction seems also to depend on size, and Gurr *et al.* (2005) [159] found that TiO_2 anatase NPs were genotoxic only when small (<200 nm). However, there is no general agreement about the genotoxicity of these materials. In fact in a recent study on human peripheral blood lymphocytes exposed to 20-200 μg/mL of 15-30 nm TiO_2 anatase NPs, the particles reached the cell nucleus but did not induce DNA double-strand breakage [160].

In vivo toxicity studies

The International Agency for Research on Cancer (IARC) classifies TiO_2 NPs as possibly carcinogenic to humans (Group 2B) on the basis of sufficient experimental evidence in animals. Induction of lung carcinoma was reported in rodents after inhalation or tracheal instillation of TiO_2 NPs [161, 162] and genotoxic (induction of MN and DNA damage) and oxidative (induction of 8-hydroxy-2 deoxyguanosine) effects were reported on mice exposed to TiO_2 NPs in drinking water [163]. Three TiO_2-based NPs (two coated rutile and one uncoated anatase) (54 μg/mouse) were instilled intra-tracheally to mice: coated TiO_2 induced DNA damage in lung lining fluid cells while uncoated TiO_2 was highly inflammatory but did not cause DNA damage [164].

2.3. Quantum dots (QDs)

In vitro studies

QDs sliding through the nuclear membrane pores may interact with the histone proteins in DNA, inducing breaks of the DNA chains, activation of p53 genes and chromatin condensation. DNA strand breaks induced by CdTe QDs in rainbow trout hepatocytes were reported by Gagne *et al.* (2008) [165]. CdTe QDs also induced yH2AX foci indicative of DNA damage in a dose-dependent manner in HUVEC after 12h exposure [166]. The addition of coating groups (e.g. ZnS) is thought to have a protective effect as it reduces cyto-genotoxicity, as highlighted in several studies [7]. Although some studies reported CdSe-ZnS QD-induced DNA damage mediated by photo-generated or surface-oxide-generated ROS, the long-term stability of the QD coating group has not yet been adequately tested [167]. If QDs are held in the organism for a very long time, the protective coating may be degraded under photolytic and oxidative conditions and they may subsequently penetrate the nuclear membrane pores and induce cyto-genotoxic effects [168].

Overall, the preparation and purification of materials plays an important role in determining the

genotoxicity of QDs. While there is some evidence of interaction between QDs and the cell nucleus, few studies have focused specifically on their genotoxicity.

***In vivo* toxicity studies**

Jacobsen *et al.* (2009) [119] used positively (QD621) and negatively (QD620) charged CdTe QDs to evaluate their genotoxic potential in broncho-alveolar lavage (BAL) fluid from ApoE-/- mice, after instillation of QDs with 63 µg Cd. BAL cells obtained 3h after QD exposure showed significantly elevated levels of DNA damage (3.3-fold), whereas there was no difference in the level of DNA damage elicited by the two different types of QDs. The study also assessed the genotoxicity of other NMs such as carbon black, fullerene C60, SWCNT and Au NPs, and found that the QDs had a greater DNA damaging effect than the other types of NPs. Another study examined the genotoxicity of mercaptoacetic acid (MAA) surface-modified CdSe QDs administered orally to mice at doses of 500-2000 mg/kg; after 7 days of treatment there was DNA damage, MN induction and generation of DNA adducts at the highest dose [169].

3. Implications for occupational health

Although there is no information as yet on adverse health effects in humans, mainly because of the lack of validated methods for environmental and biological monitoring of potential exposure to engineered NMs, particularly in the occupational field, studies have highlighted the potential cytotoxic and genotoxic-oxidative effects at cellular level and the respiratory, dermal, immunologic, neurotoxic and cardiovascular effects caused by NMs. Thus, as the production and use of engineered NMs rapidly increase, with the consequent potential exposure of workers and consumers, standardized procedures must be developed to monitor personal and environmental exposure to these substances. In the last few years the potential risks and hazards associated with human exposure to engineered NMs have been increasingly discussed [170-172]. Studies on workers' exposure and on what measurement techniques should be used to monitor occupational exposure [173, 174] are starting to be available. Exposure assessment protocols involving NP classification, the identification of biologically relevant characteristics and physical exposure metrics are now among the main aims of the scientific community involved in nanotechnology.

CONCLUSIONS

The information we have today on cytotoxicity of NMs shows that:

- carbon nanotubes induce cytotoxic and apoptotic effects but much depends on the state of aggregation, the presence of metal catalysts, functionalization groups, purity degree, length and diameter;
- fullerenes appear to be less cytotoxic though the response depends on the cell types: they are not cytotoxic for macrophages but are for other cells;
- metal NPs show a wide range of cytotoxic responses relating to the type of metal: some effects have been observed for silver-, copper-, zinc-, molybdenum- and aluminium-based NPs;
- cytotoxic effects of QDs depend on the size and type of coatings.

As regards the potential genotoxicity of engineered NMs, most *in vitro* studies have been conducted by:

- *Comet assay* - evaluating direct or oxidative DNA damage - which has given positive results for fullerenes, SWCNTs, MWCNTs, gold NPs, TiO_2, ZnO and CuO NPs, CdTe and CdSe/ZnS QDs;
- *MN test* - evaluating the clastogenic and aneugenic effects - that have given positive results for TiO_2, SiO_2, Co-Cr, ZnO NPs and TiO_2 or ZnO+ UV-visible irradiation.

Until now high concentrations of NMs have been used in most cyto-genotoxicity studies, so more studies using lower concentrations of the most common NMs, which will be relevant in terms of occupational exposure, are still needed. In the last few years studies using low concentrations and longer exposure times have been started.

In vivo studies, mainly on rodents, involve in most cases:

- carbon nanotubes, which may induce oxidative stress, inflammation, fibrosis and mouse lung granuloma;

- fullerenes, responsible for oxidative stress and DNA damage in rats;
- TiO_2 NPs, which have genotoxic and oxidative effects in mice.

Until now most *in vivo* toxicity studies have used tracheal instillation of high concentrations of NMs, therefore here too more studies should soon be reported using exposure conditions closer to real exposure (e.g. inhalation, which is the most likely route of exposure) with low concentrations, more like possible occupational exposure. In the last few years more studies using inhalation and lower doses have been started.

The contradictory findings of studies so far reflect the lack of detailed information on the chemico-physical characteristics and production process of the materials under investigation but also on the dispersion media and treatments, which may influence cell uptake, interactions with biological macromolecules and, as a result, toxicity. In addition, further genotoxicity studies using multiple tests simultaneously are needed, to investigate also how NMs interact with biological fluids, dispersion media, coloring agents and other reagents that may influence the results.

Investigating occupational exposure to NMs by establishing the best monitoring techniques, and the identification of the potential human health effects of these materials are a research priority for the scientific community involved in nanotechnology.

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