A REVIEW ON CONCEPTS OF NANOTOXICOLOGY

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Summary

The article describes the toxicity of nanosized particles in in-vivo and in-vitro models. As the application of nanomaterials expanded, the potential exposure will also increase and they become airborne. The workers of the industries will exposed to these aerosol or airborne nanomaterials, resulting in inhalation or ingestion of nanomaterials take place. In lungs of inhaled workers, they create oxidative stress causing damage or inflammation in bronchioles. It is also possible for translocation into blood stream and deposition in other vital organs like kidneys, liver, pancreas etc. In order to assess the toxicity of these nanoparticles, it is advisable to use *in vivo* and *in vitro* models.
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Keywords: Nanoparticles, carbon nanotubes, nanotoxicology

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Introduction

This manuscript reviews a number of issues that must be dealt with to assess toxicity of engineered carbon nanomaterials *in vivo* and *in vitro* models. Engineered nanomaterial is a term used to describe inorganic materials of high uniformity, with at least one critical dimension below 100 nm, specifically engineered for applications (1). Nanotechnology, as defined by the United States (US) Nanotechnology Initiative, is 'the understanding and control of matter at dimensions of roughly 1–100 nanometers, where unique phenomena enable novel applications'. In the last decade, engineered nanoparticles have become an important class of new materials with several properties that make them very attractive for commercial development (2). The unique physical properties of nanoparticles (conductivity, reactivity) compared to larger microparticles enable these novel engineering applications. One of the principal attributes of nanoparticles enabling development is their unique catalytic properties. Engineered nanomaterials can have very different shapes, *e.g*., spheres, fibers, tubes, rings, planes. Traditional methods for producing macroscopic quantities (mg or more) of SWNTs involve growth from carbon vapor produced either by arc evaporation of metal-doped carbon electrodes (3-5) or by laser vaporization of metal-doped carbon targets (6). SWNTs can also be grown by catalytic decomposition of molecules such as C2H4 and CO (7) and CH4 (8) on supported metal particles. SWNTs are produced by flowing CO mixed with a small amount of Fe (CO)5 through a heated reactor (9).

Despite the wide application of nanomaterials there is a serious lack of information on their impact on human health and the environment. Engineered nanomaterials are currently found in diverse products: personal care items, sunscreens, abrasion-resistant materials, environmental catalysts, anti-fouling and anti-microbial coatings, in wood preservation, fuel cells, UV-attenuation, scratch resistant and charge dissipating coatings, and even food products (10**).** President Clinton established the National Nanotechnology Initiative in 2000 to lead this country into the next industrial revolution (11). Nanomaterials are the building blocks of this new industry. One of the major objectives of the initiative calls for "developing materials that are 10 times stronger than steel, but a fraction of the weight for making all kinds of land, sea, air and space vehicles lighter and more fuel efficient." This statement specifically implicates carbon nanotubes, a novel and lightweight material with the strongest tensile strength of all synthetic fibers (12). The presidential initiative directs NASA to search for applications of carbon nanotubes and other nanomaterials in aerospace. Carbon nanotubes structurally resemble rolled-up graphite sheets with one end capped. These tiny tubes can have single or multiple walls. Single-wall carbon nanotubes (NTs), unlike graphite or carbon black, possess highly desirable electrical, mechanical, and thermal properties (13-14) and have many potential applications in the electronics, computer, and aerospace industries. As stated by Ajayan *et al*. (15), "It is rare to come across a material that has such a range of remarkable properties." Enormous research efforts have been channeled into discovering applications of this novel material. Dr. R. Smalley (a Nobel laureate and a pioneer in carbon nanotube research) predicted that hundreds or thousands of tons of NTs could be produced in 5 to 10 years and "in time, millions of tonnes of nanotubes will be produced worldwide every year" (10, 16). As the production and applications of NTs expand, potential human exposures will also increase.

Nanoparticle exposure- in occupational settings

Procedures for the handling of CNTs can result in aerosol release of these materials into the surroundings (17). How many people be exposed to engineered nanoparticles and in what quantities? Before interpreting toxicological data, it is thus essential to characterize the expected concentrations of Nanoparticle that may be present in air, water and soil. The exposure of workers making and using nanoparticles in manufacturing plants is growing as the nanotechnology industry increases demand for small, size-controlled particles. Many new and established companies producing nanoscale particles for diverse applications. MWCNT aerosols generally have diameters between 20 nm to > 200 nm, lengths from 1,000 nm to > 106 nm, and different shapes (straight, partly rigid, bent, curled, and partly flexible) that may appear single or in clumps or ropes (18). Only one published study has investigated the potential for SWCNTs to become airborne. A laboratory study by Maynard et al. (17) investigating the physical nature of the aerosol formed during mechanical agitation was complemented by a field study of SWCNT release during handling of unrefined SWCNTs. The authors found that sufficient agitation of unrefined SWCNT material can release fine particles into the air, but the concentrations generated while handling the material in the field were very low (< 53 μg/m3). The laboratory study also revealed that different SWCNT production methods produced different types of aerosols. The laser ablation process generated a more compact aerosol that was difficult to break down into smaller particles, whereas the HiPCO (high-pressure carbon monoxide) process generated a more extended material that was easier to break down into smaller particles and appeared to lead to higher airborne concentrations. Maynard et al. (17) also found glove deposits of SWCNTs during handling that were estimated at between 0.2 and 6 mg per hand and thus concluded that large SWCNT containing clumps had the propensity to become airborne and could remain so for long periods. This may cause dermal exposure and health risks even in less well-protected areas.

Portals for Nanoparticles

The size of nanoparticles makes them highly mobile in both humans and the environment. Therefore, they can enter the body through several ports. Translocation can then occur via the blood stream leading to an accumulation in many tissues including the brain and testes (19**).** Most of the toxicity research on nano-sized particle (NSP) *in vivo* has been carried out in mammalian systems, with a focus on respiratory system exposures for testing the hypothesis that airborne Ultra fine particles (UFP) cause significant health effects. With respect to NP, other exposure routes, *via* skin and GI tract, also need to be considered as potential portals of entry. Portal of entry specific defense mechanisms protect the mammalian organism from harmful materials. In essence, toxicokinetics look at how a particle may get into the body, how it is circulated and distributed within it, and how it may be metabolised and excreted. Understanding this is important as it allows consideration of the important target organs that may or may not be affected, to predict realistic exposure doses and to understand how the body responds to nanoparticle exposure in terms of metabolism and excretion. To date, few, if any, adsorption, distribution, metabolism and excretion (ADME) studies have been conducted for nanoparticles.

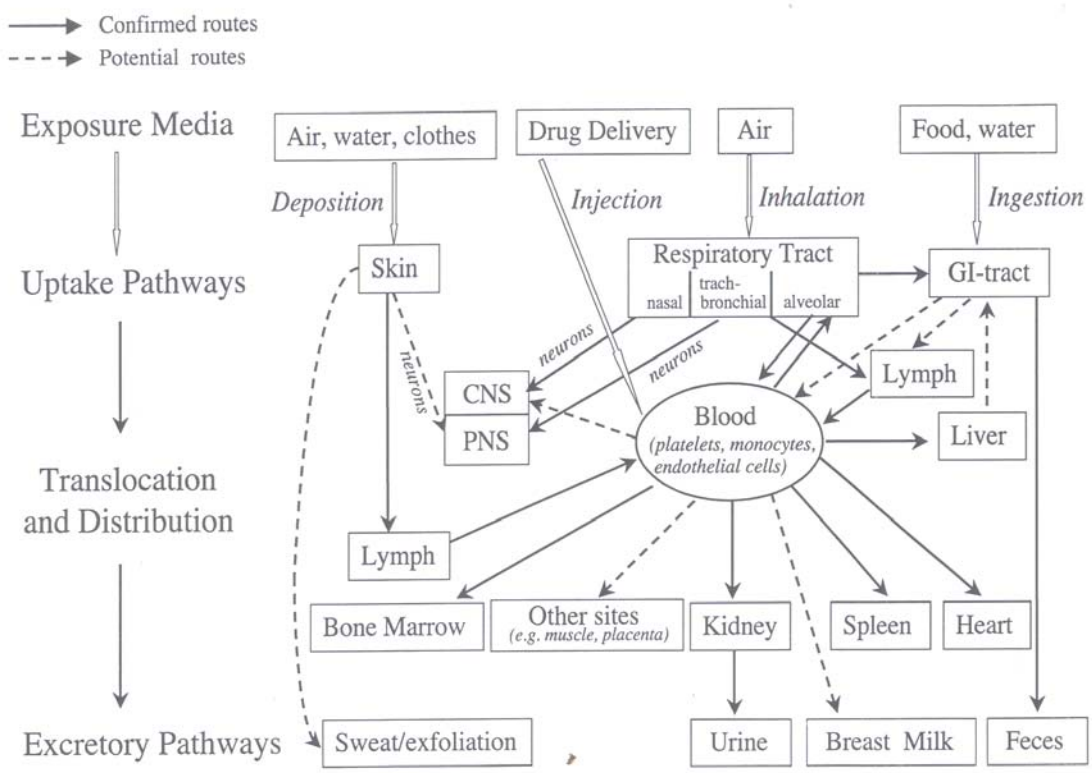

Fig 1: Summary of the hypothetical Biokinetic pathways for Nanoparticles (20**)**

Bio-kinetics of NSP:

1. The bio-kinetics of nano-sized particles are different from larger particles:

– when inhaled:

- they are efficiently deposited in all regions of the respiratory tract;
- they evade specific defense mechanisms;
- they can translocate out of the respiratory tract via different pathways
- (endocytosis and transcytosis)

– when in contact with skin:

- there is evidence of penetration to the dermis;
- they translocate *via* lymph to regional lymph nodes;
- a possible uptake into sensory nerves needs to be investigated

– when ingested:

- there appears to be little uptake into the organism, mostly excreted *via* feces

– when in blood circulation:

- they can distribute throughout the organism,
- they are taken up into liver, spleen, bone marrow, heart, and other organs

– in general, translocation rates are largely unknown, they are probably very low but are likely to change in a compromised/diseased state

2) The biological activity and biokinetics are dependent on many parameters:
- size; shape; chemistry; crystallinity;
- surface properties (area, porosity, charge, surface modifications, weathering of coating)
- agglomeration state; biopersistence; dose

3) These parameters are likely to modify responses and cell interactions such as:
- a greater inflammatory potential than larger particles per given mass
- translocation across epithelia from portal-of-entry to other organs
- translocation along axons and dendrites of neurons
- induction of oxidative stress
- pro-oxidant and antioxidant activity of NSP in environmentally-relevant species
- binding to proteins, receptors
- -localization in mitochondria.
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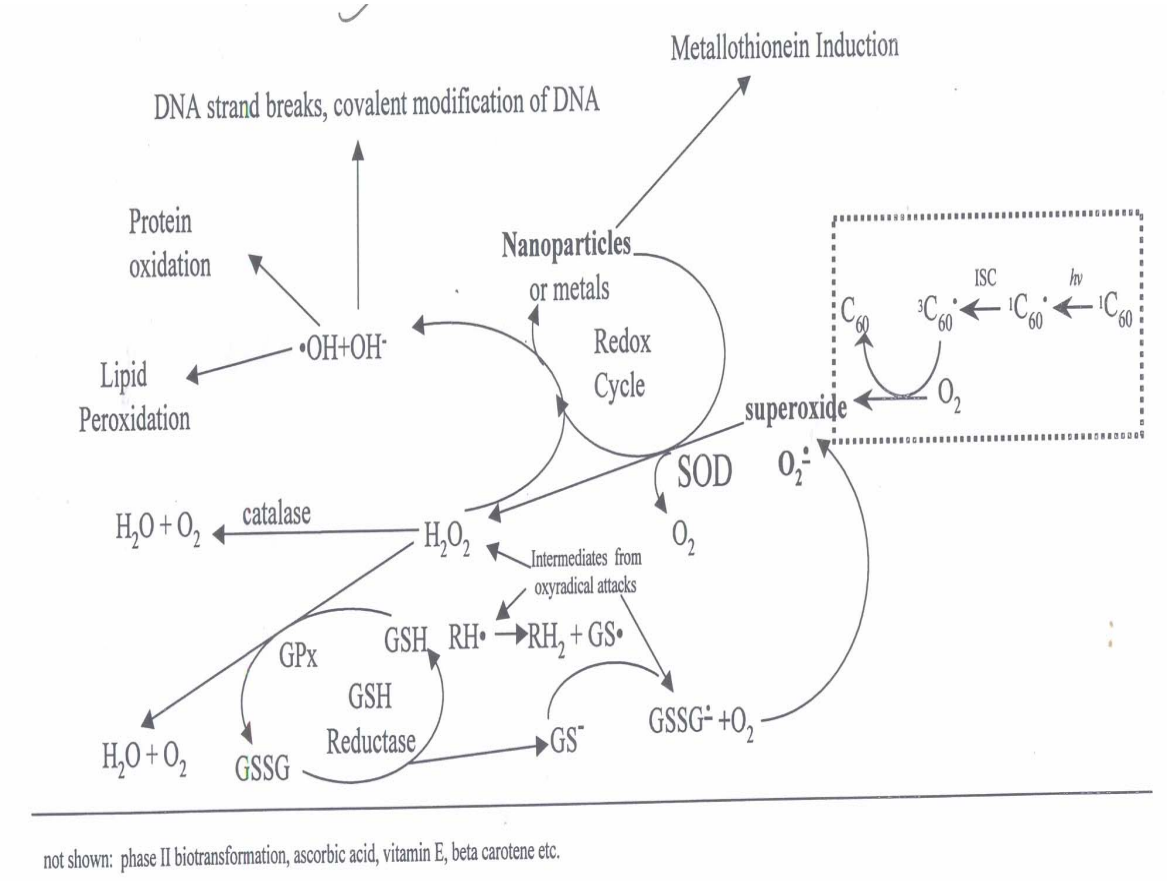
In particular, transport of nanoparticles across membranes both between and within cells (e.g. into mitochondria) and an understanding of their toxic effects (e.g. oxidative stress, genotoxicity, inflammatory cytokine production, apoptosis) are important (21).

ROS Mechanisms of Nano-sized Particle Toxicity

Both *in vivo* and *in vitro*, NSP of various chemistries have been shown to create reactive oxygen species (ROS). ROS production has been found in NP as diverse as C60 fullerenes, SWNT, quantum dots, and UFP, especially under concomitant exposure to light, UV or transition metals. (22-23). It has been demonstrated that NSP of various sizes and various chemical compositions preferentially mobilize to mitochondria (24-25). Since mitochondria are redox active organelles, there is a likelihood of altering ROS production and thereby over-loading or interfering with anti-oxidant defenses (Fig. 2).

The figure 2 explained that the engineered NP have been shown to release oxyradicals (pictured here is the mechanism of C60 as determined by Yamakoshi et al. (2003), which can interact with the antioxidant defense system. In addition to fullerenes, metals such as Cd, Fe, or Ni quantum dots, or Fe from SWNT manufacturing, could also act in Fenton-type reactions. The exact mechanism by which each of these diverse NP cause ROS is not yet fully understood, but suggested mechanisms include i) photo excitation of fullerenes and SWNT causing Inter-System Crossing (ISC) to create free electrons; ii) metabolism of NP to create redox active intermediates, especially if metabolism is *via* cytochrome P450s; iii) inflammation responses *in vivo* may cause oxyradical release by macrophages (20**)**.



(Abbreviations: SOD = superoxide dismutase; GSH = reduced glutathione; GSSG = oxidized glutathione; GPx = glutathione peroxidase; R = any organic molecule)

Fig 2: ROS Mechanisms of Nano-sized Particle Toxicity **(**20**)**

Exposure Dose–Response Considerations

A careful evaluation of exposure-dose-response relationships is critical to the toxicological assessment of NSP. This includes not only questions about the dosemetric – mass or number or surface of the particles as discussed before - but most importantly also the relevance of dose levels (20**)**. The intratracheal instillation of several hundred µg into a rat does not resemble a relevant *in vivo* inhalation exposure, both dose and dose rate cause high bolus dose artifacts. *Therefore, in vivo* and *in vitro* studies will only provide useful data on the toxicity and mode of action of NSP provided that justifiable concentration/doses are considered when designing such studies. This approach is particularly important for the proper identification of the dose-response curve. Study designs should include doses that most closely reflect the expected exposure levels. A combination of *in vitro* and *in vivo* studies with relevant dose levels will be most useful in identifying the potential hazards of engineered NP, and a thorough discussion and justification of selected dose levels should be mandatory (20**)**.

Nanomaterial- health effects

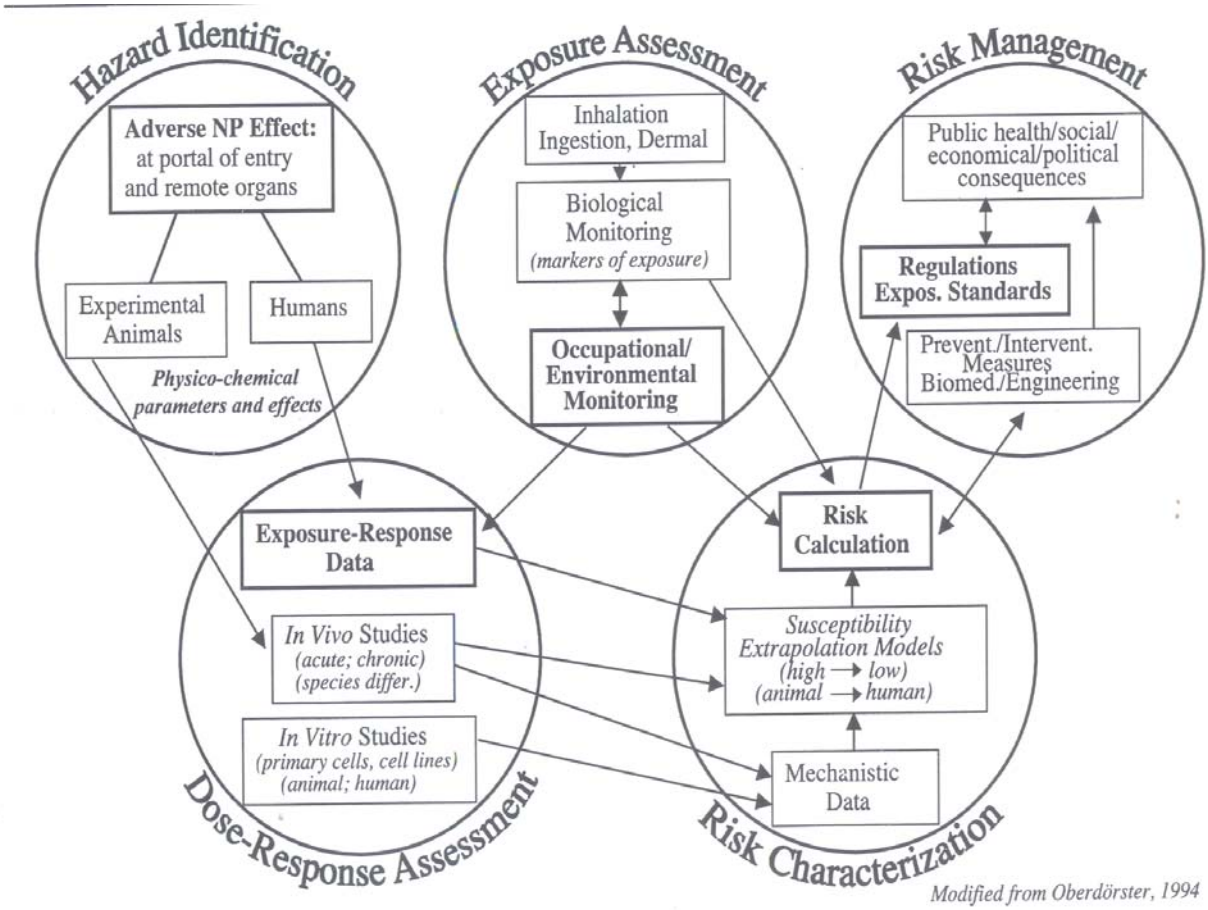


Fig 3: Risk assessment *(NRC 1983)* and risk management paradigm for engineered nanoparticles (NP) (20**)**.

IN VIVO STUDIES

Pulmonary toxicity

Upon intratracheal instillation of CNT induced dose-dependent lung lesions characterized chiefly by interstitial granulomas (26). NT-induced granulomas were also observed in a similar study in which rats were intratracheally instilled with a laser produced NT product sonicated and suspended in Tween 80 (27). The NT dosage of 5 mg/kg in Warheit's rat study was comparable to that given to our low-dose (0.1 mg/30 g mouse or 3.3 mg/kg) groups of mice. At this dose we saw granulomas in the mice treated with the HiPco products (RNT and PNT), but not with the CNT. SWCNT soot generated by the laser ablation method and intratracheally instilled in rats produced transient inflammation, cell injury effects, and a subsequent non-dose dependent series of multifocal granulomas (28). In comparison, equal doses of quartz produced sustained pulmonary inflammation, cytotoxicity, and fibrosis in a dose-dependent fashion. In an intratracheal instillation study of mice, three types of SWCNTs were investigated (29)—

two types made by the HiPCO method and one by the electric arc method. The results from all three types showed that regardless of the amount of metal impurities, dose-dependent lung lesions were characterized chiefly by interstitial granulomas. This study also showed that both SWCNTs and ultrafine carbon black were taken up by alveolar macrophages, but that the effects of these materials were different. Macrophages containing carbon black were homogeneously distributed over the alveolar space, but macrophages containing SWCNTs clustered to form granulomas in centrilobular locations. In comparison, quartz induced mild to moderate pulmonary inflammation, which was considered less severe than that induced with SWCNTs.

Initially, Shvedova et al. (30) associated the interstitial fibrosis with deposition of dispersed SWCNT structures and pointed out that the mechanism for this response differs from the classic fibrogenic particle response in that it is not driven by chronic inflammation and chronic activation of alveolar macrophages. In a pharyngeal aspiration study of mice by Shvedova et al. (30), the effects of HiPCO produced SWCNTs purified to a carbon content > 99% were investigated. The SWCNT aggregate depositions were correlated with granulomatous inflammation, whereas interstitial fibrosis with alveolar wall thickening was observed to be greater at 60 days than at 28 days postexposure in lung regions distant from the SWCNT aggregates. CNTs, which were made from graphite and may contain more graphite impurities than the CO-derived HiPco-NTs (31), actually were less potent in producing granulomas One may ask the relationship of these intratracheal-instillation NT doses that produced lung lesions to an inhalation exposure. NT particles are neither soluble nor biodegradable, and particles in the interstitium could not be removed from the lung by the macrophage-mucociliary clearance mechanism. Therefore, the lung burden from an intratracheal dose could be used to roughly estimate a burden that could be achieved by inhalation exposures (32).

Dermal toxicity

There is only one published study *in vivo* on the dermal toxicity of fullerene soot containing CNTs (33). Forty volunteers were subjected to a patch test, and four albino rabbits were subjected to an eye test. The study found no evidence of the induction of any response; thus, the authors concluded that soot containing CNTs is not associated with any risks (34). However, information is insufficient on CNT material characterization and the study design for adequate validation of these results.

IN VITRO STUDIES

The methodology of how the toxicity of CNTs is evaluated depends strongly on how CNTs are administered to the cells (i.e., homogenously dispersed or not, with or without surfactant, type and concentration of the surfactant).

Pulmonary cytotoxicity

According to Muller et al. (34), the adverse effects of CNTs depend on the length of the material used in vivo. Similar to their in vivo study, the authors found that long untreated

tubes using Triton X-100 as the vehicle evoked an LDH release of nearly 20% at a concentration of 100 μg MWCNTs/one 24-well of a 24-well plate, whereas the short ground MWCNTs induced a dose-dependent LDH release up to 35% greater than the corresponding vehicle (Triton X-100) treatment. This suggests that also in vitro the short MWCNTs are more toxic than the long ones. Interestingly, TNF-α was induced in vivo (measured in BALF) as well as in vitro (measured in preactivated peritoneal macrophages), indicating that TNF--α could be a reliable marker for future in vitro studies. A comparative in vitro cytotoxicity study of several manufactured nanoparticles and nanotubes revealed that aggregated SWCNTs/ ropes, MWCNT raw material, and MWCNT aggregated tubes suspended in 5 μg/mL dimethylsulfoxide (DMSO) affected cell viability in murine lung alveolar macrophages at nearly similar concentrations (35). In addition to the size, shape, and novel physical and chemical properties, the degree and type of agglomeration are important factors in the cytotoxicologic assessment of CNTs. Well-dispersed SWCNTs were less cytotoxic than micrometer-sized agglomerates of SWCNTs (36).

Dermal cytotoxicity

Shvedova et al. (37) exposed human keratinocytes to HiPCO-produced SWCNT material containing 30% by weight of iron. One of the first reactions on SWCNT treatment, which occurred 18 hr after incubation, was an increased oxidative stress, as indicated by the presence of free radicals. The decrease of the total antioxidant reserve and the reduction of vitamin E as well as the increase of the lipid peroxidation products compared with the control cell culture support strongly the presence and the damage of the oxidative stress within the cell cultures. Shvedova et al. (37) claimed that this oxidative stress was the reason for the reduced cell viability. To emphasize the dermal toxicity of CNTs, Monteiro-Riviere et al. (38) exposed human epidermal keratinocytes to MWCNTs that were produced without further purification processes. These particles were taken up by the keratinocytes in vacuoles where MWCNTs retained their structure, as demonstrated by transmission electron microscopy analysis. The cell viability parameters were reduced by 400 μg/mL MWCNTs to as much as 20% after a 24-hr exposure. The expression of interleukin (IL)-8 was increased up to 6 times (400 μg MWCNTs/mL) in a dose-dependent manner after that period compared with the corresponding control cell cultures (38). Dosing keratinocytes and bronchial epithelial cells *in vitro* with single walled carbon nanotubes resulted in oxidative stress, as evidenced by the formation of free radicals, accumulation of peroxidative products, and depletion of cell antioxidants (39).

***In vitro* cytotoxicity studies in different cell types.** For human embryo kidney (HEK293) cells it was reported that in 0.5% DMSO, suspended SWCNTs are able to inhibit cell proliferation and to decrease cell adhesive ability in a dose- and time-dependent manner (40). In conclusion, CNTs were taken up by different cell types and evoked diverse effects in the cells. A first and fast reaction is the formation of free radicals (oxidative stress), which has been suggested as a key factor in further cell reactions (41).

Conclusion

It is concluded that, both *in vivo* and *in vitro* studies will only provide useful data on the toxicity and mode of action of NSP provided that justifiable concentration/doses are considered when designing such studies. Further research work should be done in both *in vivo* and *in vitro models* to assess the toxicokinetics of nanomaterials.

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List of abbreviations

C60 = fullerene (60)
GSH = glutathione
μg = microgram
NP = engineered nanoparticles
NSP = nano-sized particles
ROS = Reactive Oxygen Species
SWNT = Single-walled Carbon Nanotube
MWNT = Multi-walled Carbon Nanotube
UFP = ultrafine particle