Toxicology Letters 262 (2016) 123–134

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Evaluation of the cytotoxic and genotoxic effects of benchmark multi-walled carbon nanotubes in relation to their physicochemical properties

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HIGHLIGHTS

- Cyto- and genotoxic effects of carbon nanotubes (MWCNT) in A549 and BEAS-2B cells.
- Correlation of the MWCNTs average size in cell culture medium with cytotoxicity.
- No induction of DNA damage for any MWCNTs in any cell line by comet assay.
- Only NM-401 and NM-402 were genotoxic in A549 cells, using micronucleus assay.
- Relation of biological effects and physicochemical characteristics is discussed.

ARTICLE INFO

Article history:
Received 27 July 2016
Received in revised form 29 September 2016
Accepted 30 September 2016
Available online 1 October 2016

Keywords:
Carbon nanotubes
Genotoxicity
Cytotoxicity
Physicochemical characteristics

ABSTRACT

To contribute with scientific evidence to the grouping strategy for the safety assessment of multi-walled carbon nanotubes (MWCNTs), this work describes the investigation of the cytotoxic and genotoxic effects of four benchmark MWCNTs in relation to their physicochemical characteristics, using two types of human respiratory cells.

The cytotoxic effects were analysed using the clonogenic assay and replication index determination. A 48h-exposure of cells revealed that NM-401 was the only cytotoxic MWCNT in both cell lines, but after 8-days exposure, the clonogenic assay in A549 cells showed cytotoxic effects for all the tested MWCNTs. Correlation analysis suggested an association between the MWCNTs size in cell culture medium and cytotoxicity.

No induction of DNA damage was observed after any MWCNTs in any cell line by the comet assay, while the micronucleus assay revealed that both NM-401 and NM-402 were genotoxic in A549 cells. NM-401 and NM-402 are the two longest MWCNTs analyzed in this work, suggesting that length may be determinant for genotoxicity. No induction of micronuclei was observed in BBEAS-2Beas-2B cell line and the different effect in both cell lines is explained in view of the size-distribution of MWCNTs in the cell culture medium, rather than cell's specificities.

1. Introduction

Toxicological information on nanomaterials (NMs) is of major importance for their safety assessment, since NMs are already used in many consumer products and in biomedicine and will continue to have a number of innovative applications in the future. Multi-walled carbon nanotubes (MWCNTs) are among the most

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http://dx.doi.org/10.1016/j.toxlet.2016.09.016
0378-4274/

commonly utilized NMs and have been applied, for example, as structural composites, in energy applications and electronics (De Volder et al., 2013; Wijnhoven et al., 2010). Recently, the IARC working group published a paper on the carcinogenicity of fluoro-edenite, silicon carbide (SiC) fibres and whiskers, and carbon nanotubes (CNTs) including single-walled carbon nanotubes (SWCNTs) and MWCNTs (Grosse et al., 2014). The main conclusions were that the lack of coherent evidence across the various distinct CNTs precluded generalization to other types of CNTs and only Mitsui MWCNT-7 was classified as possibly carcinogenic to humans (Group 2B; Grosse et al., 2014). Other CNTs were categorized as not classifiable as to their carcinogenicity to humans (Group 3; Grosse et al., 2014). This classification reflects the contradictory results that have been reported in the scientific literature about the toxicity of CNTs, either showing induction of DNA damage, gene mutations, micronuclei, and chromosomal aberrations in different types of cells (reviewed in Cveticanin et al., 2010) or negligible effects (Szendi and Varga, 2008) for several CNTs. Further research suggested that, besides Mitsui MWCNT-7, other types of MWCNTs may be carcinogenic as well and that straight MWCNTs appear to have a greater potency to induce mesothelioma than tangled MWCNTs (Rittinghausen et al., 2014).

Since the discrepancies found among the studies evaluating the toxicity of CNTs probably relate to variations in NM physicochemical properties, methods of NM dispersion, cell systems, and the genotoxicity testing itself, the use of well characterized NMs, standardized dispersion methods, and validated genotoxicity tests is crucial for the correct understanding of nanotoxicity. In fact, the toxicity of MWCNTs can be affected by a wide range of factors. Surface coating, impurities such as metal catalysts, but also their size, shape and rigidity have been pointed as factors affecting CNTs' toxicity (reviewed in Madani et al., 2013). One report has shown that straight MWCNTs induce DNA damage *in vitro* and induce both DNA damage and micronucleus formation in mouse lungs, while tangled MWCNTs show only a slight increase in DNA damage (Catalan et al., 2016). However, no associations between physicochemical parameters and the biological effects were identified, using principal component analysis, in the study of Jackson et al. (2015), that analysed the effects of 15 different MWCNT, including NM-401, NM-402 and NM-403, in the FE1 Muta^{TM}-Mouse lung epithelial cell line. The latter authors also could not find a link between the dispersion quality of the stock dispersion and any of the tested toxicological effects (Jackson et al., 2015). In another study, no correlation could be found between CNTs' genotoxicity and metal impurities, length, surface area, or induction of cellular oxidative stress, but genotoxicity was seen to increase with width (Darne et al., 2014). In agreement with this view, Poulsen et al. (2016), using 10 commercial MWCNTs from Cheap Tubes, demonstrated that the diameter significantly predicted genotoxicity in mouse BAL fluid cells and lung tissue, thus showing that a lower surface area or a correspondingly larger diameter was associated with increased genotoxicity. Additionally, many physicochemical properties of NMs are inter-related and thus cannot be varied systematically in isolation from others, *e.g.* increasing surface charge may impact on hydrophobicity, or changing the shape of a NM may introduce defects or alter the atomic configuration of the surface (Lynch et al., 2014). These facts create a problem in the context of hazard assessment. It is presently not clear how to predict which type of MWCNT is actually harmful, therefore establishing a grouping strategy for the expanding number of MWCNTs is hardly possible. However, at present a case-by-case approach to the risks of each MWCNT seems an unreasonably extensive task while high-throughput screening tools adapted for studying the toxicity of these NMs are still in progress.

Previously, we have used a standardized procedure to ensure the dispersion of a set of six well-characterized MWCNTs developed by Jensen et al. (2011) and performed the cytokinesis-block micronucleus assay in human lymphocytes exposed *in vitro* (Tavares et al., 2014). We reported significant increases in the frequencies of micronucleated binucleated cells (MNBNC) for two MWCNTs: NRCWE-006 (Mitsui MWCNT-7, Mitsui&Co., Ltd., provided as sub-samples by the National Research Centre for the Working and Environment, NRCWE) and NM-403 (Joint Research Centre Repository). While no cytoxicity was observed, the differences observed in genotoxicity among closely related MWCNT could not be simply explained by the variation in tube length or diameter (Tavares et al., 2014). In fact, in human lymphocytes the thick and long NRCWE-006, but also the thin and short NM-403, were genotoxic. However, NRCWE-007 (Cheap tubes, Brattle, VT, USA; provided as sub-samples by NRCWE) and NM-400, two other similarly thin and short MWCNTs, did not induce chromosome breakage or loss (Tavares et al., 2014).

The objective of the present work was to contribute to the comprehensive investigation of the cytotoxic and genotoxic effects of a set of benchmark MWCNTs and to relate the effects observed with their physicochemical characteristics, as well as with their properties in the cellular moiety, in order to contribute to a grouping strategy. Four benchmark MWCNTs were used (NM-400, NM-401, NM-402, NM-403, from the Joint Research Centre repository) and have been previously characterized with respect to their physicochemical properties, presenting different thicknesses, lengths, surface areas, aspect ratios and morphologies (Tavares et al., 2014; Jensen, 2013; De Temmerman et al., 2012; Rasmussen et al., 2014). Considering that inhalation is the most probable route of human exposure to these NMs, two types of human respiratory cells were selected for *in vitro* complementary analysis: a bronchial epithelial cell line (BEAS-2B) and a lung adenocarcinoma epithelial cell line (A549). Bronchial epithelial cells play an important role as a physical barrier protecting the underlying tissue and maintaining the local environment of the airway. The BEAS-2B cell line is derived from normal human bronchial epithelium obtained from non-cancerous individuals and is able to generate and release mediators of inflammation (Atsuta et al., 1997). On the other hand, A549 cells mimics function of Type II pneumocytes, retaining the endocytic ability of the pulmonary epithelium and localization of cytochrome P450 systems (Foster et al., 1998). However, according to some authors, transformed cell lines such as A549 cells are frequently referred to as being more resistant to toxic insults compared to cells derived from normal tissue, possibly due to higher glutathione and catalase antioxidant activities (Herzog et al., 2007).

The cytotoxic effects were analysed using the clonogenic assay and replication index determination, while the genotoxicity was addressed by the conventional and FPG-modified comet assay and the *in vitro* micronucleus assay, that allow detecting different types of DNA damage. The comet assay detects primary DNA lesions – single or double strand breaks and alkali-labile sites – that can be repaired by specific enzymes (Collins, 2014). With the introduction of enzymatic treatment, the modified comet assay can also analyze specific types of genetic damage, particularly oxidative damage, using DNA repair endonucleases (Azqueta et al., 2013). The micronucleus assay, on the other hand, detects irreversible DNA lesions – chromosomal breaks or losses. Aneugenic but not clastogenic compounds, for this reason, produce positive results in the micronucleus assay, but not in the comet assay (Hartmann et al., 2001). Furthermore, the *in vitro* micronucleus assay is a validated method accepted for regulatory purposes (OECD, 2010).

The biological endpoints were analysed in relation to the previously determined physicochemical characteristics of each