Regulatory Toxicology and Pharmacology 64 (2012) 124–129

Contents lists available at SciVerse ScienceDirect

Regulatory Toxicology and Pharmacology

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

# *In vivo* genotoxicity study of single-wall carbon nanotubes using comet assay following intratracheal instillation in rats

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ARTICLE INFO

*Article history:*
Received 28 February 2012
Available online 23 June 2012

*Keywords:*
Single-wall carbon nanotubes
Nanomaterials
Genotoxicity
*In vivo*

ABSTRACT

The genotoxicity of single-wall carbon nanotubes (SWCNTs) was evaluated *in vivo* using the comet assay after intratracheal instillation in rats. The SWCNTs were instilled at a dosage of 0.2 or 1.0 mg/kg body weight (single instillation group) and 0.04 or 0.2 mg/kg body weight once a week for 5 weeks (repeated instillation group). As a negative control, 1% Tween 80 was instilled in a similar manner. As a positive control, ethyl methanesulfonate (EMS) at 500 mg/kg was administered once orally 3 h prior to dissection. Histopathologically, inflammation in the lung was observed for all the SWCNTs in both single and repeated groups. In the comet assay, there was no increase in% tail DNA in any of the SWCNT-treated groups. In the EMS-treated groups, there was a significant increase in% tail DNA compared with the negative control group. The present study indicated that a single intratracheal instillation of SWCNTs (1.0 mg/kg) or repeated intratracheal instillation (0.2 mg/kg) once a week for five weeks induced a clear inflammatory response (hemorrhage in the alveolus, infiltration of alveolar macrophages and neutrophiles), but no DNA damage, in the lungs in rats. Under the conditions of the test, SWCNTs were not genotoxic in the comet assay following intratracheal instillation in rats.



## 1. Introduction

Carbon nanotubes (CNTs) are attractive nanomaterials because of their unique physical and chemical characteristics. Manufactured CNTs exist in many forms, including single-wall carbon nanotubes (SWCNTs), double-wall carbon nanotubes (DWCNTs), and multi-wall carbon nanotubes (MWCNTs). Biological and toxicological responses to CNTs can vary, depending on form, manufacturing process, route of exposure, and dosage. There is little information about the possible human health and environmental impacts of manufactured CNTs. Early studies suggested that SWCNTs did not have mutagenic effects in *Salmonella typhimurium* YG1024 and YG1029 strains (Kisin et al., 2007), and MWCNTs did not have mutagenic effects in the bacterial reverse mutation assay (Di Sotto et al., 2009; Wirnitzer et al., 2009). In an *in vitro* comet assay (single cell gel electrophoresis), a mixture of SWCNTs and other CNTs increased the extent of DNA damage in human bronchial epithelial cells in a dose-dependent manner (Lindberg et al., 2009). In an *in vivo* micronucleus assay, MWCNTs induced an increase in micro-nucleated pneumocytes (MNPNCEs) in rats (Muller et al., 2008). MWCNTs induced mesothelioma in p53+/− mice (Takagi et al., 2008) or asbestos-like pathological changes in wild-type mice (Poland et al., 2008). However, the existing information on the genotoxicity of CNTs is limited and remains inconclusive (Landsiedel et al., 2009; Singh et al., 2009). To assess the toxicity of nanoparticles and manage their risks, it is important to understand whether nanoparticles are more toxic than micron-sized particles. Our recent study showed that high-purity and well-dispersed samples of SWCNTs were not genotoxic under the conditions of the *in vitro* bacterial reverse mutation assay, chromosomal aberration assay, or *in vivo* bone marrow micronucleus test (Naya et al., 2011; Ema et al., 2012). In genotoxicity testing, an *in vivo* comet assay is useful for follow-up testing of positive *in vitro* findings and for the evaluation of local genotoxicity. In the present study, the *in vivo* comet assay using lung tissue following an intratracheal instillation in rats was performed for the assessment of genotoxic effects in human populations exposed to well-dispersed samples of SWCNTs in the environment or at the workplace.

## 2. Materials and methods

The experiments were performed at the Biosafety Research Center, Foods, Drugs and Pesticides (BSRC, Shizuoka, Japan) in

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http://dx.doi.org/10.1016/j.yrtph.2012.05.020

compliance with the Law Concerning the Protection and Control of Animals (1973), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (1980) and Guidelines for Animal Experimentation, Biosafety Research Center, Foods, Drugs and Pesticides. The study was performed in accordance with the ethics criteria contained in the bylaws of the Committee of National Institute of Advanced Industrial Science and Technology (AIST).

### 2.1. Chemicals

SWCNTs were obtained from Nikkiso Co., Ltd. (Tokyo, Japan). The SWCNTs were synthesized by the chemical vapor deposition (CVD) method. As a dispersant for the SWCNTs, Tween 80 (Polyoxyethylene sorbitan monooleate, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was applied at 10 mg/mL and ultrasonicated using an ultrasonic bath (5510J-MT, Branson Ultrasonics Div. of Emerson Japan, Ltd., Kanagawa, Japan). PBS (10 mM) was then added to the ultrasonicated SWCNT suspension. The SWCNT suspensions were used for intratracheal instillation. Ethyl methanesulfonate (EMS, Sigma–Aldrich Corporation, USA) was used as the positive control. Dulbecco's phosphate-buffered saline, regular melting agarose and triton-X were obtained from Sigma–Aldrich Corporation, and low melting agarose was obtained from Lonza Rockland, Inc., USA. Ethylenediaminetetraacetic acid (EDTA) disodium salt was obtained from DOJINDO LABORATORIES, Japan. Hanks's balanced salt solutions and SYBR® Gold nucleic acid gel stain were obtained from Life Technologies Corporation, USA. Dimethyl sulfoxide (DMSO), tris(hydroxymethyl)aminomethane and sodium *N*-lauroyl sarcosinate were purchased from Wako Pure Chemical Industries, Ltd., Japan. TE buffer solution (pH 8.0) was obtained from Nippon Gene, Japan.

### 2.2. Characterization of SWCNTs

For the bulk SWCNTs and dispersed SWCNTs in the test suspension, tube morphology was evaluated on the basis of observations using a transmission electron microscope (TEM; JEM-1010; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 100 kV. The Brunauer, Emmett, Teller (BET) specific surface area of the bulk SWCNTs was measured by the $N_2$ gas adsorption method (AUTOSORB-1-C; Quantachrome Instruments, USA). The metal impurities of the bulk SWCNTs were determined by quantitative analysis of 28 elements with an inductively coupled plasma-mass spectrometer (ICP-MS; Agilent Technologies 7500a, Tokyo, Japan) after microwave-assisted acid digestion of the CNTs.

### 2.3. Animals and treatment

Sixty-one male Crl: CD (SD) rats (7 weeks old) were obtained from Charles River Laboratories, Japan, Inc. (Yokohama, Japan). The rats were kept individually in a positive-pressure air-conditioned unit (20–26 °C, 35–75% relative humidity) for animal housing on a 12:12-h light/dark cycle. After a 6-day acclimation, 55 rats were assigned to the study. A standard rodent pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were provided *ad libitum*.

The experimental design was decided in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the Japanese Center for the Validation of Alternative Methods (JaCVAM, 2009). For clarifying the relationship between inflammatory responses and positive findings of the comet assay, the dosage was selected to induce lung inflammation or not. Based on the results of the dose-finding test, 1.0 mg/kg SWCNT was used for the high dosage group, expected to induce lung inflammation, and 0.2 mg/kg was used for the low dosage group, expected not to induce inflammation, in a single instillation study. In the repeated (intermittent) instillation study, a dosage of 0.2 or 0.04 mg/kg body weight once a week for 5 weeks was selected because it was expected to induce sub-acute lung inflammation or not. Suspensions of SWCNTs were dispersed in 1% Tween 80 and instilled in a volume of 1.0 mL/kg body weight. As a negative control, 1% Tween 80 was instilled intratracheally by single or intermittent administration in a similar manner. EMS was used for a positive control. In our pilot study, intratracheal instillation of EMS did not given good results, but a single oral administration of EMS did, in the lung epithelial comet assay. Therefore, 500 mg/kg of EMS was administered orally once 3 h before sacrifice in both the single and repeated study. In the single instillation group, rats were anesthetized and sacrificed 3 or 24 h after the treatment, while in the repeated instillation group, rats were anesthetized and sacrificed 3 h after the last treatment. Five rats per group were used for each time point throughout the study. The lungs were excised immediately after sacrifice. The left lobe was used for the histopathological examination, and the right lobe, for the comet assay.

### 2.4. Histopathological examination

The left lobes of the lungs were inflated and fixed in 10% neutral buffered formalin. All fixed tissues were routinely processed, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin (H&E) for light microscopic examination. The slides were scored double blind.

### 2.5. Comet assay

The comet assay was conducted in accordance with the standard protocol "International Validation of the *In Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens" issued by the JaCVAM. Briefly, the right lobes of the lungs were washed out with homogenizing buffer (Hanks's balanced salt solution containing 25 mmol/L EDTA-2Na and 10% v/v DMSO) and then homogenized in 5 mL of the homogenizing buffer using a Dounce-type tissue grinder (Wheaton Science Products, New Jersey, USA). Cell suspensions were chilled on ice for 5 min and centrifuged at 800 rpm for 5 min. After the supernatant was removed, the cells were re-suspended in homogenizing buffer. Ten microliters of the single cell suspension was mixed with 90 μL of 0.5% low-melting agarose gel, and 90 μL of the mixture was placed on a slide precoated with 1.0% agarose gel. Another 90 μL of low melting agarose was added. Two slides were prepared from each rat. The slides were transferred to lysing solution (2.5 mol/L NaCl, 100 mmol/L EDTA-2Na, 10 mmol/L, pH 10 Tris buffer, 10 vol.% DMSO and 1 vol.% Triton X-100) for at least one night at 4 °C in the dark. The slides were next covered with chilled electrophoresis buffer (pH > 13) for 20 min to allow DNA to unwind. Electrophoresis was then conducted at a constant voltage of 0.7 V/cm (25 V) (current at the start: 300 mA) for 20 min. The slides were transferred into neutralization buffer and left to stand for about 10 min. Subsequently, they were dehydrated with ethanol, and air-dried. The slides were stained with SYBR® Gold nucleic acid gel stain which was diluted 5000-fold with TE buffer solution. Images of DNA migration were examined using a fluorescence microscope (Olympus Corporation, Tokyo, Japan). The final magnification was 200×. The images were analyzed using a Comet assay analyzer (Comet Assay IV system, Perceptive Instruments Ltd., Suffolk, UK). The comet parameter to measure DNA damage in the cells was the percentage of DNA in the tail (% Tail DNA), because% Tail DNA could be considered meaningful and easy to conceptualize (Kumaravel and Jha, 2006). Images of 100 (50 × 2) cells per rat were analyzed. The mean of the % Tail DNA value (mean value for 100 cells) of each group was calculated.