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ENV/JM/MONO(2015)13/PART2

Organisation de Coopération et de Développement Économiques
Organisation for Economic Co-operation and Development

05-Jun-2015

English - Or. English

**ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY**

**DOSSIER ON SINGLE-WALLED CARBON NANOTUBES (SWCNT)
- PART 2 -**

**Series on the Safety of Manufactured Nanomaterials
No. 50**

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OECD Environment, Health and Safety Publications

Series on the Safety of Manufactured Nanomaterials

No. 50

**DOSSIER ON SINGLE-WALLED CARBON NANOTUBES (SWCNT)
- PART 2 -**



INTER-ORGANIZATION PROGRAMME FOR THE SOUND MANAGEMENT OF CHEMICALS

A cooperative agreement among FAO, ILO, UNDP, UNEP, UNIDO, UNITAR, WHO, World Bank and OECD

**Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris, 2015**

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or contact:

**OECD Environment Directorate,
Environment, Health and Safety Division
2 rue André-Pascal
75775 Paris Cedex 16
France**

Fax: (33-1) 44 30 61 80

E-mail: ehscont@oecd.org

PREAMBLE

In November 2007, OECD's Working Party on Manufactured Nanomaterials (WPMN) launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The outcomes of the Testing Programme were intended to assess the applicability of the existing **test guidelines**¹ to nanomaterials, as well as to provide useful information on any intrinsic properties of MNs, which are different from the same bulk material with greater external dimensions. Understanding the properties of NMs is crucial to choose appropriate strategies for hazard identification, risk assessment or risk management measures. The Testing Programme involved delegations from OECD member countries, some non-member economies and other stakeholders. The broad international representation, from a range of delegations enabled the programme to pool expertise and resources without which this programme would not have been possible.

Before launching the Testing Programme, the WPMN first identified a broad list of possible nanomaterials, and the list was later adjusted to a final selection of eleven MNs for testing². This list comprised: i) fullerenes (C60); ii) single-walled carbon nanotubes (SWCNTs); iii) multi-walled carbon nanotubes (MWCNTs); iv) silver nanoparticles; v) titanium dioxide; vi) cerium oxide; vii) zinc oxide; viii) silicon dioxide; ix) dendrimers; x) nanoclays; and xi) gold nanoparticles. One fundamental criterion for selecting these materials was that they should be either in commercial use at the time or expected to be in the near future. At the same time, other considerations were also given attention, such as the production volume of the materials, the likely availability of such materials for testing and the existing information that would readily be available on the materials.

It was also agreed that 59 endpoints would be addressed³ for each material corresponding to the following categories: i) nanomaterial information/ identification; ii) physical-chemical properties and material characterisation; iii) environmental fate; iv) toxicological and eco-toxicological effects; v) environmental toxicology; vi) mammalian toxicology; and vii) material safety. These endpoints were judged to be most important based largely on the general experience of testing chemicals, while taking into account the potentially different or new properties of nanomaterials. It is worth noticing that it was not expected that testing for all of the listed endpoints would be necessary for each of the selected MNs.

To assist with the Testing Programme, the WPMN developed two documents: i) a Preliminary Review of OECD Test Guidelines for their Applicability to Manufactured Nanomaterials [ENV/JM/MONO(2009)21]; and ii) Guidance Manual for the Testing of Manufactured Nanomaterials: OECD's Sponsorship Programme (Guidance Manual) in 2009, which was subsequently updated in 2010

¹ The OECD Test Guidelines are a collection of internationally agreed test methods used by government, industry and independent laboratories. They are used to determine the safety of chemicals.

<http://www.oecd.org/chemicalsafety/testing/oecdguidelinesforhetestingofchemicals.htm>

² Originally Iron nanoparticles, Aluminium, Carbon black, and Polystyrene were suggested but later withdrawn and replaced by gold nanoparticles.

³ As specified in the Guidance Manual, "address" includes the term "completed" which provides that all dossiers will contain the identified endpoint information. Note that for some endpoints (for example, solubility) it is specified that the endpoint must be "completed". In such instances "completed" means that all Dossiers will be providing this endpoint information.

[ENV/JM/MONO(2009)20/REV]⁴. The objective of this Guidance Manual was to guide sponsors⁵ in the testing of the materials while ensuring that the information collected was reliable, accurate, consistent and therefore also comparable. The Guidance Manual addressed a whole range of issues including the organisation of the work.

The *Guidance Manual* contains detailed information on the selected endpoints for testing and recommendations on sample preparation and dosimetry.

The *Guidance Manual* also described the development of *Dossier Development Plans* (DDPs). These plans were prepared by Lead sponsors, Co-sponsors together with contributors to describe the specific plan for the testing of each nanomaterial including when and where the testing will be undertaken and by whom. The DDPs also included information on the materials to be tested as well as information on issues such as sample preparation and dosimetry. Each of the DDPs was prepared and reviewed by the WPMN before testing work began.

Based on the lessons learned during the Testing Programme, the WPMN also developed *Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials* [ENV/JM/MONO(2012)40]. This latter document is an update of an earlier text first published in 2010.

The work on OECD's Testing Programme was completed by the end of 2013. In June 2014 the WPMN agreed that for each nanomaterial the dataset would be published in IUCLID printed format^{6 7}. The document will include the protocols and methods to allow their wider use (regulators and researchers).

The dataset in this document has been declassified and made publicly available and it is expected regulators and researchers will wish to use it. Due to a broad dissemination of the data and the exploratory setting in which they were developed there are a number of limitations in using the data of which potential users should be aware. The programme focused on answering scientific questions in the field of the OECD test guidelines but not to provide conclusions on the hazard or risk of the materials selected. The absence of data for some endpoints may be a gap for some endpoints but for other end points there may not if the data was not considered necessary. Although the programme ensured a broad participation of many stakeholders it was not intended to arrive at any pre-defined regulatory datasets requirements or risk assessment decisions. It was recognised from the beginning that the exploratory nature of the work would require subsequent follow-up work for example to review the specific needs that may arise when performing risk assessment of nanomaterials. In this context, the programme's ultimate goal, to add to the knowledge of the properties of nanomaterials, would form a cornerstone.

⁴ It is worth noting that while the *Guidance Manual for Sponsors* was primarily intended as a guide to WPMN's Testing Programme, it is also expected that it will be of value to anyone involved in testing NMs.

⁵ The Guidance Manual noted, for example, that there could be three levels of participation to the programme. Lead sponsors, who would assume responsibility for conducting or coordinating all of the testing, determined to be appropriate for each of the endpoints for a specific nanomaterial. In some cases, "joint lead" arrangements were developed. Co-sponsors conducted some of the testing determined to be appropriate and feasible to address the endpoints for a specific listed nanomaterial. Contributors provided test data, reference or testing materials or other relevant information to the lead and co-sponsors.

⁶ IUCLID is a software programme for the administration of data on chemical substances. Although it was originally developed to fulfill requirements in the EU for the evaluation and control of the risks of existing chemical substances, it is used by many others.

⁷ SIAR = SIDS Initial Assessment Report (SIDS = Screening Information Data Set)

FOREWORD

As part of its Programme on the Safety of Manufactured Nanomaterials, OECD launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (hereafter the Testing Programme). The objective was to conduct specific tests, relevant to human health and environmental safety endpoints, on a variety of manufactured nanomaterials (MN). The Testing Programme mainly aimed to assess the applicability of the existing test guidelines to nanomaterials, as well as to provide useful information on any intrinsic properties of MNs, which are different from the same bulk material with greater external dimensions.

This document presents the Dossier of the carbon nanomaterials: Single-Walled Carbon Nanotubes (SWCNT), Multiwalled Carbon Nanotubes (MWCNT) and Fullerenes (C60), which was prepared under the leadership of Japan and the United States. This nanomaterial has been tested for a number of endpoints for: i) Nanomaterials Information / Identification; ii) Physical-Chemical Properties; iii) Environmental Fate; iv) Environmental Toxicology; and v) Mammalian Toxicology. They have been analysed using OECD Guidelines for the Testing of Chemicals (TG)⁸. The data is presented in an IUCLID⁹ style format and includes the protocols and methods used (see Preamble).

Japan and the United States led the Testing Programme on carbon nanomaterials. This included the determination of the tests that were appropriate for carbon nanomaterials, performing a number of tests, as well as coordinating tests performed and inputs provided by other participating country and stakeholder, from Korea and the Business and Industry Advisory Committee to the OECD (BIAC).

Due to the large amount of information generated throughout the OECD Testing Programme on carbon nanomaterials, each dossier has been split into several parts, as follows:

- Single-Walled Carbon Nanotubes (SWCNT): 2 parts
- Multiwalled Carbon Nanotubes (MWCNT): 3 parts
- Fullerenes (C60): 2 parts

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

⁸ <http://www.oecd.org/env/testguidelines>

⁹ IUCLID is a software programme for the administration of data on chemical substances. It was originally developed to fulfil requirements in the EU for the evaluation and control of the risks of existing chemical substances. It is specifically relevant in the context of an international programme for the initial assessment of chemical substances.

ACKNOWLEDGMENTS

The OECD Secretariat and the WPMN is thankful to Japan and to the United States for leading the Testing Programme on Carbons, including Single-Walled Carbon Nanotubes (SWCNT), Multiwalled Carbon Nanotubes (MWCNT) and Fullerenes (C60). They are specifically grateful to Hiroyuki Hanawa from the Ministry of Economy, Trade and Industry of Japan, and to Philip Sayre from the Environment Protection Agency, USA. In addition, we appreciate the efforts made by other participating countries/ organisations, and in particular to those that coordinated the efforts within their respective delegations: Dr. Kyunghye CHOI from the Korean Ministry of Environment, as well as Dr. Daniel Bernard (Arkema, France) and Dr. Jacques Ragot (Bayer material Science, Germany) from the Business Advisory Committee to the OECD (BIAC).

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7. TOXICOLOGICAL INFORMATION

7.1 Toxicokinetics, metabolism and distribution

7.1.1 Basic toxicokinetics

Endpoint study record: SWCNTs by arc-charge method Basic toxicokinetics.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	H. Wang et al.	2004	Biodistribution of Carbon Single-Wall Carbon Nanotubes in Mice	J Nanoscience and Nanotechnology, Vol.4, No8, p1019-1024					

Materials and methods

Type of method

in vivo

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

no

Test material identity

Identifier	Identity
other:	

Radiolabelling

yes

Details on test material

The raw soot of SWCNTs with a diameter of ca 1.4nm was made by arc-charge method and purified to a purity of 90%. SWNTols were synthesized and separated, following a solid-phase mechanochemical

method, with some modifications. Purified SWNTs and KOH(1:20, w:w) were transferred into a sealed plastic vial containing small stainless steel balls. The vial was agitated vigorously for 1 h at room temperature. Thereafter, the reaction mixture was dissolved in deionized water. After centrifugation at 12,000g for 15 min, the supernatant was collected and centrifuged again until no residue was found at the bottom of the vial. Then, a size-exclusion chromatographic column(diameter 26mm, length 28cm) of fine Sephadex G-25 was used to obtain pure SWNTols from the final supernatant, taking deionized water as amobile phase. The first dark brown fraction was assigned to SWNTols by micro-FTIR, macroscopic confocal Raman spectrum and TEM. The length distribution of SWNTols in water was estimated by hydrodynamic radius (length equals two times hydrodynamic radius) via laser scattering characterization. Estimated average length was 300nm.SWNTols were labeled with iodine-125 by the chloramine-T(N-chloro-p-toluenesulfonamide)method.

Test animals

Species

mouse

Strain

other: KM

Sex

male

Details on test animals and environmental conditions

Male KM mice(~25g) was obtained from the Institute of zoology, Chinese Academy of Sciences.

Administration / exposure

Route of administration

intraperitoneal

Vehicle

water

Details on exposure

Effect of 4 different administration modes (intraperitoneal, gavage, subcutaneous, intravenous) was studied.

Duration and frequency of treatment / exposure

Single administration

Doses / concentrations

100µl of 125I-SWNTols solution(3,520,000cpm/ml, 15µg/ml))

No. of animals per sex per dose

5 mice/sex/dose

Control animals

no

Details on study design

In the study of distribution of ¹²⁵I-SWNTols, each mouse in all eight groups (five mice per group) was intraperitoneally (ip) injected with 0.1ml of the ¹²⁵I-SWNTols solution (3,520,000cpm/ml, 0.015mg/ml). They were sacrificed at different intervals post dosing. Their tissues, including skin, muscle (leg), bone (shank), brain, heart, lungs, liver, kidneys, spleen, stomach (emptied) and intestine (emptied) were immediately dissected, and the blood was collected. Each tissue was wrapped in Al foil, weighed and counted for ¹²⁵I activity. Data were corrected for the physical decay of radioactivity. The distribution in the tissues was represented by ng SWNTols per gram wet tissue, which could be calculated by multiplying the total weight of SWNTols injected (0.015 mg) with the percent of dose injected (tissue activity/total activity dosed) per gram of wet tissue. Studying the influence of four different administration modes on the distribution of ¹²⁵I-SWNTols, four groups of mice were administered 0.1ml of the ¹²⁵I-SWNTols solution (3,520,000cpm/ml, 0.015 mg/ml) in four different modes; intraperitoneal injection (ip), subcutaneous injection, stomach intubation (gavage) and intravenous injection. Mice were sacrificed at 3 h post dosing and their tissues were treated as described in the experimental section. Results were expressed as ng SWNTols per gram wet tissue, in a mean of five mice per time point. To compare the behaviors of ¹²⁵I-SWNTols and inorganic Na¹²⁵I, three groups of mice were ip injected with 0.015ml of a carrier-free Na¹²⁵I solution (5.56 x 10⁶ cpm/ml) and sacrificed at 6 h, 3 d and 6 d post injection, respectively. Their tissues were treated as described before. The data were represented by the percent dose injected per gram wet tissue, in a mean of five mice per time point. The excretion of SWNTols from mice was investigated by counting ¹²⁵I in urine and feces of mice at different time intervals from 0 to 11 d post dosing. Results were expressed as ng SWNTols, which is calculated by multiplying the total weight of the SWNTols injected (1.5 fJ/g) with the percent dose injected (tissue activity/total activity dosed), in a mean of five mice per time point.

Overall remarks, attachments

Remarks on results including tables and figures

Figure 6 shows the distribution histogram of the ¹²⁵I-SWNTols in mice by ip injection at different time intervals. The data demonstrate that ¹²⁵I-SWNTols distribute in measurable levels throughout the entire body quickly, except for the brain. This shows that they can easily pass through a number of compartments. SWNTols accumulate readily and are retained for long time periods in the bone. Besides, the distribution of SWNTols is not markedly influenced by the administration modes, including ip, subcutaneous injection, stomach intubation and intravenous injection (Fig. 7). These results demonstrate that SWNTols can move freely and rapidly to reach proper targets in the whole mouse body, behaving as typical small molecules. However, the mean apparent molecular weight of SWNTols (one tube of 300 nm long and 1.4 nm in diameter) is ca 600,000. The novel biological behavior of SWNTols may be attributed to their exceptionally compact network structure and good biocompatibility. Following the distribution, we could calculate the weight of SWNTols accumulated in each tissue.

The very different distribution profiles and elimination rates of the two radiolabels at three time intervals are shown in Fig. 8. From these very different profiles, we may arrive at a very important point; that the distribution of ¹²⁵I-SWNTols (Fig. 6) is mainly contributed by SWNTols *per se*, but not the inorganic ¹²⁵I ions.

The excretion experiment demonstrates that over 11 days, ca 80% of the total dosing radiolabel was collected from feces and urine. The excretion percentage via urine is 94% and 6% via feces, so SWNTols excreted predominantly via urine (Fig. 9). Again, this suggests that SWNTols behave like small molecules in the process of excretion.

Illustration (picture/graph)

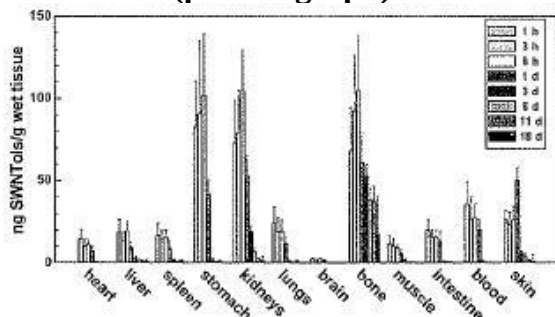


Fig. 6. Biodistribution histogram of ^{125}I -SWNTols (3.52×10^6 cpm/ml, $15 \mu\text{g/ml}$) in mice at eight different time intervals. Data represent mean $\pm$ S.D. ($n = 5$).

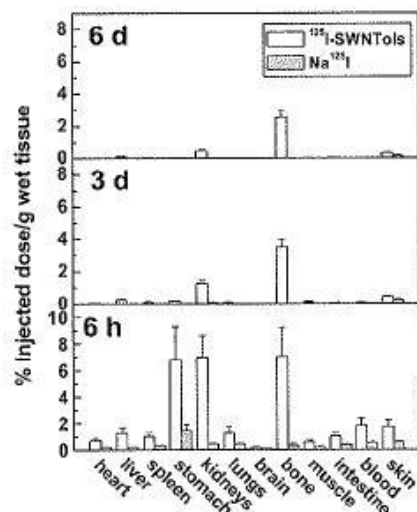


Fig. 8. Comparison of biodistribution and elimination between ^{125}I -SWNTols and Na^{125}I in mice at 6 h, 3 d and 6 d post ip dosing. Data represent mean $\pm$ S.D. ($n = 5$).

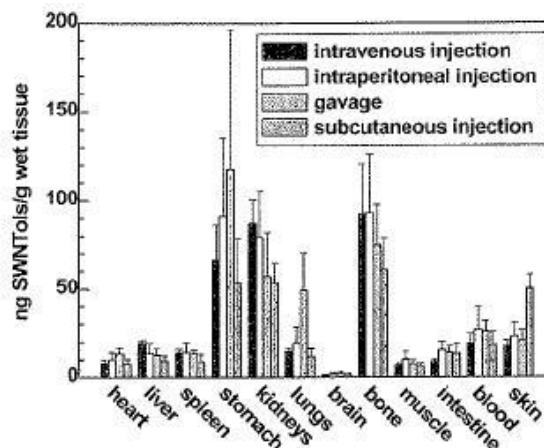


Fig. 7. The effect of four different administration modes on the distribution of ^{125}I -SWNTols in mice at 3 h post dosing. Data represent mean $\pm$ S.D. ($n = 5$).

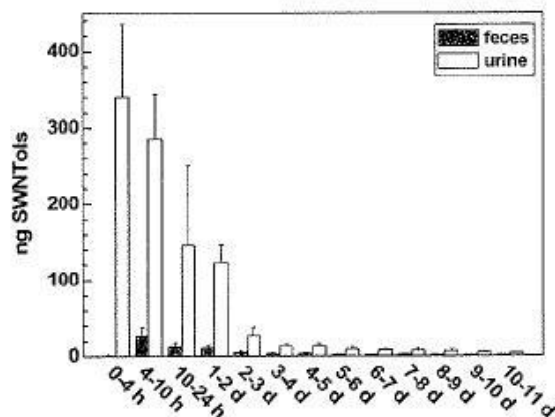


Fig. 9. Excretion of ^{125}I -SWNTols from mice via feces and urine over 11 days. Data represent mean $\pm$ S.D. ($n = 5$).

Applicant's summary and conclusion

Executive summary

The water-soluble ^{125}I -labeled SWNTols have been successfully prepared, and the distribution and excretion of ^{125}I -SWNTols in mice have been studied. The very different distribution profiles and elimination rates between ^{125}I -SWNTols and Na^{125}I *in vivo* indicate that the observed distribution of radioactivity was contributed by the ^{125}I -SWNTols *per se*, but not the free ^{125}I ions. SWNTols transported easily among the different compartments and tissues, and were excreted mainly via urine.

Endpoint study record: SWCNTs by HiPco method Basic toxicokinetics.001**Administrative Data**

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	P. Cherukuri et al.	2006	Mammalian pharmacokinetics of carbon nanotubes using intrinsic near-infrared Fluorescence	PNAS vol.103, no.50, 18882-18886					

Materials and methods**Type of method**

in vivo

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

no

Test material identity

Identifier	Identity
other:	

Radiolabelling

other: intrinsic near infrared fluorescence

Details on test material

Because as-produced carbon nanotubes are strongly aggregated and highly hydrophobic, they required processing to form stable aqueous suspensions suitable for i.v. administration. Raw SWNTs grown by the high-pressure carbon monoxide (HiPco) method were suspended by intense, direct contact ultrasonic agitation in an aqueous 1% solution of pharmaceutical-grade Pluronic F108, a nonionic poloxamer surfactant. This processing exfoliates many individual nanotubes from bundles bound by van der Waals forces and allows the debundled individuals to be stabilized and suspended by physisorbed surfactant molecules. The resulting suspension was centrifuged for 4 h at 100,000 g to remove impurities and obtain a supernatant fraction enriched in individual SWNTs at a concentration of 10 g/ml. Finally, the SWNT suspension was autoclaved at 121°C for 60 min before use to ensure sterility. Proper dispersion of the resulting sample was confirmed by its strong near-IR fluorescence, which arises from individual but not

from aggregated SWNTs.

Test animals

Species

rabbit

Strain

other: Newzealand White rabbit

Sex

no data

Details on test animals and environmental conditions

Adult Newzealand White rabbits (3-4 kg) were used.

Administration / exposure

Route of administration

intravenous

Vehicle

water (1% Pluronic F-18 solution)

Details on exposure

Adult New Zealand White rabbits (3–4 kg) were sedated with intramuscular injection of ketamine (30–50 mg/kg) and acepromazine (1–3 mg/kg). The rabbits were then intubated and maintained with isoflurane (1–3%) inhalational anesthesia. The neck of each animal was shaved and prepped with betadine surgical scrub. After cutdown, the jugular vein was cannulated with a 5 French catheter. The catheter was sutured to the vein, tunneled s.c. to the posterior neck of the animal, and affixed. Once the catheter was in place, an initial pretreatment blood sample was drawn. Then, 7.5 ml of SWNTs in 1% Pluronic F-108 was injected through the catheter over 2 min. Catheter patency was maintained with heparinized saline (100 units/ml). The surgical wounds were reapproximated with suture. The rabbits were then allowed to emerge from anesthesia.

Duration and frequency of treatment / exposure

Single administration

Doses / concentrations

The dose of SWNTs was 0.075mg (in 7.5ml solution) , corresponding to 0.020mg per kg of body mass.

No. of animals per sex per dose

5 mice/sex/dose

Control animals

yes, concurrent vehicle

Details on study design

The near-IR emission spectrum of each blood serum specimen was measured by using 658-nm diode laser excitation in a model NS1 NanoSpectralyzer (Applied NanoFluorescence, Houston, TX). Near-IR

fluorescence microscopy was performed using a custom-built apparatus that differed from the one described in ref. 21 only by the addition of a 785-nm diode laser excitation source to supplement the 658-nm laser. Studies involving the identification of individual SWNTs used 785-nm excitation with a 60 oil immersion objective and a 946-nm long-pass filter in the collection path. For the biodistribution observations, 12 different organ systems and tissues were analyzed: brain, heart, lung, liver, kidney, spleen, stomach, pancreas, small intestine, muscle, skin, and bone. Bright-field images were taken of these tissue sections by using a 20 objective, and the tissue was surveyed for SWNT emission with 658-nm excitation. SWNT emission was recognized by its dependence on excitation polarization and characteristic spectral signature. Fluorescence imaging of individual nanotubes was performed by scanning the sample position while exciting with the 658-nm laser and collecting emission with a 60 or 100 oil-immersion objective followed by a 1,125-nm longpass filter. Spectra were normally acquired only in case of an ambiguous outcome in the test based on modulation of emission intensity through rotation of the excitation beam polarization axis.

Details on dosing and sampling

Blood samples were extracted from the indwelling catheter at the following time points: 30 min, 60 min, 90 min, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h, and 24 h. After collection, the blood samples were stored in heparinized tubes at 4°C until the time of analysis, 48 h later. At 24 h after SWNT injection, each rabbit was given 1 ml of IV Beuthanasia-D. During necropsy, the following tissue samples were obtained from representative animals: brain, spinal cord, heart, lung, liver, spleen, kidney, pancreas, stomach, small intestine, skin, muscle, and bone. The tissue samples were preserved in 10% formalin. After formalin fixation, tissue blocks were embedded in paraffin. Sections were cut from 3 to 5 mm thick. Unstained samples were mounted on fused quartz slides for evaluation by visible and near-IR fluorescence microscopy. Hematoxylin and eosin-stained specimens were mounted on glass slides and evaluated by using visible light microscopy.

Results and discussions

Preliminary studies

In separate in vitro experiments at room temperature, the displacement kinetics of a Pluronic surface coating by serum proteins through time-dependent fluorescence spectrometry were monitored. When serum proteins were added to a Pluronic suspension of SWNTs, nearly complete spectral shifts occurred within our experimental time resolution of 0.7 s. Subsequent, more subtle changes in peak positions and intensity were observed on a longer time scale. These data can be adequately fit by a biexponential decay plus asymptotic baseline model in which the two kinetic components are of comparable magnitude and have half-lives of 20 and 310 s. It is suggested that these slower processes may reflect structural relaxation of the protein coating on the nanotube surface. In circulating blood at physiological temperature, one would expect the Pluronic displacement processes to be accelerated, so we conclude that the injected nanotubes' initial environment is replaced by a relatively stable coating of blood proteins within a period of seconds. Our pharmacokinetics results therefore reveal the fate of SWNTs surrounded by endogenous proteins rather than a synthetic surfactant.

Pharmacokinetic studies

Details on distribution in tissues

The biodistribution of SWNTs among organ systems after elimination from the systemic circulation were examined by performing near-IR fluorescence microscopy on tissue specimens. Debundled single-walled carbon nanotubes are high contrast near-infrared fluorophores that can be sensitively and selectively tracked in mammalian tissues using optical methods. At 24 h after i.v. administration, significant concentrations of nanotubes were found only in the liver. Tissue specimens from the kidneys, lungs,

spleen, heart, brain, spinal cord, bone, muscle, pancreas, intestine, and skin revealed far fewer or no nanotubes, and the control specimens showed no emissive features identifiable as SWNTs. It is concluded that, at 24 h after i.v. injection, the only significant SWNT concentration is in the liver.

Details on excretion

To quantify the kinetics of SWNT elimination from blood circulation, spectrally integrated SWNT fluorescence intensities were measured from specimens representing different time points. A plot of serum SWNT content vs. circulation time, averaged over measurements from four rabbits were obtained. The concentration scale was calibrated by assigning the initial signal strength to the injected mass of SWNTs divided by the estimated blood volume (120 ml). The data are well modeled by first-order (exponential) decay with a half-life of 1.0 $\pm$ 0.1 h. The absence of biexponential or multiexponential kinetic components indicates that there was no significant temporary accumulation of nanotubes in tissues that could act as reversible reservoirs.

Bioaccessibility

Any other information on results incl. tables

After i.v. injection, the SWNT-exposed rabbits recovered uneventfully. They exhibited normal behavior, including food and water intake, before being killed at 24 h. Gross examination of the organs at necropsy failed to reveal any abnormality after SWNT injection. Hematoxylin and eosin microscopic evaluation detected no pathological differences between the experimental and control animals.

Overall remarks, attachments

Illustration (picture/graph)

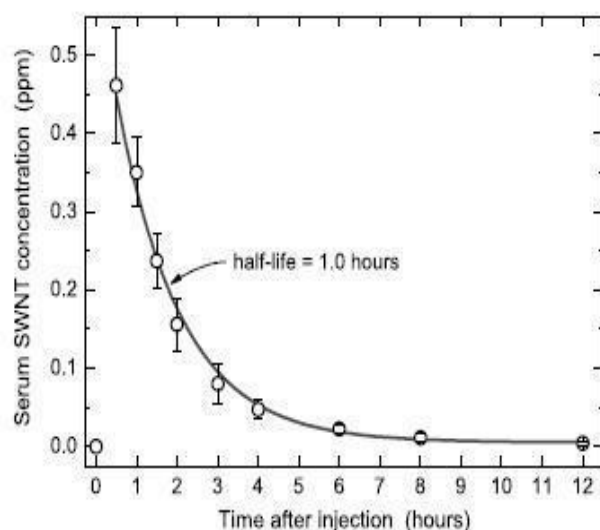


Fig. 3. Time dependence of blood serum SWNT concentration after injection, as measured for four rabbits. Each data point is the averaged emission intensity spectrally integrated above a linear baseline connecting the minima at 1,100 and 1,250 nm. This baseline construction captures a major SWNT fluorescence feature while avoiding systematic errors from an underlying background of weak autofluorescence. Error bars show standard errors of the mean. The solid curve is a first-order kinetic fit to the data.

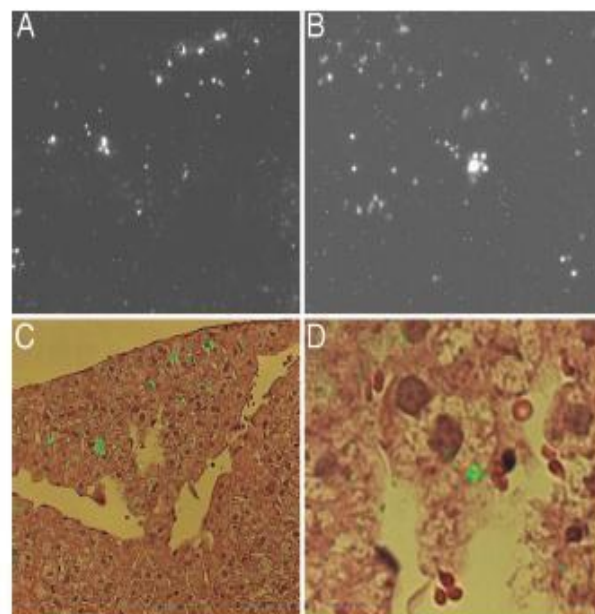


Fig. 4. Micrographs at two magnifications of liver tissue from rabbits killed 24 h after i.v. administration of suspended SWNTs. (A and B) Near-IR SWNT fluorescence images with field widths of 390 μm (A) and 83 μm (B). Scattered isolated bright pixels are artifacts from defective sensor elements in the near-IR camera; all larger features represent emission from SWNTs. In C and D, the SWNT fluorescence from A and B is shown overlaid as false-color green onto visible bright-field images from adjacent 3- μm -thick specimen slices that had been stained with hematoxylin and eosin.

Applicant's summary and conclusion

Executive summary

Individualized, chemically pristine single-walled carbon nanotubes have been intravenously administered to rabbits and monitored through their characteristic near-infrared fluorescence. Spectra indicated that blood proteins displaced the nanotube coating of synthetic surfactant molecules within seconds. The nanotube concentration in the blood serum decreased exponentially with a half-life of 1.0 $\pm$ 0.1 h. No adverse effects from low-level nanotube exposure could be detected from behavior or pathological examination. At 24 h after i.v. administration, significant concentrations of nanotubes were found only in the liver. These results demonstrate that debundled single-walled carbon nanotubes are high-contrast near-infrared fluorophores that can be sensitively and selectively tracked in mammalian tissues using optical methods.

7.1.2 Dermal absorption**7.2 Acute Toxicity****7.2.1 Acute toxicity: oral*****Endpoint study record: Nikkiso SWCNT Acute toxicity: oral.001*****Administrative Data**

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	National Institute of Health Sciences (NIHS)	2011	Acute Oral Toxicity Study of SWCNT in rats		Bozo Research Center Inc.	B-6930			
publication	Matsumoto et al.	2012	No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats	J Toxicol Sci. 2012;37(3):463-74					

Materials and methods**Test type**

acute toxic class method

Limit test

yes

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 423 (Acute Oral toxicity - Acute Toxic Class Method)	yes (The maximum dose of 2000 mg/kg required by the guideline was impracticable because of very high specific volume of SWCNT)

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals**Species**

rat

Strain

other: Crl:CD(SD)

Sex

female

Details on test animals and environmental conditions

Crl:CD(SD) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Female rats were 8 weeks old. Rats were individually housed in metallic cages with wire mesh bottoms and reared on a basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. Rats were maintained at room temperature, a humidity of 50± 20%, 10-15 air changes per hour and a 12 hr dark/12 hr light cycle. Body weights were 191-200g when administered.

Administration / exposure**Route of administration**

oral: gavage

Vehicle

other: 5% gum acacia of aqueous solution

Details on oral exposure

Test material were accurately weighed and added to gum acacia (vehicle). This vehicle was chosen based on the results of the preliminary investigation with commonly used vehicles, in which CNTs showed the best dispersion state in 5% gum acacia of aqueous solution. The mixture of test materials was homogenized using an ultrasonic homogenizer (UR-200P, TOMY Seiko Co., Ltd., Tokyo, Japan) and a compact ultrasonic cleaning bath (SWCNT: US-1, As One Co., Ltd., Tokyo, Japan). The homogeneity of test suspensions was confirmed microscopically. The concentration of test substance was

0.625mg/mL. Volume of administration was 80 mL/kg bw. 20 mL/kg bw was administered 4 times at the interval of c.a. 15 minutes. 50 mg/kg bw at 1st step

Doses

50 mg/kg bw at 1st step and 50 mg/kg bw at 2nd step

No. of animals per sex per dose

3 animals/dose

Control animals

no

Details on study design

Rats were observed for 14 days. Clinical observation was performed consecutively for several hours after administration and once or twice a day from the next day of administration. Animals were weighed just prior to administration, and 1, 3, 7 and 14 days (SWCNT) after administration. Necropsy was performed 14 days after administration.

Statistics

Continuous data from the administration period were analyzed by the Bartlett test for homogeneity of distribution. When homogeneity was recognized, data were analyzed by the Dunnett test, whereas heterogeneous data were analyzed by the Dunnett-type mean rank test between the control groups and individual treatment groups.

Results and discussions

Effect levels

Sex	Endpoint	Effect level	Based on	95% CL	Remarks
female	LD50	> 50 mg/kg bw			

Mortality

No deaths occurred after administering the test article at 50 mg/kg b.w. in either dose step.

Clinical signs

No toxicological effects on clinical observation were observed

Body weight

No effects on body weights were observed

Gross pathology

There were no abnormal macroscopic findings in the external appearance or the cranial, thoracic or abdominal organs / tissues.

Overall remarks, attachments

Illustration (picture/graph)

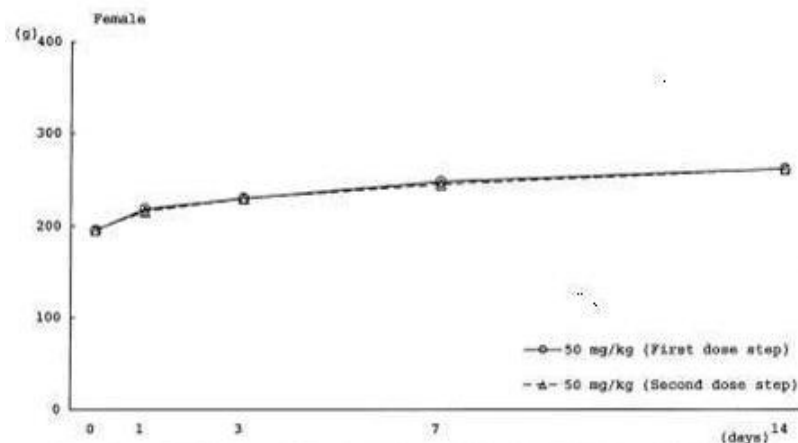


Fig.1 Acute oral toxicity study of Single-walled Carbon Nanotubes in rats

— Body weight —

Table 1 Acute oral toxicity study of Single-walled Carbon Nanotubes in rats
Mortality and LD₅₀ values

Sex		Female																						
Dose (mg/kg)	Number of animals	Distribution of Death														Mortality	LD ₅₀ (mg/kg)							
		minutes			hours				days															
		1-5	15	30	1	2	4	6	1	2	3	4	5	6	7			8	9	10	11	12	13	14
50 (1)	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/3	>50
50 (2)	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0/3	

(1): First dose step

(2): Second dose step

1 : Immediately after administration

Applicant's summary and conclusion

Conclusions

An acute oral toxicity study was conducted based on OECD TG 423. Three female Crl:CD(SD) rats were gavage dosed with Nikkiso SWCNT (suspended in 5% arabic-gum aqueous solution) at a total dose of 50 mg/kg bw (four equally divided doses at one-hour intervals). No deaths occurred, and no abnormalities were observed in the clinical condition during the observation period in any animals. The LD₅₀ of Nikkiso SWCNT was considered to be greater than 50 mg/kg bw in rats.

Endpoint study record: Nikkiso SWCNT Acute toxicity: oral.002

Administrative Data

other:OECD/WPMN

Purpose flag

supporting study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011		NEDO [#NN-30] cited in Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (Final Reports issued on August 17, 2011) (in Japanese). English summary is available at http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Corp.	ZT10112			

Cross-reference to same study

7.6.2 Nikkiso SWCNT Micronucleus test.Genetic toxicity in vivo 001.

Materials and methods

Test type

other: micronucleus assay

Test guideline

Qualifier	Guideline	Deviations
no guideline followed	other guideline: OECD Guideline 474 (Mammalian Erythrocyte Micronucleus Test)	

Principles of method if other than guideline

Test was conducted by the observation of animals in the course of testing OECD TG 474

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals

Species

mouse

Strain

other: Crlj:CDI(ICR)

Sex

male

Administration / exposure**Route of administration**

oral: gavage

Vehicle

other: CNTs were suspended in injection water and diluted with 0.3% CMC

Doses

20mL/kg/day; 5,10 and 20 mg/kg/day

No. of animals per sex per dose

6 animals/dose

Control animals

yes

Any other information on materials and methods incl. tables

No death or no indicative of abnormality is observed in each group

Results and discussions**Effect levels**

Sex	Endpoint	Effect level	Based on	95% CL	Remarks
female	LD50	> 50 mg/kg bw			

Mortality

No deaths were observed

Clinical signs

No toxicological effects on clinical observation were observed

Body weight

No effects on body weights were observed

Gross pathology

No effects on body weights were observed

Any other information on results incl. tables

Clinical observations of male mice - Main test

Compound	Dose (mg/kg)	Animal No	Day 1 of dosing			Day 2 of dosing			Day of specimen preparation
			IB	IA	1h	IB	IA	1h	
0.3%CMC ^{a)}	0	LS1M01	N	N	N	N	N	N	N
		LS1M02	N	N	N	N	N	N	N
		LS1M03	N	N	N	N	N	N	N
		LS1M04	N	N	N	N	N	N	N
		LS1M05	N	N	N	N	N	N	N
		LS1M06	N	N	N	N	N	N	N
SWCNT	5	LS2M01	N	N	N	N	N	N	N
		LS2M02	N	N	N	N	N	N	N
		LS2M03	N	N	N	N	N	N	N
		LS2M04	N	N	N	N	N	N	N
		LS2M05	N	N	N	N	N	N	N
		LS2M06	N	N	N	N	N	N	N
SWCNT	10	LS3M01	N	N	N	N	N	N	N
		LS3M02	N	N	N	N	N	N	N
		LS3M03	N	N	N	N	N	N	N
		LS3M04	N	N	N	N	N	N	N
		LS3M05	N	N	N	N	N	N	N
		LS3M06	N	N	N	N	N	N	N
SWCNT	20	LS4M01	N	N	N	N	N	N	N
		LS4M02	N	N	N	N	N	N	N
		LS4M03	N	N	N	N	N	N	N
		LS4M04	N	N	N	N	N	N	N
		LS4M05	N	N	N	N	N	N	N
		LS4M06	N	N	N	N	N	N	N

Compound	Dose (mg/kg)	Animal No	Day -1	Day of dosing			Day of specimen preparation
				IB	IA	1h	
Mitomicyn ^{c)}	5	LS4M01	N	N	N	N	N
		LS4M02	N	N	N	N	N
		LS4M03	N	N	N	N	N
		LS4M04	N	N	N	N	N
		LS4M05	N	N	N	N	N
		LS4M06	N	N	N	N	N

IB: Immediately before dosing IA: Immediately after dosing 1h: 1 hour post-dosing**Day -1: One day prior to dosing****N: No abnormalities****a) Negative control****b) Positive control****Applicant's summary and conclusion****Conclusions**

No death was observed by gavage of 5,10 and 20 mg/kg/day SWCNT in mice

Cross-reference to other study

7.6.2 Super Growth SWCNT Micronucleus test, Genetic toxicity in vivo.001

Endpoint study record: Super Growth Acute toxicity: oral.001**Administrative Data**

other:OECD/WPMN

Purpose flag

supporting study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG**Data source****Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	NEDO Project P06041, Risk Assessments of Manufactured Nanomaterials - Carbon Nanotubes (CNT)-	http://www.aist-riss.jp/main/modules/product/nano_rad.html?ml_lang=en					
publication	Naya, et al.	2011	Evaluation of the genotoxic potential of single-walled carbon nanotubes by using a battery of in vitro and in vivo genotoxicity assays.	Regul Toxicol Pharmacol 61(2):192-198					

Cross-reference to same study

7.6.2 Genetic toxicity in vivo;Super Growth SWCNT Micronucleus test;Genetic toxicity in vivo.001

Materials and methods

Test type

acute toxic class method

Limit test

yes

Test guideline

Qualifier	Guideline	Deviations
no guideline followed	other guideline: pre-study of OECD TG474	

Principles of method if other than guideline

Test was conducted by the observation of animals in the course of testing OECD TG474

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Super Growth

Test animals

Species

mouse

Strain

other: Crtj:CD1(ICR)

Sex

male

Administration / exposure

Route of administration

oral: gavage

Doses

Twice dose of 60 or 200 mg/kg bw in an interval of 24hrs

No. of animals per sex per dose

5 animals/dose

Control animals

no

Any other information on materials and methods incl. tables

The test was done using 5 male Crlj:CD1(ICR) mice of six weeks of age in each dose group. The CNTs, which were suspended in the phosphate buffered saline with 1% of Tween 80, is gavage-administered two times in the interval of 24 hours at the dose of 60 and 200 mg/kg/day. The observation is carried out after 24 hours of final dose.

Mortality in toxicity and micronucleus tests

Test	Substances	Dose (mg/kg)	Mortality	
			Male	Female
Toxicity test	SG-SWCNT	60 x 2	0 / 3	0 / 3
		200 x 2	0 / 3	0 / 3
Micronucleus test	Vehicle	0 x 2	0 / 5	
	SG-SWCNT	60 x 2	0 / 5	
		200 x 2	0 / 5	
	Mitomycin C	10 x 1	0 / 5	

Mortality: Number of death / Number of animals dosed.

Vehicle: 1% Tween 80.

Individual clinical observation in toxicity test

Dose (mg/kg)	Sex	Animal No.	Time after the administration (hr)				
			1	3	5	24	48
60 x 2	male	11	-	-	-	-	-
		12	-	-	-	-	-
		13	-	-	-	-	-
	female	1011	-	-	-	-	-
		1012	-	-	-	-	-
		1013	-	-	-	-	-
			-	-	-	-	-
200 x 2	male	14	-	-	-	-	-
		15	-	-	-	-	-
		16	-	-	-	-	-
	female	1014	-	-	-	-	-
		1015	-	-	-	-	-
		1016	-	-	-	-	-

-: No abnormality detected

Results and discussions

Effect levels

Sex	Endpoint	Effect level	Based on	95% CL	Remarks
female	LD50	> 50 mg/kg bw			

Any other information on results incl. tables

No death or no indicative of abnormality is observed in each group

Applicant's summary and conclusion**Conclusions**

No death was observed by twice gavage dose of 200 mg/kg/day SWCNT in mice.

7.2.2 Acute toxicity: inhalation***Endpoint study record: CNI SWCNT Acute toxicity: inhalation.001*****Administrative Data**

other:OECD/WPMN

Purpose flag supporting study
Study result type experimental result
Reliability 2 (reliable with restrictions)

Rationale for reliability incl. deficiencies Meets generally accepted scientific standards, well documented and acceptable for assessment.

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Shvedova et al.	2008	Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis	AJP - Lung Physiol October 2008 vol. 295 no. 4 L552-L565					

Materials and methods**Test materials****Test material identity**

Identifier	Identity
other:	CNI SWCNT

Test animals**Species**

mouse

Sex

female

Details on test animals and environmental conditions

Specific pathogen-free adult female C57BL/6 mice (8–10 wk) were supplied by The Jackson Laboratory

(Bar Harbor, ME) and weighed 20.0 +/- 1.9 g when used. Animals were housed one mouse per cage receiving HEPA filtered air in Association for Assessment and Accreditation of Laboratory Animal Care-approved National Institute for Occupational Safety and Health (NIOSH) animal facilities. All animals were acclimated in the animal facility under controlled temperature and humidity for 1 wk before use. Animals were supplied with water and certified chow 5020 (Purina Mills, Richmond, IN) ad libitum in accordance with guidelines and policy set forth by the Institute of Laboratory Animals Resources, National Research Council. All experimental procedures were conducted in accordance with a protocol approved by the NIOSH Institutional Animal Care and Use Committee.

Administration / exposure**Route of administration**

inhalation

Type of inhalation exposure

whole body

Vehicle

clean air

Concentrations

4 days, 5h/day at 5.52 ± 1.37 mg/m³

No. of animals per sex per dose

12 mice/group

Any other information on materials and methods incl. tables

Animals were exposed in a whole body inhalation exposure chambers with individual steel mesh compartments for 4 consecutive days, 5 h/day. Aerosolized SWCNT were sampled from the breathing zone within each chamber for characterization during exposure. Mice from the control group were exposed to filtered air. After the last exposure, animals were removed from the chambers and euthanized 1, 7, and 28 days postinhalation

Overall remarks, attachments**Remarks on results including tables and figures**

Followings are observed; Transient inflammation and persistent fibrosis.

SWCNT inhalation was more effective than aspiration in causing inflammatory response, oxidative stress, collagen deposition, and fibrosis as well as mutations of *K-ras* gene locus in the lung of mice

7.3 Irritation / corrosion

7.3.1 Skin irritation / corrosion

Endpoint study record: Nikkiso SWCNT Skin irritation / corrosion.001

Administrative Data

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	NEDO Project P06041, Risk Assessments of Manufactured Nanomaterials - Carbon Nanotubes (CNT)-	http://www.aist-riss.jp/main/modules/product/nano_rad.html?ml_lang=enriss.jp/main/modules/product/nano_rad.html	Ina Research Corp.	ZT10113			
publication	Em et al.	2011	Evaluation of dermal and eye irritation and skin sensitization due to carbon nanotubes.	Reg Toxicol pharmacol 61: 276-281					

Materials and methods

Type of method

in vivo

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 404 (Acute Dermal Irritation / Corrosion)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso

Test animals**Species**

rabbit

Strain

other: Kbl:NZW

Details on test animals and environmental conditions

male 16 weeks old, administered after 1 week of quarantine and acclimation period

Test system**Type of coverage**

occlusive

Preparation of test site

shaved

Vehicle

other: olive oil

Amount/concentration applied

0.5 g of 1 wt% solution

Duration of treatment / exposure

4 h

Number of animals

1,24,48 and 72 hours after the lint removal

Control animals

no

Details on study design

0.5g of 1 wt% of CNTs solution, which is prepared at the maximum concentration allowing a uniform mixture in a media of non-irritating olive oil, was applied on a lint of 2.5 x 2.5 cm. The lint was occlusive dressed to the pelage removed back skin of the rabbits. In four hours the lint was removed and the adhered part was cleaned with warm and wet gauze. In 1, 24, 48 and 72 hours after the lint removal, the red macular, the crust formation, the edematous formation, etc. are observed.

Results and discussions**Irritation / corrosion results**

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
primary dermal irritation index (PDII)	animal #3		ca. 0			

Other effects

No indicative of abnormality was observed for all the rabbits. Also no feature to suggest irritation was not observed in all observation periods and all dose group.

Overall remarks, attachments

Illustration (picture/graph)

Table 1 Primary Skin Irritation Study of Carbon nanotube A in Rabbits
Observation of Skin

Sex: Male								
Animal No.	Skin signs	Score of skin reaction ⁴¹						
		First test			After removal of patches			
		3 min	1 h	4 h	60 min	24 h	48 h	72 h
NS1M01	Erythema and eschar formation	0	0	0	0	0	0	0
	Oedema formation	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
NS1M02	Erythema and eschar formation	-	-	-	0	0	0	0
	Oedema formation	-	-	-	0	0	0	0
	Total				0	0	0	0
NS1M03	Erythema and eschar formation	-	-	-	0	0	0	0
	Oedema formation	-	-	-	0	0	0	0
	Total				0	0	0	0
Mean					0.0	0.0	0.0	0.0
P.I.I. ⁴²							0.0	

⁴¹ Evaluations of skin reactions were scored as follows [OECD TG 404 (2002)]

Erythema and Eschar Formation: 0 = No erythema, 1 = Very slight erythema (barely perceptible), 2 = Well defined erythema, 3 = Moderate to severe erythema,

4 = Severe erythema (boof redness) to eschar formation precluding grading of erythema

Oedema Formation: 0 = No oedema, 1 = Very slight oedema (barely perceptible), 2 = Slight oedema (edges of area well defined by definite raising),

3 = Moderate oedema (raised approximately 1 mm), 4 = Severe oedema (raised more than 1 mm and extending beyond area of exposure)

⁴² P.I.I. (Primary Irritation Index) = (24 h mean scores + 48 h mean scores + 72 h mean scores) / 3

Criteria for primary skin irritation: 0 < P.I.I. ≤ 2 = Mildly irritating, 2 < P.I.I. ≤ 5 = Moderately irritating, 5 < P.I.I. = Severely irritating

When no skin reactions were observed in any application site during the observation period, the test formulation was judged to be "Not irritating".

-: Not examined

Endpoint study record: Nikkiso SWCNT Skin irritation / corrosion.002

Administrative Data

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

Conducted according to OECD TG under SOP.

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	NIHS	2011	Skin irritation study of CNT in rabbits		DIMS Institute of Medical Science Inc.	11102			

Materials and methods

Type of method

in vivo

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 404 (Acute Dermal Irritation / Corrosion)	no

GLP compliance

no (according to SOP)

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Details on test material

Lot No. SW1860

Test animals

Species

rabbit

Strain

other: Kbl:JW

Details on test animals and environmental conditions

9 weeks old, 10 weeks old at the start of the test. 1.900~2.099 Kg

Test system

Type of coverage

occlusive

Preparation of test site

shaved

Vehicle

other: silicone oil

Amount/concentration applied

0.02g SWCNT wetted with 0.5 mL silicone oil or 0.5 mL of 0.3% SWCNT ultra-sonically homogenised dispersion in silicone oil on lint cut to 2.5×2.5 cm. Control is only silicone oil.

Duration of treatment / exposure

4 hours

Observation period

1, 24, 48, 72 hours

Number of animals

3 animals

Control animals

not required

Details on study design

Each animal was applied with 3 patches, directly applied, dispersion dropped and vehicle dropped

Results and discussions**Irritation / corrosion results**

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
overall irritation score (edema and erythema score)		1, 24, 48, 72 hrs	0			

Any other information on results incl. tables**SKIN IRRITATION STUDY OF CNT IN RABBITS; THE TEST SITE OBSERVATION DATA**

GRO UP	TEST CHEMIC AL	ANIM AL	Findin gs	Time after remov al					Tot al	Avera ge	Irritati on	Irritati on
NO.		NO.		of the closed patch					Sco re	Score	Score	Index
				1h	24 h	48 h	72 h					
1	Silicone oil	111020 01	Er. ^a	0	0	0	0		0	0.0	0.0	0.0
			Ed. ^b	0	0	0	0					
		111020 02	Er.	0	0	0	0		0	0.0		

			Ed.	0	0	0	0					
		111020 03	Er.	0	0	0	0		0	0.0		
			Ed.	0	0	0	0					
	0.02g CNT	111020 01	Er.	0	0	0	0		0	0.0	0.0	0.0
			Ed.	0	0	0	0					
		111020 02	Er.	0	0	0	0		0	0.0		
			Ed.	0	0	0	0					
		111020 03	Er.	0	0	0	0		0	0.0		
			Ed.	0	0	0	0					
	0.3% CNT	111020 01	Er.	0	0	0	0		0	0.0	0.0	0.0
			Ed.	0	0	0	0					
		111020 02	Er.	0	0	0	0		0	0.0		
			Ed.	0	0	0	0					
		111020 03	Er.	0	0	0	0		0	0.0		
			Ed.	0	0	0	0					
a : Erythema and eschar formation 0: No erythema 1: Very slight erythema (barely perceptible) 2: Well defined erythema 3: Moderate to severe erythema 4: Severe erythema (beet redness) to eschar formation preventing grading of erythema b : Edema formation 0: No edema 1: Very slight edema (barely perceptible) 2: Slight edema (edges of area well defined by definite raising) 3: Moderate edema (raised approximately 1 mm) 4: Severe edema (raised more than 1 mm and extending beyond area of exposure)												

Overall remarks, attachments

Remarks on results including tables and figures

SKIN IRRITATION STUDY OF CNT IN RABBITS; GENERAL CONDITION DATA

ROUP	TEST CHEMICAL	ANIMAL	Experimental day			
NO.		NO.	1	2	3	4
1	CNT	11102001	N	N	N	N
		11102002	N	N	N	N
		11102003	N	N	N	N
N : Normal						

SKIN IRRITATION STUDY OF CNT IN RABBITS; BODY WEIGHT DATA

GROUP	TEST CHEMICAL	ANIMAL	Experimental day	
NO.		NO.	1	4

1	CNT	11102001	1900	2031
		11102002	2099	2254
		11102003	2016	2140
		N	3	3
		MEAN	2005.0	2141.7
		S.D.	100.0	111.5

Endpoint study record: Super Growth SWCNT Skin irritation / corrosion.001

Administrative Data

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	NEDO Project P06041, Risk Assessments of Manufactured Nanomaterials - Carbon Nanotubes (CNT)-	http://www.aist-riss.jp/main/modules/product/nano_rad.html?ml_lang=en	Ina Research Corp.	ZT10137			
publication	Ema et al.	2011	Evaluation of dermal and eye irritation and skin sensitization due to carbon nanotubes	Reg Toxicol pharmacol 61: 276-281					

Materials and methods

Type of method

in vivo

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 404 (Acute Dermal Irritation / Corrosion)	

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Super Growth

Test animals

Species

rabbit

Strain

other: Kbl:NZW

Details on test animals and environmental conditions

male 16 weeks old, administered after 1 week of quarantine and acclimation period

Test system

Type of coverage

occlusive

Preparation of test site

shaved

Vehicle

other: olive oil

Amount/concentration applied

0.5 g of 1 wt% solution

Duration of treatment / exposure

4 h

Number of animals

1,24,48 and 72 hours after the lint removal

Control animals

no

Details on study design

0.5g of 1 wt% of CNTs solution, which is prepared at the maximum concentration allowing a uniform mixture in a media of non-irritating olive oil, was applied on a lint of 2.5 x 2.5 cm. The lint was occlusive dressed to the pelageremoved back skin of the rabbits. In four hours the lint was removed and the adhered part was cleaned with warm and wet gauze. In 1, 24, 48 and 72 hours after the lint removal, the red macular, the crust formation, the edematous formation, etc. are observed.

Results and discussions**Irritation / corrosion results**

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
primary dermal irritation index (PDII)	animal #3		0	0		

Other effects

No indicative of abnormality was observed for all the rabbits. Also no feature to suggest irritation was not observed in all observation periods and all dose group.

Overall remarks, attachments**Illustration (picture/graph)**

Table 1 Primary Skin Irritation Study of Carbon nanotube D in Rabbits
Observation of Skin

Sex: Male		Score of skin reaction ^{a)}						
Animal No.	Skin signs	First test			After removal of patches			
		3 min	1 h	4 h	60 min	24 h	48 h	72 h
QT1 M01	Erythema and eschar formation	0	0	0	0	0	0	0
	Oedema formation	0	0	0	0	0	0	0
	Total	0	0	0	0	0	0	0
QT1 M02	Erythema and eschar formation	-	-	-	0	0	0	0
	Oedema formation	-	-	-	0	0	0	0
	Total	0	0	0	0	0	0	0
QT1 M03	Erythema and eschar formation	-	-	-	0	0	0	0
	Oedema formation	-	-	-	0	0	0	0
	Total	0	0	0	0	0	0	0
Mean		0.0			0.0			
P.I.I. ^{b)}					0.0			

^{a)} Evaluations of skin reactions were scored as follows [OECD TG 404 (2002)]

Erythema and Eschar Formation; 0 = No erythema, 1 = Very slight erythema (barely perceptible), 2 = Well defined erythema, 3 = Moderate to severe erythema, 4 = Severe erythema (heef redness) to eschar formation preventing grading of erythema

Oedema Formation; 0 = No oedema, 1 = Very slight oedema (barely perceptible), 2 = Slight oedema (edges of area well defined by definite raising), 3 = Moderate oedema (raised approximately 1 mm), 4 = Severe oedema (raised more than 1 mm and extending beyond area of exposure)

^{b)} P.I.I. (Primary Irritation Index) = (24 h mean scores + 48 h mean scores + 72 h mean scores) / 3

Criteria for primary skin irritation: 0 < P.I.I. ≤ 2 = Mildly irritating, 2 < P.I.I. ≤ 5 = Moderately irritating, 5 < P.I.I. = Severely irritating

When no skin reactions were observed in any application site during the observation period, the test formulation was judged to be "Not irritating".

-: Not examined

Endpoint study record: CNI SWCNT Skin irritation / corrosion.001

Administrative Data

other:OECD/WPMN

Purpose flag

supporting study

Study result type

experimental result

Reliability

2 (reliable with restrictions)

Rationale for reliability incl. deficiencies

Meets generally accepted scientific standards, well documented and acceptable

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Murray AR et al.	2009	Oxidative stress and inflammatory response in dermal toxicity of single-walled carbon nanotubes	Toxicology. 2009 ;257(3):161-71					

Materials and methods**Type of method**

in vivo

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNT were from CNI by high-pressure catalytic CO conversion (HiPCO method). Unpurified SWCNT (30% iron) and partially purified SWCNT (0.23% iron) were used for tests.

Test animals**Species**

mouse

Strain

other: immune-competent hairless SKH-1 mice

Test system***Amount/concentration applied***

Topical exposure, 5 days, with daily doses of 40 microg/mouse, 80 microg/mouse, or 160 microg/mouse to unpurified SWCNT in deionized water. Twenty-four hours following the last exposure, mice were sacrificed with sodium pentobarbital, and skin was removed for biochemical and histological analysis.

Results and discussions***Irritant/corrosive response data***

Topical exposure of SKH-1 mice to unpurified SWCNT caused oxidative stress, depletion of glutathione, oxidation of protein thiols and carbonyls, elevated myeloperoxidase activity, an increase of dermal cell numbers, and skin thickening resulting from the accumulation of polymorphonuclear leukocytes (PMNs) and mast cells.

Overall remarks, attachments

Remarks on results including tables and figures

These data indicated that topical exposure to unpurified SWCNT, induced free radical generation, oxidative stress, and inflammation, thus causing dermal toxicity.

SWCNT toxicity may be dependent upon the metal (particularly iron) content of SWCNT via the metal's ability to interact with the skin, initiate oxidative stress, and induce redox-sensitive transcription factors thereby affecting/leading to inflammation.

7.3.2 Eye irritation***Endpoint study record: Nikkiso SWCNT Eye irritation.001*****Administrative Data**

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	A Primary Eye Irritation Study of Nikkiso SWCNT in Rabbits	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Corp.	ZT10114			
publication	Ema et al.	2011	Evaluation of dermal and eye irritation and skin sensitization due to carbon nanotubes	Reg Toxicol pharmacol 61: 276-281					

Materials and methods**Type of method**

in vivo

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 405 (Acute Eye Irritation / Corrosion)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals**Species**

rabbit

Strain

other: Kbl:NZW

Details on test animals and environmental conditions

male, 11 weeks of age

Test system**Vehicle**

other: olive oil

Amount/concentration applied

0.1ml of 0.1 wt% SWCNT solution

Duration of treatment / exposure

1 h

Observation period

1, 24, 48 and 72 hours after the instillation

Number of animals

3

Control animals

no

Any other information on materials and methods incl. tables

0.1ml of 0.1 wt% CNTs solution, which was prepared at the maximum concentration allowing the instillation into the eyes in a media of non-irritating olive oil, was dropped into the conjunctival sac of left eye. The test specie was rinsed off with warm saline from cornea and conjunctival sac in an hour. In 1, 24, 48 and 72 hours after the instillation, the irritation to the eye was checked.

Results and discussions**Overall irritation / corrosion results**

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
cornea score	animal #3	1,24,48 and 72 h	ca. 0			
iris score	animal #3	1,24,48 and 72 h	ca. 0			
conjunctivae score	animal #3	1,24,48 and 72 h	ca. 0			
chemosis score	animal #3	1,24,48 and 72 h	ca. 0			

Irritant/corrosive response data

Corneal staining-Scores of corneal epithelium damage, McDonald-Shadduck scoring system; At animal selection and after approximately 72 hours post-dosing, in right and left eyes fluorescein staining were 0

Overall remarks, attachments**Remarks on results including tables and figures**

The abnormality was not seen in all rabbits. A conjunctival flare, muddiness and edema, the abnormality of the iris and the conjunctival obstacle were not recognized to all rabbit at any observation period. It was judged to have no eye irritation from these views.

Illustration (picture/graph)

Table 1-1 A Primary Eye Irritation Study of Carbon nanotube A in Rabbits
 Observation of Eyes
 Gross observation - Scores of ocular irritation, Grading of ocular lesions [OECD TG 405 (2002)] -

Sex: Male											
Animal No.	Parameters	At animal selection		Post-dosing							
		R	L	1 h		24 h		48 h		72 h	
PS1M02	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0
PS1M03	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0
PS1M04	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0
Mean	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0

R: Right eye (untreated) L: Left eye (Carbon nanotube A)

Evaluations of ocular reactions were scored as follows:

Cornea:

Opacity: degree of density; 0 = No ulceration or opacity, 1 = Scattered or diffuse areas of opacity (other than slight dulling of normal lustre); details of iris clearly visible,
 2 = Easily discernible translucent area; details of iris slightly obscured, 3 = Necrotic area; no details of iris visible; size of pupil barely discernible,
 4 = Opaque cornea; iris not discernible through the opacity

Conjunctivae:

Redness; 0 = Normal, 1 = Some blood vessels hyperaemic (injected), 2 = Diffuse, crimson colour; individual vessels not easily discernible, 3 = Diffuse beefy red

Iris:

0 = Normal, 1 = Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia; or injection; iris reactive to light (a sluggish reaction is considered to be an effect),
 2 = Hemorrhage, gross destruction, or no reaction to light

Chemosis:

Swelling; 0 = Normal, 1 = Some swelling above normal, 2 = Obvious swelling, with partial eversion of lids, 3 = Swelling, with lids about half closed, 4 = Swelling, with lids more than half closed

Applicant's summary and conclusion

Conclusions

Nikkiso SWCNT was not irritating to the eye of rabbits.

Endpoint study record: Super Growth SWCNT Eye irritation.001

Administrative Data

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	A Primary Eye Irritation Study of Super Growth SWCNT in Rabbits	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (Final Reports issued on August 17, 2011) (in Japanese). English summary is available at http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Corp.	ZT10138			

Materials and methods**Type of method**

in vivo

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 405 (Acute Eye Irritation / Corrosion)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Super Growth SWCNT

Test animals**Species**

rabbit

Strain

other: Kbl:NZW

Details on test animals and environmental conditions

male, 11 weeks of age

Test system**Vehicle**

other: olive oil

Amount/concentration applied

0.1ml of 0.1 wt% SWCNT solution

Duration of treatment / exposure

1 h

Observation period

1, 24, 48 and 72 hours after the instillation

Number of animals

3

Control animals

no

Any other information on materials and methods incl. tables

0.1ml of 0.1 wt% CNTs solution, which was prepared at the maximum concentration allowing the instillation into the eyes in a media of non-irritating olive oil, was dropped into the conjunctival sac of left eye. The test specie was rinsed off with warm saline from cornea and conjunctival sac in an hour. In 1, 24, 48 and 72 hours after the instillation, the irritation to the eye was checked.

Results and discussions**Overall irritation / corrosion results**

Irritation parameter	Basis	Time point	Score	Max. score	Reversibility	Remarks
cornea score	animal #3	1,24,48 and 72 h	ca. 0			
iris score	animal #3	1,24,48 and 72 h	ca. 0			
conjunctivae score	animal #3	1,24,48 and 72 h	ca. 0			
chemosis score	animal #3	1,24,48 and 72 h	ca. 0			

Irritant/corrosive response data

Corneal staining-Scores of corneal epithelium damage, McDonald-Shadduck scoring system; At animal selection and after approximately 72 hours post-dosing, in right and left eyes fluorescein staining were 0

Overall remarks, attachments

Remarks on results including tables and figures

The abnormality was not seen in all rabbits. A conjunctival flare, muddiness and edema, the abnormality of the iris and the conjunctival obstacle were not recognized to all rabbit at any observation period. It was judged to have no eye irritation from these views.

Illustration (picture/graph)

Z110158

Table 1-1 A Primary Eye Irritation Study of Carbon nanotube D in Rabbits
Observation of Eyes
Gross observation - Scores of ocular irritation, Grading of ocular lesions [OECD TG 405 (2002)] -

Sex: Male

Animal No.	Parameters	At animal selection		Post-dosing							
				1 h		24 h		48 h		72 h	
		R	L	R	L	R	L	R	L	R	L
RT1M01	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0
RT1M02	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0
RT1M03	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0
Mean	Cornea Opacity: degree of density	0	0	0	0	0	0	0	0	0	0
	Conjunctivae Redness	0	0	0	0	0	0	0	0	0	0
	Iris	0	0	0	0	0	0	0	0	0	0
	Chemosis Swelling	0	0	0	0	0	0	0	0	0	0

R: Right eye (untreated) L: Left eye (Carbon nanotube D)

Evaluations of ocular reactions were scored as follows:

Cornea:

Opacity: degree of density; 0 = No ulceration or opacity, 1 = Scattered or diffuse areas of opacity (other than slight dulling of normal lustre); details of iris clearly visible,
2 = Easily discernible translucent area; details of iris slightly obscured, 3 = Necrotic area; no details of iris visible; size of pupil barely discernible,
4 = Opaque cornea; iris not discernible through the opacity

Conjunctivae:

Redness; 0 = Normal, 1 = Some blood vessels hyperaemic (injected), 2 = Diffuse, crimson colour; individual vessels not easily discernible, 3 = Diffuse beefy red

Iris:

0 = Normal, 1 = Markedly deepened rugae, congestion, swelling, moderate circumcorneal hyperaemia; or injection; iris reactive to light (a sluggish reaction is considered to be an effect),
2 = Hemorrhage, gross destruction, or no reaction to light

Chemosis:

Swelling; 0 = Normal, 1 = Some swelling above normal, 2 = Obvious swelling, with partial eversion of lids, 3 = Swelling, with lids about half closed, 4 = Swelling, with lids more than half closed

7.4 Sensitisation

7.4.1 Skin sensitisation

Endpoint study record: Nikkiso SWCNT Skin sensitisation.001

Administrative Data

other:OECD/WPMN

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	A Skin Sensitization Study of Nikkiso SWCNT in Guinea Pig	Risk Assessments of Manufactured Nanomaterials- Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Corp.	ZT10115			
publication	Ema et al.	2011	Evaluation of dermal and eye irritation and skin sensitization due to carbon nanotubes.	Reg Toxicol pharmacol 61: 276-281					

Materials and methods

Type of method

in vivo

Type of study

Buehler test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 406 (Skin Sensitisation)	

GLP compliance

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals

Species

guinea pig

Strain

Hartley

Sex

male

Details on test animals and environmental conditions

Animals were purchased from Japan SLC Inc.(Hamamatsu, Japan) and housed individually in standard aluminum cages and acclimated to the laboratory for six days prior to the start of the experiment. Only animals found to be in good health were selected for use. the animals were reared on a diet (LRC4:Oriental Yeast Co. Ltd.,Tolyo, Japan)and tap water ad libitum, and maintained in an air-conditioned room at 19.0-25.0°C, with a relative humidity of 40-70%, a 12-h light/dark cycle,and ventilation of 15-21 air changes/h.

Test system

Traditional sensitisation test

Route of induction exposure

epicutaneous, occlusive

Route of challenge exposure

epicutaneous, occlusive

Vehicle

olive oil (for induction, white petrolatum for challenge)

Concentration

The SWCNT was moistened with a minimum amount of olive oil (for induction) or white petrolatum (for challenge) to prepare a paste for dermal application. The paste contained 1% SWCNT. This amount of cnt was maximum not leading to overflow when patched and the sample fully coated the skin.

No. of animals per dose

The animals were assigned to three groups; a control group(n=10), a CNT-treated group(n=20), and a positive control group(n=10).

Details on study design (Traditional tests)

As for sensitization, the lint (2 x 2.5 cm), on which 0.4g of sensitization specimen was applied, was occlusivedressed for 6 hours to the skin of the regio lateralis of guinea pig where the pelage had been removed on the day before (day 0). The sensitizations were performed once a week and three times in total to the area where the pelage was removed on the day before (day 7 and day 14). As for challenge, in two weeks (day 28) after the final sensitization, a lint (2 x 2 cm) on which 0.2g of challenge specimen was uniformly applied was occlusive-dressed for 6 hours to the pelage removed regio lateralis where the sensitization had not performed. In 24 and 48 hours after challenge, the skin where the challenge was applied was observed.

Challenge controls

DNCB(1-chloro-2,4-dinitrobenzene)

Positive control substance(s)

yes DNCB(1-chloro-2,4-dinitrobenzene)

Results and discussion***Positive control results***

Mean of score was 1.9 at 24 h after the end of challenge and 2.0 at 48 h after the end of challenge.

Traditional sensitisation test**Results of test (except LLNA)**

Reading	Hours after challenge	Group	Dose level	No. with + reactions	Total no. in group	Clinical observations
1st reading	24	negative control		0	10	no abnormalities
2nd reading	48	negative control		0	10	no abnormalities
1st reading	24	test group		0	20	no abnormalities
2nd reading	48	test group		0	20	no abnormalities
1st reading	24	positive control		10	10	no abnormalities
2nd reading	48	positive control		10	10	no abnormalities

Overall remarks, attachments**Remarks on results including tables and figures**

No abnormality in conjunction with the test was observed to the all guinea pigs and no challenge effect was seen in all observations.

It was judged as no sensitization.

Illustration (picture/graph)

Table 1 A Skin Sensitization Study of Carbon nanotube A in Guinea Pigs (Buehler Test)
Skin Sensitization Reactions

Sex: Male						
Group	Substance for induction	Substance for challenge	Number of animals	Time after the end of challenge (h)	Mean of score	Positive ratio ^{a)}
Negative control	Physiological saline	Petrolatum	10	24	0.0	
				48	0.0	
		1% Carbon nanotube A ^{b)}	10	24	0.0	
				48	0.0	
Test article	1% Carbon nanotube A ^{b)}	Petrolatum	20	24	0.0	0/20
				48	0.0	0/20
		1% Carbon nanotube A ^{b)}	20	24	0.0	0/20
				48	0.0	0/20
Positive control	1% DNCB ^{c)}	Acetone	10	24	0.0	0/10
				48	0.0	0/10
		0.1% DNCB ^{c)}	10	24	1.9	10/10
				48	2.0	10/10

DNCB: 1-chloro-2,4-dinitrobenzene

^{a)} Number of animals with positive / Number of animals tested

^{b)} Vehicle: Olive oil ^{c)} Vehicle: 70 vol% ethanol ^{d)} Vehicle: Petrolatum ^{e)} Vehicle: Acetone

Applicant's summary and conclusion**Conclusions**

Nikkiso SWCNT was not skin sensitizer in rabbits.

Endpoint study record: NikkisoWCNT Skin sensitisation.002**Administrative Data**

Purpose flag key study
Study result type experimental result
Reliability 1 (reliable without restriction)
Rationale for reliability incl. deficiencies Conducted according to OECD TG under SOP.

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	NIHS	2011	SKIN SENSITIZATION STUDY OF SINGLE WALL CARBONNANOTUBE IN GUINEA PIGS		DIMS Institute of Medical Science Inc.	11103			

Materials and methods**Type of method**

in vivo

Type of study

Buehler test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 406 (Skin Sensitisation)	no

GLP compliance

no (according to SOP)

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Details on test material

Lot No. SW1862

Test animals

Species

guinea pig

Strain

other: Slc:Hartley

Sex

male

Details on test animals and environmental conditions

Animals were gained from Japan SLC Corp. The experimental animal room was maintained at 21~23°C, 48~60% relative humidity. Lighting was 12 h/day, ventilation is more than 10 times/h. Animals were acclimatised for 6~11days prior to the test.

Test system

Traditional sensitisation test

Route of induction exposure

epicutaneous, occlusive

Route of challenge exposure

epicutaneous, occlusive

Vehicle

silicone oil

Concentration

0.3 w/v%

No. of animals per dose

20 animals for SWCNT treated, 10 for control(silicone oil), 5 for positive control and 5 for non-sensitized positive control.

Positive control substance(s)

yes DNCB(2,4-dinitrochlorobenzene)

Results and discussion

Positive control results

At 24 h after exposure, positive rate of positive control group is 100%, and positive rate of non-sensitized positive control group is 0%.

Overall remarks, attachments

Remarks on results including tables and figures

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SKIN SENSITIZATION STUDY OF SINGLE WALL CARBONNANOTUBE IN GUINEA PIGS; THE TEST SITE OBSERVATION DATA
IN SKIN - DETERMINATION

				Challenge				
				Time after removal of the closed patch				
Group	Sensitization	Challenge	No. of	24h			48h	
			animals	Mean	Positive		Mean	Positive
				score	rate (%)		score	rate (%)
Negative control	Silicone oil	0.3% CNT	10	0.0	0		0.0	0
Test material	0.3% CNT	0.3% CNT	20	0.0	0		0.0	0
sensitized								
Positive control	1% DNCB	0.1% DNCB	5	1.6	100		1.2	100
Non-sensitized	Acetone	0.1% DNCB	5	0.0	0		0.0	0
positive control								
Mean score = Sum (score) / number of animals Positive rate = Number of animals with skin reactions / total number of animals x 100								

SKIN SENSITIZATION STUDY OF SINGLE WALL CARBONNANOTUBE IN GUINEA PIGS;GENERAL CONDITION DATA

Group	Sensitization	Challenge		Findings	Experimental days															
					1 ^a	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Negative control	Silicone oil	0.3% CNT		Normal	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Test material	0.3% CNT	0.3% CNT		Normal	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20
sensitized																				
Positive control	1% DNCB	0.1% DNCB		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Non-sensitized	Acetone	0.1% DNCB		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
positive control																				
<i>a : First sensitization day.</i>																				

TABLE 2. SKIN SENSITIZATION STUDY OF SINGLE WALL CARBONNANOTUBE IN GUINEA PIGS GENERAL CONDITION DATA STUDY NO. 11103																				
Grou p	Sensitizat ion	Challenge		Finding s	Experimental days															
					1 7	18	19	20	21	2 2	23	24	25	26	2 7	28	29	30	31	
Nega tive contr ol	Silicone oil	0.3% CNT		Normal	1 0	10	10	10	10	1 0	10	10	10	10	1 0	10	10	10	10	
Test mate rial	0.3% CNT	0.3% CNT		Normal	2 0	20	20	20	20	2 0	20	20	20	20	2 0	20	20	20	20	
sensi tized																				
Posit ive contr ol	1% DNCB	0.1% DNCB		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
Non- sensi tized	Acetone	0.1% DNCB		Normal	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	
posit ive contr ol																				

BODY WEIGHT DATA (G)

BODY WEIGHT DATA (g)																						
Group	Sensitization	Challenge	No. of animals	Sensitization												Day of challenge				Final observation day		
			animals		First				Second			Third										
						a																
Negative control	Silicone oil	0.3% CNT	10	402.6	±	23.2		45.2	±	36.7		499.7	±	48.2		589.6	±	56.6		592.7	±	53.3
Test material	0.3% CNT	0.3% CNT	20	406.0	±	18.5		45.4.5	±	26.9		498.0	±	34.0		588.6	±	50.3		595.1	±	49.3
sensitized																						
Positive control	1% DNCB	0.1% DNCB	5	412.2	±	17.7		45.2.8	±	32.2		486.4	±	34.6		564.0	±	45.5		569.6	±	46.0
Non-sensitized	Acetone	0.1% DNCB	5	404.2	±	21.8		45.0.2	±	37.1		498.0	±	43.9		590.4	±	60.0		593.8	±	59.0
positive control																						
a : Data are mean (g) ± SD values.																						

Endpoint study record: Super Growth SWCNT Skin sensitisation.001**Administrative Data**

other:OECD/WPMN

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	A Skin Sensitization Study of Super Growth SWCNT in Guinea Pig	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Corp.	ZT10139			
publication	Ema et al.	2011	Evaluation of dermal and eye irritation and skin sensitization due to carbon nanotubes	Reg Toxicol pharmacol 61: 276-281					

Materials and methods**Type of method**

in vivo

Type of study

Buehler test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 406 (Skin Sensitisation)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Super Growth SWCNT

Test animals**Species**

guinea pig

Strain

Hartley

Sex

male

Details on test animals and environmental conditions

Animals were purchased from Japan SLC Inc.(Hamamatsu, Japan) and housed individually in standard aluminum cages and acclimated to the laboratory for six days prior to the start of the experiment. Only animals found to be in good health were selected for use. the animals were reared on a diet (LRC4:Oriental Yeast Co. Ltd.,Tolyo, Japan) and tap water ad libitum, and maintained in an air-conditioned room at 19.0-25.0°C, with a relative humidity of 40-70%, a 12-h light/dark cycle, and ventilation of 15-21 air changes/h.

Test system**Traditional sensitisation test****Route of induction exposure**

epicutaneous, occlusive

Route of challenge exposure

epicutaneous, occlusive

Vehicle

olive oil (for induction, white petrolatum for challenge)

Concentration

The SWCNT was moistened with a minimum amount of olive oil (for induction) or white petrolatum (for challenge) to prepare a paste for dermal application. The paste contained 1% SWCNT. This amount of cnt

was maximum not leading to overflow when patched and the sample fully coated the skin.

No. of animals per dose

The animals were assigned to three groups; a control group(n=10), a CNT-treated group(n=20), and a positive control group(n=10).

Details on study design (Traditional tests)

As for sensitization, the lint (2 x 2.5 cm), on which 0.4g of sensitization specimen was applied, was occlusivedressedfor 6 hours to the skin of the regio lateralis of guinea pig where the pelage had been removed on the day before (day 0). The sensitizations were performed once a week and three times in total to the area where the pelage was removed on the day before (day 7 and day 14). As for challenge, in two weeks (day 28) after the final sensitization, a lint (2 x 2 cm) on which 0.2g of challenge specimen was uniformly applied was occlusive-dressed for 6 hours to the pelage removed regio lateralis where the sensitization had not performed. In 24 and 48 hours after challenge, the skin where the challenge was applied was observed.

Challenge controls

DNCB(1-chloro-2,4-dinitrobenzene)

Positive control substance(s)

yes DNCB(1-chloro-2,4-dinitrobenzene)

Results and discussion

Positive control results

Mean of score was 1.9 at 24 h after the end of challenge and 2.0 at 48 h after the end of challenge.

Traditional sensitisation test

Results of test (except LLNA)

Reading	Hours after challenge	Group	Dose level	No. with + reactions	Total no. in group	Clinical observations
1st reading	24	negative control		0	10	no abnormalities
2nd reading	48	negative control		0	10	no abnormalities
1st reading	24	test group		0	20	no abnormalities
2nd reading	48	test group		0	20	no abnormalities
1st reading	24	positive control		10	10	no abnormalities
2nd reading	48	positive control		10	10	no abnormalities

Overall remarks, attachments

Remarks on results including tables and figures

No abnormality in conjunction with the test was observed to the all guinea pigs and no challenge effect was seen in all observations.

It was judged as no sensitization.

Illustration (picture/graph)

Table 1 A Skin Sensitization Study of Carbon nanotube A in Guinea Pigs (Buehler Test)
Skin Sensitization Reactions

Sex: Male						
Group	Substance for induction	Substance for challenge	Number of animals	Time after the end of challenge (h)	Mean of score	Positive ratio ⁴⁾
Negative control	Physiological saline	Petrolatum	10	24	0.0	
				48	0.0	
		1% Carbon nanotube A ⁴⁾	10	24	0.0	
				48	0.0	
Test article	1% Carbon nanotube A ³⁾	Petrolatum	20	24	0.0	0/20
				48	0.0	0/20
		1% Carbon nanotube A ⁴⁾	20	24	0.0	0/20
				48	0.0	0/20
Positive control	1% DNCB ⁴⁾	Acetone	10	24	0.0	0/10
				48	0.0	0/10
		0.1% DNCB ⁴⁾	10	24	1.9	10/10
				48	2.0	10/10

DNCB: 1-chloro-2,4-dinitrobenzene

⁴⁾ Number of animals with positive / Number of animals tested

¹⁾ Vehicle: Olive oil ²⁾ Vehicle: 70 vol% ethanol ³⁾ Vehicle: Petrolatum ⁴⁾ Vehicle: Acetone

7.5 Repeated dose toxicity

7.5.1 Repeated dose toxicity: oral

Endpoint study record: Nikkiso SWCNT Repeated dose toxicity: oral.001

Administrative Data

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	NIHS	2011	28 Days Repeated Oral Gavage Doses of SWCNT in Rats with 14 Days Recovery Period		Boso Research Center Inc.	B-6931			
publication	Matsumoto et al.	2012	No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats	J Toxicol Sci. 2012;37(3):463-74.					

Materials and methods**Test type**

subchronic

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 407 (Repeated Dose 28-Day Oral Toxicity in Rodents)	yes (The maximum dose of 1000mg/kg requested by the guideline was impracticable because of very high specific volume of SWCNT)

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals**Species**

rat

Strain

other: Crl:CD(SD)

Sex

male/female

Details on test animals and environmental conditions

Male and female rats of 6 weeks old were used for repeated dose toxicity studies. Rats were individually housed in metallic cages with wire mesh bottoms and reared on a basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum. Rats were maintained at room temperature, a humidity of 50± 20%, 10-15 air changes per hour and a 12 hr dark/12 hr light cycle. Body weights of male were 2012-231g and 148-173 for female at the beginning of administration.

Administration / exposure**Route of administration**

oral: gavage

Vehicle

other: 5% gum acasia of aqueous solution

Duration of treatment / exposure

28days

Frequency of treatment

1 time/day

No. of animals per sex per dose

5 animals/sex/dose

Details on study design

Five or ten rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days. Five animals/sex at 0 and 12.5 mg/kg bw/day were used as the recovery groups and were observed for 14 days after the administration period.

Examinations***Observations and examinations performed and frequency***

All males and females were observed three times per day, every day during the administration and once a day during the recovery period. A detailed clinical observation was carried out one day before

administration, and once a week. A functional examination was carried out in the fourth week of the administration period and in the second week of the recovery period. Body weight was measured on days 1, 4, 7, 10, 14, 17, 21, 24 and 28 of the administration period and on days 1, 3, 7, 10 and 14 of the recovery period. Food consumption was measured on days 1, 7, 14, 21 and 28 of the administration period, and on days 7 and 14 of the recovery period. Hematological examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Clinical chemistry examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Necropsy was performed under anesthesia on the day following the end of the administration or recovery period. The external surfaces of rats were examined and a gross internal examination was performed. Organ weights were measured and histopathological evaluations were performed on the organs. Urinary samples were collected for 3 or 4, and 20 hr in the fourth week of the administration period, and in the second week of the recovery period. Urine volume was calculated and a urinary examination was conducted and histopathological evaluations were performed on the organs. Urinary samples were collected for 3 or 4, and 20 hr in the fourth week of the administration period, and in the second week of the recovery period. Urine volume was calculated and a urinary examination was conducted.

Results and discussions

Effect levels

Endpoint	Effect level	Based on	Sex	Basis for effect level / Remarks
NOAEL	> 12.5	test mat.	male/female	

Results of examinations

Clinical signs and mortality

no effects

Body weight and weight gain

no effects

Food efficiency

no effects

Ophthalmoscopic examination

not examined

Haematology

no effects

Clinical chemistry

no effects

Urinalysis

no effects

Neurobehaviour

not examined

Organ weights

no effects

Gross pathology

no effects

Details on results

Histopathology No effects There were no differences in the clinical observation, detailed clinical observation, body weight and food consumption (Table 1) or histopathological examination (Table 2). In the functional examination, significantly low values of landing foot splay were observed in all the treatment groups in males at the end of the administration period (90 ± 11 , 61 ± 15 , 64 ± 18 and 75 ± 10 mm at 0, 0.125, 1.25 and 12.5 mg/kg bw/day, respectively). However, it was due to the high value in the control group and was not considered to be toxicological effects. In urinalysis, a significantly low urine volume was observed during the administration period in females at 1.25 mg/kg bw/day and above (Table 3). However, these values were within the historical background data of the test facility (Mean $\pm$ S.D.: 8.3 ± 4.0 ml/24 hr), and these were not dose dependent. In the hematological examination, significantly high erythrocyte counts in females and lymphocyte counts and basophil counts in males were observed at 12.5 mg/kg bw/day at the end of the administration period (Table 4). However, these changes were considered to be incidental because there were no changes in related parameters. In the serum biochemistry examination, significantly high alanine aminotransferase and triglyceride levels were observed in females in the 0.125 mg/kg bw/day group but not in the high dose groups at the end of the administration period (Table 5). In organ weight measurements, a significantly low absolute weight of the spleen was observed in males in the 12.5 mg/kg group at the end of the administration period (Table 6). However, there were no changes in the relative weight and histopathological examination of the spleen. A significantly high relative weight of the heart was observed in females in the 0.125 mg/kg group but not in the high dose groups at the end of the administration period. At necropsy, a dark red focus in the lung was observed sporadically in the control group and 12.5 mg/kg bw/day group at the end of the administration and recovery periods. At the histopathological examination, focal hemorrhage in the lung was observed in the animals showed a dark red focus. Blackish contents were observed in the cecum of animals in the treatment groups at the end of administration period. However, the histopathological examination revealed no effects in these organs. The contents in the intestines were not observed after the recovery period. Significant differences noted at the end of administration period all disappeared by the end of the recovery period.

Any other information on results incl. tables**Body weight and food consumption in rats dosed with SWCNT by gavage for 28 days**

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Body weight (g)								
Day 1	216 $\pm$ 7	212 $\pm$ 6	214 $\pm$ 8	214 $\pm$ 8	162 $\pm$ 5	165 $\pm$ 6	160 $\pm$ 7	161 $\pm$ 7
Day 4	243 $\pm$ 8	238 $\pm$ 8	241 $\pm$ 11	241 $\pm$ 10	174 $\pm$ 8	175 $\pm$ 5	172 $\pm$ 8	172 $\pm$ 7
Day 7	274 $\pm$ 12	267 $\pm$ 9	273 $\pm$ 16	270 $\pm$ 14	187 $\pm$ 9	186 $\pm$ 6	181 $\pm$ 9	184 $\pm$ 8
Day 14	335 $\pm$ 18	322 $\pm$ 16	337 $\pm$ 29	331 $\pm$ 20	211 $\pm$ 14	210 $\pm$ 10	198 $\pm$ 12	209 $\pm$ 10
Day 21	390 $\pm$ 26	368 $\pm$ 27	388 $\pm$ 34	378 $\pm$ 24	231 $\pm$ 18	234 $\pm$ 11	213 $\pm$ 17	227 $\pm$ 15
Day 28	426 $\pm$ 31	399 $\pm$ 34	426 $\pm$ 39	413 $\pm$ 30	250 $\pm$ 22	246 $\pm$ 15	227 $\pm$ 18	245 $\pm$ 17

Food consumption (g/rat/day)								
Day 0 - 1	25 ± 2	24 ± 1	25 ± 2	25 ± 1	18 ± 2	20 ± 1	18 ± 2	19 ± 2
Day 1 - 7	27 ± 2	26 ± 1	26 ± 2	26 ± 1	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 7 - 14	29 ± 3	28 ± 2	29 ± 3	29 ± 2	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 14 - 21	29 ± 3	28 ± 3	29 ± 3	29 ± 2	20 ± 2	20 ± 2	18 ± 1	19 ± 2
Day 21 - 28	29 ± 4	27 ± 3	29 ± 2	28 ± 2	20 ± 3	20 ± 2	18 ± 1	20 ± 2

Histopathological findings of rats dosed with SWCNT by gavage for 28 days

Sex		Male				Female			
Dose (mg/kg bw/day)		0	0.125	1.25	12.5	0	0.125	1.25	12.5
	Grade								
Number of animals examined (a)		5	0	0	5	5	5	5	5
Kidney									
Tubular regeneration	+	2			1	0	1	0	0
Liver									
Vacuolation of midzonal hepatocyte	+	0			1	0			0
Vacuolation of periportal hepatocyte	+	0			0	2			3
	++	0			0	1			0
Focal necrosis	+	1			0	0			1
Microgranuloma	+	5			3	5			5
Lung (bronchus)									
Focal hemorrhage	+	1			1	0			0
Cell infiltration	+	0			1	0			0
Accumulation of foamy cell	+	0			1	0			0
Spleen									
Lymphoid hyperplasia	++	1			0	0			0

Grade; +: slight change; ++: mild change, -: Not applicable

(a) :The histopathological examination was carried out in 5 animals at 0 and 12.5 mg/kg bw/day and the kidney was examined in 5 females at 0.125 and 12.5 mg/kg bw/day.

Hematology in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
RBC (x104/111)	841 ± 32	845 ± 30	819 ± 26	816 ± 83	810 ± 43	841 ± 13	858 ± 18	859 ± 34*
Hemoglobin (g/dl)	15.5 ± 0.6	15.6 ± 0.4	15.7 ± 0.6	15.2 ± 1.6	15.4 ± 0.7	15.9 ± 0.4	15.9 ± 0.4	16.2 ± 0.7
Hematocrit (%)	45.7 ± 1.4	45.5 ± 1.3	46 ± 1.8	44.8 ± 4.7	43.6 ± 2.3	45.5 ± 1	45.4 ± 1.1	46.3 ± 1.9
MCV (fl)	54.3 ± 1.3	53.8 ± 0.9	56.1 ± 1	54.9 ± 2	53.9 ± 2.5	54.1 ± 1.2	52.9 ± 0.9	53.9 ± 1.3
MCH (pg)	18.5 ± 0.5	18.5 ± 0.4	19.2 ± 0.3	18.6 ± 0.8	19 ± 0.8	19 ± 0.5	18.5 ± 0.4	18.8 ± 0.4
MCHC (g/dl)	34 ± 0.3	34.4 ± 0.1	34.2 ± 0.2	34 ± 0.2	35.3 ± 0.3	35 ± 0.3	35 ± 0.4	34.9 ± 0.1
Reticulocyte (%)	2.4 ± 0.4	1.9 ± 0.5	2.6 ±	2.8 ± 1.5	1.8 ± 0.3	1.5 ± 0.2	1.5 ± 0.1	1.7 ± 0.4

			0.3					
WBC (x 102/[d])	89.1 ± 14.0	110.5 ± 24.2	105.5 ± 23.7	123.8 ± 34.8	70 ± 22.4	67.8 ± 11.4	81.0 ± 30.8	64.1 ± 10.9
Differential leukocyte counts (x10 ² /μl)								
Lymphocyte	70.6 ± 9.7	86 ± 14.8	76.7 ± 13.6	99.9 ± 25.2*	52.4 ± 18	53.3 ± 13.7	64.1 ± 31.2	48.9 ± 11.3
Neutrophil	14.8 ± 3.2	20.1 ± 8.9	24.6 ± 16.3	19.2 ± 8.2	14.7 ± 3.4	11.8 ± 2.8	14.1 ± 5.4	12.7 ± 4.7
Eosinophil	1.0 ± 0.8	1.1 ± 0.7	0.9 ± 0.1	0.9 ± 0.4	1.0 ± 0.7	0.9 ± 0.2	0.9 ± 0.4	1.0 ± 0.3
Basophil	0.3 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.3*	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.1 ± 0.1
Monocyte	1.9 ± 0.8	2.3 ± 1.5	2.4 ± 1	2.6 ± 1.8	1.3 ± 0.7	1.3 ± 0.4	1.2 ± 0.5	1.2 ± 0.3

RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.

***: Significantly different from the control group ($p < 0.05$).**

Serum biochemistry in rats dosed with SWCNT by gavage for 28 days

	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
AST (IU/1)	68 ± 4	68 ± 5	62 ± 4	64 ± 6	60 ± 7	86 ± 23	79 ± 29	72 ± 11
ALT (IU/1)	28 ± 5	30 ± 4	25 ± 3	28 ± 7	19 ± 2	34 ± 10**	27 ± 10	22 ± 1
LDH (IU/1)	54 ± 8	53 ± 7	52 ± 5	54 ± 6	52 ± 3	63 ± 16	57 ± 12	56 ± 9
γ-GTP (IU/1)	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 1	1 ± 0	1 ± 0
ALP (IU/1)	647 ± 198	686 ± 82	655 ± 73	663 ± 84	415 ± 127	359 ± 102	378 ± 34	470 ± 102
T. bile acid (μmol/l)	15.1 ± 12.7	12.5 ± 4.9	10.5 ± 7.5	7.6 ± 2.9	12.2 ± 2.3	15.4 ± 4.9	11.3 ± 3.1	11.6 ± 6.3
T. cholesterol (mg/dl)	50 ± 5	48 ± 14	52 ± 7	50 ± 6	55 ± 7	57 ± 14	66 ± 16	50 ± 17
Triglyceride (mg/dl)	56 ± 19	55 ± 22	50 ± 18	59 ± 24	14 ± 3	29 ± 12*	21 ± 11	14 ± 6
Phospholipid (mg/dl)	93 ± 8	92 ± 14	94 ± 10	93 ± 10	100 ± 11	108 ± 18	123 ± 20	95 ± 26
T. bilirubin (mg/dl)	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0	0.1 ± 0
Glucose (mg/dl)	126 ± 13	121 ± 5	135 ± 18	141 ± 12	113 ± 11	106 ± 11	122 ± 8	123 ± 23
BUN (mg/dl)	13 ± 2	14 ± 3	12 ± 1	14 ± 2	16 ± 2	16 ± 2	15 ± 2	16 ± 2
Creatinine (mg/dl)	0.24 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	0.25 ± 0.03	0.27 ± 0.02	0.26 ± 0.04	0.27 ± 0.02	0.28 ± 0.01

Sodium (mmo1/1)	144 ± 1	144 ± 1	144 ± 1	143 ± 1	144 ± 1	143 ± 1	143 ± 1	143 ± 2
Potassium (mmo1/1)	4.6 ± 0.1	4.6 ± 0.1	4.6 ± 0.1	4.7 ± 0.2	4.3 ± 0.2	4.4 ± 0.3	4.2 ± 0.2	4.2 ± 0.2
Chloride (mmo1/1)	105 ± 1	106 ± 1	105 ± 1	106 ± 1	107 ± 1	107 ± 1	107 ± 2	107 ± 1
Calcium (mg/dl)	10 ± 0.2	9.8 ± 0.2	10.2 ± 0.3	9.8 ± 0.2	10 ± 0.3	10 ± 0.1	9.9 ± 0.1	10 ± 0.1
I. phosphorus (mg/dl)	7.8 ± 0.3	7.5 ± 0.4	7.9 ± 0.5	7.5 ± 0.6	7 ± 0.3	7 ± 0.6	6.9 ± 0.9	6.9 ± 0.8
T. protein (g/dl)	5.8 ± 0.1	6 ± 0.1	6 ± 0.1	5.9 ± 0.2	6.4 ± 0.3	6.2 ± 0	6.2 ± 0.2	6.1 ± 0.1
Albumin (g/dl)	3.2 ± 0	3.2 ± 0.1	3.2 ± 0.1	3.2 ± 0.1	3.6 ± 0.2	3.5 ± 0.1	3.6 ± 0.1	3.5 ± 0.1
Albumin/Globlin	1.21 ± 0.09	1.14 ± 0.07	1.15 ± 0.08	1.23 ± 0.06	1.31 ± 0.12	1.31 ± 0.09	1.43 ± 0.08	1.35 ± 0.07

AST: Aspartate aminotransferase; **ALT:** Alanine aminotransferase; **LDH:** lactate dehydrogenase;
ALP: Alkaline phosphatase;
BUN: Blood urea nitrogen

Overall remarks, attachments

Remarks on results including tables and figures

No treatment related changes of body weight, behavioral and blood biochemical parameters were observed. A few minor changes with statistical significance in white blood cells composition, organ weights and urine volume were detected. However no relevant histopathological changes were observed. Based on the above findings, the NOAEL of repeated oral dose toxicity of the SWCNT was considered to be 12.5 mg/kg bw/day (the highest dose tested) in rats.

Attached full study report

Attached full study report
Acute and Repeated Oral Toxicity of Nikkiso SWCNT and MWCNT (TG423-407).pdf / 901.1 KB (application/pdf): Annexe 1

7.5.2 Repeated dose toxicity: inhalation

Endpoint study record: Nikkiso SWCNT Repeated dose toxicity: inhalation.001

Administrative Data

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

Guide line study

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Evaluating Risks associated with Manufactured Nanomaterials. Developing Toxicity Evaluating Methods by Inhalation Exposure	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	University of Occupational and Environmental Health, Japan(UOEH)	06990625-0-1 (Report to NEDO)			

Materials and methods**Test type**

subacute

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 412 (Repeated Dose Inhalation Toxicity: 28/14-Day)	

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals**Species**

rat

Strain

Wistar

Sex

male

Details on test animals and environmental conditions

Age: 9 weeks(at the start of test),

Weight: 300g

Administration / exposure

Route of administration

inhalation: aerosol

Type of inhalation exposure

whole body

Details on inhalation exposure

Exposure chamber. SWCNT was suspended in the air using the pressurized nebuliser, and its aqueous component (distilled water including Triton) was removed by mist dryer. The size and number concentration of SWCNTs in the chamber were analysed in-line using a particle spectrometer consisting of a differential mobility analyser and a condensation particle counter through the exposure period.

Analytical verification of doses or concentrations

yes

Details on analytical verification of doses or concentrations

Mass concentration of the particles were determined by carbon analysis and were 0.08 ± 0.014 mg/m³ for lower concentration and 0.40 ± 0.11 mg/m³ for higher concentration. Triton concentration as negative control were 1.0 ± 0.4 mg/m³.

Duration of treatment / exposure

Exposure period: 6h/d*5d*4w

No. of animals per sex per dose

15 rats / dose / observation period

Details on study design

Animal: 15 male Wistar rats (9 weeks old at the start of the test) / each observation periods. Body weight was recorded in observation periods, and wet organ weights of the lung, brain, nasal cavity, testis, liver, kidney and spleen were recorded on the day of autopsy. Histopathological examination was carried out on these organs. White blood cells and neutrophil cells were counted as biomarkers representing inflammation in blood, number of total cells of BALF, number of neutrophil cells of BALF, and concentration of HO-1 were recorded. Pulmonary inflammation and collagen deposits (fibrosis) were analyzed by the point counting method. Urinary examination was carried out for 8-OHdG levels.

Positive control

nano-sized nickel oxide negative control; Triton

Examinations***Observations and examinations performed and frequency***

Post exposure observation period: 3d, 1m, 3m

Sacrifice and pathology

Sacrificed 3 days, 1 month and 3 months after exposure

Results and discussions**Results of examinations*****Clinical signs and mortality***

no effects

Body weight and weight gain

no effects

Organ weights

no effects

Gross pathology

no effects

Histopathology: non-neoplastic

no effects

Any other information on results incl. tables

Wet lung weight was not significantly changed in the treatment groups (see Table), and other organs (brain, nasal cavity, testis, liver, kidney and spleen) were not also changed.

	negative cont.	low	high	positive cont.
3 d	1.31±0.06	1.38±0.23	1.37±0.07	1.80±0.11**
1 m	1.47 ± 0.08	1.55 ± 0.07	1.58 ± 0.08*	1.76 ± 0.07**
3 m	1.66 ± 0.14	1.62 ± 0.04	1.60 ± 0.	1.63 ± 0.07

for negative control; * p<0.05, **p<0.01

Neutrophil cells in blood were slightly but significantly increased only at 3 month after the administration in the high concentration group. No effects were observed in BALF samples by SWCNT while the positive control increased a total number cells and neutrophil cells in BALF. No differences were found the results of the point counting method between the negative control and treatment groups. No histopathological effects were found in the lung and other organs. No changes were observed in urinary 8-OHdG levels.

Applicant's summary and conclusion

Executive summary

Wistar rats were exposed to Nikkiso SWCNT (long-CNT) for 4 weeks (6 hours/day, 5 days/week) at a level of 0.08 +/- 0.014 mg/m³ or 0.40 +/- 0.11 mg/m³ of the particle weight concentration in the exposure chamber. Inflammation and fibrotic response were examined at three days, one month or three months after the last exposure. Only observed effects were increased neutrophil cells in blood at 3 month after the administration in the high concentration group. Neither the low concentration exposure group nor the high concentration exposure group showed increase of the pulmonary wet weight, the infiltration of the inflammatory cell and increase of the HO-1 gene expression.

Endpoint study record: Super Growth Repeated dose toxicity: inhalation.001

Administrative Data

other:OECD/WPMN

Purpose flag key study

Study result type experimental result

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Risk Assessments of Manufactured Nanomaterials- Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html		University of Occupational and Environmental Health, Japan				
publication	Morimoto et al.	2012	Pulmonary toxicity of well-dispersed single-walled carbon nanotubes after inhalation	Nanotoxicology, 6(7):766-75					

Materials and methods

Test type

subacute

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 412 (Repeated Dose Inhalation Toxicity: 28/14-Day)	

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Super Growth

Test animals**Species**

rat

Strain

Wistar

Sex

male

Details on test animals and environmental conditions

Male Wistar rats (8 weeks old) were purchased from Kyudo Co., Ltd. (Kumamoto, Japan). The animals were kept in the Laboratory Animal Research Center of the University of Occupational and Environmental Health for a week with access to free-feeding of commercial diet and water. All procedures and animal handling were done according to the guideline described in the Japanese Guide for care and Use of Laboratory animals as approved by the Animal Care and Use committee, UOEH Japan. the guidelines described in the Japanese Guide for the Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee, University of Occupational and Environmental Health Japan, Kitakyushu, Japan.

Administration / exposure**Route of administration**

inhalation: aerosol

Type of inhalation exposure

whole body

Vehicle

clean air

Details on inhalation exposure

The raw material was dispersed in 1% Tween 80 solution with a bath sonicator (40 kHz, 240W) for 9 h. The dispersed SWCNT fibres were then collected on a membrane filter and re-dispersed into distilled water in order to reduce the Tween 80 concentration in the dispersion medium. TEM observation showed that the SWCNT fibres were suspended in bundled form. Each bundle consisted of several to a few tens

of single SWCNT fibres, creating a rope-like shape. The length of the bundle was determined to be 0.2 μm in median and c.a.1 μm in the maximum by atomic force microscope (AFM) image analysis. The whole-body exposure system consisted of a pressurised nebuliser and a mist dryer and was connected to an exposure chamber (volume:0.52m³). SWCNT was suspended in the air using the pressurised nebuliser, and its aqueous component (distilled water including Tween 80) was removed by a mist dryer. The size and number concentrations of SWCNTs in the chamber were analysed in-line using a particle spectrometer consisting of a differential mobility analyser and a condensation particle counter (Model 1000XP WPS, MSP Corp., Shoreview, MN, USA) throughout the exposure period. The average diameter and number concentration of the particles in the chamber were measured throughout all exposure days.

Details on analytical verification of doses or concentrations

The SWCNTs and Tween 80 aerosol in the chamber were collected with quartz fibre filter (QAT-UP, Pall Sciences, Ann Arbor, MI, USA), the elemental carbon amounts were determined by an organic carbon (OC)/elemental carbon (EC) aerosol analyser (Sunset Laboratory, Forest Grove, OR, USA), and the concentrations in the chamber were calculated. Carbon analysis of the particles showed that the mass concentration of SWCNTs in the aerosol particles delivered to the low and high concentration rat groups was 0.03 ± 0.003 and 0.13 ± 0.03 mg/m³, respectively. The particle number concentrations in the two groups were $5.0 \pm 0.7 \times 10^4$ and $6.6 \pm 2.1 \times 10^4$ particles/cm³, respectively.

Duration of treatment / exposure

The rats inhaled the aerosol 6 h/day, 5 days/week, for 4 weeks in a whole-body exposure chamber.

MMAD / GSD

The geometric mean value and geometric standard deviation of the particle length, the length measured along the particle shape, were 0.7 and 1.7 μm , respectively. Those for the width of the particles, the maximum size perpendicular to the length, were 0.2 and 1.7 μm , respectively.

No. of animals per sex per dose

Nine-week-old male Wistar rats were divided into three groups: low SWCNT concentration, high SWCNT concentration, and Tween 80. There were 10 rats in each group.

Positive control

Positive control was not reported. The Tween-exposed rats, the negative control, were designed to inhale Tween aerosol particles (average diameter: 62.3 nm), prepared by using Tween 80 solution and another set of a nebuliser and mist dryer, in a same-sized chamber at an average mass and particle number concentration of 0.38 ± 0.06 mg/m³ and $5.6 \pm 0.8 \times 10^4$ particles/cm³, respectively.

Examinations

Sacrifice and pathology

After the exposure period and recovery, rats were injected intraperitoneally with a fatal overdose of phenobarbital at 3 days, 1 month, and 3 months, and dissected. Each group of 10 animals was divided into 2 subgroups of 5 animals for lung tissue analysis. The first subgroup provided bronchoalveolar lavage, which was collected using physiological saline that was injected through a cannula inserted into the respiratory tract, into the right lung. Three to 10 ml of physiological saline was infused in the right lung each time and, up to 50 ml in total of lavage fluid was collected. On the other hand, the left lung was inflated and fixed by 4% paraformaldehyde at 25 cm H₂O pressure. The lungs of the second subgroup

(five rats) were homogenised to extract protein. The lung tissue was homogenised with a T-PER tissue protein extraction reagent, and then centrifuged (1500xg for 10 min). The protein concentration of the supernatant was measured by a BCA Protein Assay Kit (PIERCE, Rockford, IL, USA) using bovine serum albumin as a standard. The total protein concentration was adjusted to a final concentration of 500 µg/ml for cytokine-induced neutrophil chemoattractant-1 (CINC-1) and CINC-2, 4000 µg/ml for CINC-3, and 2000 µg/ml for heme oxygenase-1 (HO-1). Chemokine and HO-1 concentration in the lung tissue was determined by Quantikine Rat CINC-1, CINC-2, CINC-3 (R&D Systems, Minneapolis, MN, USA) (Cat.#RCN100, #RCN200, and #RCN300) and an HO-1 ELISA Kit (Stressgen Bioreagents, Ann Arbor, MI, USA). These cytokines and the HO-1 concentration in the bronchoalveolar lavage fluid (BALF) supernatant were also measured. Alkaline phosphatase (ALP) released in the BALF supernatant was determined by a LabAssay™ ALP (Wako Pure Chemical Industries, Ltd., Japan). The lungs (total left lobe), which were inflated and fixed by 4% paraformaldehyde, and the trachea were resected from the surrounding tissue. The lung tissue was embedded in paraffin and 5 µm thick sections were cut from the lobe. The samples were then sectioned and stained with H&E.

Statistics

Statistical analysis was carried out using the Mann-Whitney U test, with differences of $p < 0.05$ considered to be statistically significant.

Results and discussions

Effect levels

Endpoint	Effect level	Based on	Sex	Basis for effect level / Remarks
NOAEC	ca. 0.13 mg/m ³ air	test mat.	male	

Results of examinations

Clinical signs and mortality

no effects

Body weight and weight gain

no effects

Haematology

no effects

Clinical chemistry

no effects

Urinalysis

no effects

Gross pathology

no effects

Histopathology: neoplastic

no effects

Details on results

Cell count and ALP release in BALF; The total cell and neutrophil counts in the high and low concentrations of SWCNTs did not increase significantly more than in the Tween-exposed groups (negative control) during the observation period. No significant release of ALP was observed in the SWCNT-exposed groups. Concentration of CINC in lung; There were no differences in the CINC-1, CINC-2 and CINC-3 concentrations in the lung tissue between the SWCNT-exposed groups and the Tween-exposed group during the observation period. HO-1 concentration in lung and BALF; Regarding the concentration of HO-1 in lung tissue, there were no significant increases in HO-1 level in either concentration of SWCNT during the observation period. Although transient decreases in the concentration of HO-1 in BALF in the SWCNT-exposed groups were observed in the time course, no consistent changes in the concentration of HO-1 were found during the observation period. Histopathological changes in lungs; No neutrophil infiltrations into the alveolar space, granulomatous lesion, interstitial collagen deposition or emphysematous changes were found during the observation period in either of the SWCNT-exposed groups. Alveolar macrophages that ingested SWCNTs were seen to a slight extent. There were no pathological changes in the Tween-exposed group throughout the period. Morphological feature of alveolar macrophage by TEM; SWCNTs were found in a plurality of phagolysosomes in an alveolar macrophage. Individual and agglomerated SWCNTs were also identified in the alveolar macrophages. No SWCNTs were observed in the nuclei or organelles.

Overall remarks, attachments

Attached background material

Applicant's summary and conclusion

Conclusions

In a 4-week inhalation study of well-dispersed purified SWCNTs, neither low nor high concentrations of SWCNTs induced inflammation of mainly neutrophils or the concentration of CINC or HO-1 in the lung. Well-dispersed SWCNTs did not induce neutrophil inflammation in the lung under the conditions in the present study.

7.5.3 Repeated dose toxicity: dermal

7.5.4 Repeated dose toxicity: other routes

7.6 Genetic toxicity

7.6.1 Genetic toxicity in vitro

Endpoint study record: Nikkiso SWCNT Ames Test; Genetic toxicity in vitro.001

Administrative Data

other:OECD/WPMN

Purpose flag key study

Study result type experimental result

Reliability 1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Bacterial reverse mutation test of Nikkiso SWCNT	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Inc.	ZT10110			

Materials and methods

Type of genotoxicity

gene mutation

Type of study

bacterial reverse mutation assay (e.g. Ames test)

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 471 (Bacterial Reverse Mutation Assay)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Method**Species/strain****Species/strain** S. typhimurium TA 1535, TA 1537, TA 98, TA 100 and E. coli WP2**Metabolic activation** with and without**Metabolic activation system** S9 mix**Test concentrations**

Test 11.563, 3.125, 6.25, 12.5, 25, and 50 µg/plate (-S9; all strains).1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/plate (+S9; TA100 and WP2)1.563, 3.125, 6.25, 12.5, 25, and 50 µg/plate (+S9; TA1535 and TA1537)0.391, 0.781, 1.563, 3.125, 6.25, 12.5, and 25 µg/plate (+S9; TA98)Test 2-11.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/plate (-S9; all strains).1.563, 3.125, 6.25, 12.5, 25, and 50 µg/plate (+S9; TA100, WP2 TA1535 and TA1537)0.781, 1.563, 3.125, 6.25, 12.5, and 25 µg/plate (+S9; TA98)Test 2-2 (additional study)1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/plate (-S9; all strains).1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/plate (+S9; TA100, WP2 TA1535 and TA1537)0.781, 1.563, 3.125, 6.25, 12.5, and 25 µg/plate (+S9; TA98)Test 31.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/plate (-S9; all strains).

Vehicle

CNTs were suspended in the water for injection and was diluted with 0.3% CMC-Na solution after the sonication to each concentration

Controls**Negative controls** no**Solvent / vehicle controls** yes**True negative controls** no**Positive controls** yes

Positive control substance other: S9 mix; 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide(TA100, TA98, WP2 uvrA), sodium azide (TA1535) and 9-aminoacridine (TA1537). +S9 mix; 2-aminoanthracene (five strains)

Details on test system and conditions

The mutation test was performed using S. typhimurium (TA98, TA100, TA1535, TA1537) and E. coli (WP2uvrA)according to OECD TG471 under a metabolism activation existence and non-existence, via S9 mix. S9 fraction.METHOD OF APPLICATION: preincubationDURATION- Preincubation period:20 min- Exposure duration: 48 hNUMBER OF REPLICATIONS: 3DETERMINATION OF

CYTOTOXICITY- Method: colony count

Evaluation criteria

The test substance was determined to be positive if the following criteria were met: the number of the revertant colonies increased more than two-fold of the vehicle control value in any test strain, either in the presence or absence of S9 mix; the test substance induced a dose-dependent increase of the number of revertant colonies; the dose-dependent increase was reproducible.

Results and discussions

Test results

Species/strain	S. typhimurium TA 1535, TA 1537, TA 98, TA 100 and E. coli WP2
Metabolic activation	with and without
Test system	all strains/cell types tested
Genotoxicity	negative
Cytotoxicity	yes
Vehicle controls valid	yes
Positive controls valid	yes

Any other information on results incl. tables

In the 1st test, the positive results were obtained for all strains, but the 2nd tests and 3rd test showed negative results for all strains. Therefore, it was concluded SWCNT did not induce mutagenicity. Precipitation was observed at 25 µg/plate and higher for all strains. Slight growth inhibition was sporadically observed at 25 µg/plate and higher (see Tables).

Table2-1. Bacteria reverse mutation of SWCNT without S9mix

Dose (µg /plate)	Number of revertant colonies / plate (Mean + S.D.)				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA98	TA1537
0 a)	109 ±16.0	7 ± 1.0	24 ± 1.5	19 ± 2.1	11 ± 3.6
1.563	120 ±12.0	18 ± 5.6	32 ± 2.9	57 ± 5.0	28 ± 3.5
3.125	190 ± 17.1	30 ± 1.7	31 ± 1.2	98 ± 16.1	47 ± 4.0
6.25	263 ± 49.3	67 ± 3.8	37 ± 5.6	169 ± 14.7	58 ± 4.4
12.5	323 ± 29.5	143 ± 36.1	90 ± 15.5	417 ± 42.9	269 ± 24.1
25	549 ± 95.0	250 ± 18.6	180 ± 45.1	702± 12.1	460 ± 132.0
50	651 ± 67.2	470 ± 73.7	491 ± 129.6	717± 101.7	594 ± 34.9
Substance	AF-2 b)	NaN3 c)	AF-2	AF-2	9AA d)
Dose (µg/plate)	0.01	0.5	0.01	0.1	80
	647±44.2	567 ± 73.8	109 ± 15.0	465 ± 68.6	141 ± 26.9

a) Negative control, 0.3% CMC

b) 2-(2FuyI)-3-(5-nitro-2fuyI)acrylamide

c) Sodium azide

d) 9-Aminoacridine hydrochloride

Table2-1 (Cont.) Bacteria reverse mutation of SWCNT with S9mix

Dose (µg /plate)	Number of revertant colonies / plate (Mean + S.D.)				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA98	TA1537
0 a)	117±15.0	7±3.8	23±8.0	28±4.4	16±4.2
0.391	NT	NT	NT	34±6.6	NT
0.781	NT	NT	NT	44±4.7	NT
1.563	136±8.6	78±8.7	74±11.9	68±8.7	78±3.6
3.125	164±10.1	142±9.3	108±14.2	101±8.7	153±6.2
6.25	209±3.5	275±48.8	156±11.5	269±14.6	262±2.9
12.5	334±47.5	612±152.2	306±35.9	392±58.6	464±63.0
25	730±53.8	1034±59.8	755±57.8	736±50.1	734±107.1
50	937±115.7	1073±129.7	904±41.9	NT	980±23.1
100	1582±163.7	NT	1255±161.1	NT	NT
Substance	2AA b)	2AA	2AA	2AA	2AA
Dose (µg/plate)	1	2	10	0.5	2
	772±92.3	256±55.6	580±152.0	373±43.0	146±2.6

a) Negative control, 0.3% CMC**b) 22-Aminoanthracene****Table2-2. Bacteria reverse mutation of SWCNT without S9mix**

Dose (µg /plate)	Number of revertant colonies / plate (Mean + S.D.)				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA98	TA1537
0 a)	113±8.0	8±3.0	22±7.8	16±4.0	6±1.0
1.563	106±2.5	12±1.5	24±3.1	15±5.5	5±1.2
3.125	105±5.5	6±1.5	19±4.6	14±2.6	7±2.1
6.25	117±15.6	10±2.0	18±4.2	16±2.3	8±2.0
12.5	94±11.9	7±4.0	21±1.5	14±5.5	7±3.1
25	110±20.7	7±3.2	18±2.9	14±4.9	7±3.1
50	114±12.9	10±2.5	21±2.6	17±2.1	6±4.6
Substance	AF-2 b)	NaN3 c)	AF-2	AF-2	9AA d)
Dose (µg/plate)	0.01	0.5	0.01	0.1	80
	487±40.4	433±17.8	85±11.8	436±76.9	231±26.1

a) Negative control, 0.3% CMC**b) 2-(2FuyI)-3-(5-nitro-2fuyI)acrylamide****c) Sodium azide****d) 9-Aminoacridine hydrochloride****Table2-2 (Cont.). Bacteria reverse mutation of SWCNT with S9mix**

Dose (µg /plate)	Number of revertant colonies / plate (Mean + S.D.)				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA98	TA1537
0 a)	95±11.9	9±1.0	20±1.0	26±6.4	15±4.0
0.781	NT	NT	NT	25±1.2	NT
1.563	110±2.3	7±2.0	20±4.7	27±5.9	13±3.1
3.125	112±11.8	7±3.5	19±2.6	26±1.0	10±0.6
6.25	116±14.7	7±0.6	15±2.1	22±3.0	11±2.1

12.5	124±13.9	8±1.0	22±2.5	27±6.5	15±4.5
25	118±21.6	8±2.0	21±4.2	32±3.2	16±2.9
50	124±8.7	9±2.1	26±7.6	NT	14±2.1
100	125±5.5	NT	28±8.4	NT	NT
Substance	2AA b)	2AA	2AA	2AA	2AA
Dose (µg/plate)	1	2	10	0.5	2
	862±12.0	239±24.0	653±18.1	394±11.8	98±14.0

a) Negative control, 0.3% CMC

b) 22-Aminoanthracene

Table2-3. Bacteria reverse mutation of SWCNT without S9mix

Dose (µg /plate)	Number of revertant colonies / plate (Mean + S.D.)				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA98	TA1537
0 a)	118±13.8	11±1.5	25±5.9	19±2.6	10±0.6
1.563	118±8.0	6±2.0	26±4.6	15±2.6	9±4.0
3.125	120±9.5	7±2.5	26±6.2	14±2.5	9±2.1
6.25	121±1.2	7±1.2	23±3.2	16±3.8	9±3.8
12.5	111±7.0	9±1.5	22±7.2	16±3.2	8±1.2
25	116±11.0	7±3.1	22±3.0	14±2.6	7±3.5
50	126±3.5	6±1.5	21±1.2	19±5.5	7±1.5
50	114±1.0	8±2.1	19±2.1	16±1.7	7±3.2
Substance	AF-2 b)	NaN3 c)	AF-2	AF-2	9AA d)
Dose (µg/plate)	0.01	0.5	0.01	0.1	80
	459±32.6	437±56.0	106±17.0	479±14.2	369±60.7

a) Negative control, 0.3% CMC

b) 2-(2FuyI)-3-(5-nitro-2fuyI)acrylamide

c) Sodium azide d) 9-Aminoacridne hydrochloride

Table2-3 (Cont.). Bacteria reverse mutation of SWCNT with S9mix

Dose (µg /plate)	Number of revertant colonies / plate (Mean + S.D.)				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA98	TA1537
0 a)	119±19.7	10±3.1	27±1.5	30±0.6	15±2.6
0.781	NT	NT	NT	24±4.7	NT
1.563	126±9.5	9±3.1	25±1.0	25±2.3	11±2.6
3.125	131±2.1	8±4.0	31±3.0	23±1.5	16±1.7
6.25	136±3.5	7±2.0	26±2.5	25±4.0	17±2.6
12.5	137±7.0	8±2.5	28±4.4	22±4.0	11±3.1
25	112±11.5	8±3.5	24±3.6	21±4.5	12±3.8
50	125±5.1	8±2.0	30±4.0	NT	10±4.0
100	134±10.5	8±2.3	28±4.7	NT	12±1.2
Substance	2AA b)	2AA	2AA	2AA	2AA
Dose (µg/plate)	1	2	10	0.5	2
	999±72.9	198±38.2	555±115.6	413±20.4	142±21.5

a) Negative control, 0.3% CMC

b) 22-Aminoanthracene

Table2-4. Bacteria reverse mutation of SWCNT without S9mix

Dose (µg /plate)	Number of revertant colonies / plate (Mean + S.D.)				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA98	TA1537
0 a)	105±17.2	9±0.6	18±4.5	18±1.5	10±1.5
1.563	113±4.5	7±3.1	22±3.6	22±1.5	8±4.4
3.125	112±3.6	9±4.0	25±2.1	20±0.6	11±4.7
6.25	114±8.7	9±2.6	18±2.9	17±2.1	8±5.0
12.5	127±6.2	8±2.1	21±1.5	16±3.8	7±2.5
25	122±8.5	8±2.6	21±1.0	15±3.1	10±2.6
50	129±6.4	8±1.2	21±3.1	14±2.6	11±0.6
100	131±15.9	7±1.7	20±2.1	13±2.5	8±4.2
Substance	AF-2 b)	NaN3 c)	AF-2	AF-2	9AA d)
Dose (µg/plate)	0.01	0.5	0.01	0.1	80
	529±59.7	505±21.4	142±19.3	533±25.3	290±85.5

a) Negative control, 0.3% CMC

b) 2-(2FuyI)-3-(5-nitro-2fuyI)acrylamide

c) Sodium azide

d) 9-Aminoacridine hydrochloride

Overall remarks, attachments

Remarks on results including tables and figures

No mutation induction was observed under a metabolism activation existence and non-existence in all the tested concentration and in all the strain.

Applicant's summary and conclusion

Interpretation of results

negative

Endpoint study record: Nikkiso SWCNT Chromosomal Aberration Test; Genetic toxicity in vitro.002

Administrative Data

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Bacterial reverse mutation test of Nikkiso SWCNT	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Inc.	ZT10111			

Materials and methods**Type of genotoxicity**

chromosome aberration

Type of study

in vitro mammalian chromosome aberration test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 473 (In vitro Mammalian Chromosome Aberration Test)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Method**Test concentrations**

6.25, 12.5, 25, 50 and 100 µg/plate.

Vehicle

CNTs were suspended in the water for injection and was diluted with 0.3% CMC-Na solution after the

sonication to each concentration

Controls

Negative controls	yes
Solvent / vehicle controls	yes
True negative controls	no
Positive controls	yes
Positive control substance	other: -S9:Benzo(a) pyrene +S9:Mitomycin C

Details on test system and conditions

The short term test for 6 hours under a metabolism activation existence and non-existence and the long term test for 24 hours under a metabolism activation non-existence were performed using CHL/IU according to the OECD TG473.

Evaluation criteria

Negative: chromosome aberration <5%Equivocal: Chromosome aberration 5-10%Positive: chromosome aberration >10%Concentration dependency was also considered for judgment of results

Results and discussions

Test results

Species/strain	other: Chinese hamster CHL/IU
Metabolic activation	with and without
Genotoxicity	negative
Cytotoxicity	no
Vehicle controls valid	yes
Negative controls valid	yes
Positive controls valid	yes

Overall remarks, attachments

Remarks on results including tables and figures

In both short term tests (6 hours) under the metabolism activation existence and non-existence and long term tests (24 hours) under the metabolism activation non-existence, the expressions of structural chromosomal aberration and polyploidy were below 5% .

The chromosome aberration was judged to be negative.

Illustration (picture/graph)**Table 2** In vitro chromosomal aberration test of Carbon nanotube A with cultured mammalian cells

- Direct method -

Treatment time ^{a)} (h)	Dose (µg/mL)	Growth rate (%)	No. of cells	Number of cells with structural aberrations ^{b)}						Total (%)		Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap	-gap	
6-18	0 ^{c)}	100	200	0	0	0	0	0	0	0.0	0.0	0.5
	6.25	[101]	200	1	0	0	0	0	0	0.5	0.0	0.5
	12.5	[112]	200	1	0	0	0	0	0	0.5	0.0	1.5
	25	[114] ‡	200 ‡	2	0	0	0	0	0	1.0	0.0	1.0
	50	[116] ‡	200 ‡	0	0	0	0	0	0	0.0	0.0	0.5
	100	[110] ‡	200 ‡	1	2	0	1	0	0	2.0	1.5	0.0
	MMC 0.12 ^{d)}	-	200	0	64	81	3	2	0	58.0	58.0	0.5
24-0	0 ^{c)}	100	200	1	0	0	0	0	0	0.5	0.0	0.0
	6.25	[119]	200	0	0	0	0	0	0	0.0	0.0	1.0
	12.5	[114]	200	0	1	0	0	0	0	0.5	0.5	0.0
	25	[118] ‡	200 ‡	0	0	0	2	0	0	1.0	1.0	1.0
	50	[118] ‡	200 ‡	0	0	0	0	0	0	0.0	0.0	0.5
	100	[112] ‡	- ‡	(a few metaphases)						-	-	-
	MMC 0.06 ^{d)}	-	200	3	44	70	0	1	0	49.5	49.0	0.0

- Metabolic activation method -

Treatment time ^{a)} (h)	Dose (µg/mL)	Growth rate (%)	No. of cells	Number of cells with structural aberrations ^{b)}						Total (%)		Number of polyploid cells (%)
				gap	ctb	cte	csb	cse	oth	+gap	-gap	
6-18	0 ^{c)}	100	200	2	0	1	1	0	0	2.0	1.0	0.5
	6.25	[110]	200	1	0	0	0	0	0	0.5	0.0	0.0
	12.5	[117]	200	1	1	0	0	0	0	1.0	0.5	1.5
	25	[96] ‡	200 ‡	4	0	0	0	0	0	2.0	0.0	0.0
	50	[112] ‡	200 ‡	0	1	0	0	1	0	1.0	1.0	0.0
	100	[103] ‡	200 ‡	1	0	1	0	0	0	1.0	0.5	1.0
	B[a]P 30 ^{e)}	-	200	2	35	97	3	2	0	59.0	58.0	0.0

^{a)} Treatment time - Recovery time^{b)} gap; Chromatid and chromosome gap, ctb; Chromatid break, cte; Chromatid exchange, csb; Chromosome break, cse; Chromosome exchange, oth; Multiple aberrant cell, fragmentation, etc.^{c)} Negative control, 0.3% CMC^{d)} Positive control, Mitomycin C^{e)} Positive control, Benzo[a]pyrene

‡: Precipitation (at the start and end of treatment)

[]: Mean growth rate of 2 plates

Applicant's summary and conclusion**Interpretation of results**

negative

Conclusions

The expressions of structural chromosomal aberration and polyploidy were not increased.

Endpoint study record: Super Growth SWCNT Ames Test; Genetic toxicity in vitro.001**Administrative Data**

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Super Growth SWCNT:Bacterial Reverse Mutation Assay	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)-(August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	The Institute of Environmental Toxicology (Ibaraki, Japan)	IET 07-0095			
publication	Naya et al.	2011	Evaluation of the genotoxic potential of single-walled carbon nanotubes by using a battery of in vitro and in vivo genotoxicity assays	Regulatory Toxicology and Pharmacology,61(2011),192-198					

Materials and methods**Type of genotoxicity**

gene mutation

Type of study

bacterial reverse mutation assay (e.g. Ames test)

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 471 (Bacterial Reverse Mutation Assay)	
according to	JAPAN: Guidelines for Screening Mutagenicity Testing Of Chemicals	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Super Growth SWCNT

Method**Test concentrations**

12.5, 25, 50, 100 and 500µg/plate.

Vehicle

CNTs were suspended in the distilled water with 0.1% CMC-Na and the solution was used for the negative control and vehicle control.

Controls**Negative controls** no**Solvent / vehicle controls** yes**True negative controls** no**Positive controls** yes

Positive control substance other: -S9 mix; 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide(TA100, TA98, WP2 uvrA), sodium azide (TA1535) and 9-aminoacridine (TA1537). +S9 mix; 2-aminoanthracene (five strains)

Details on test system and conditions

S. typhimurium strains TA97, TA98, TA100, and TA1535 and E. coli mutant WP2 uvrA/pKM101 were cultured in a nutrient broth at 37 °C with shaking. SWCNTs were insoluble in the preparation vehicle for the bacterial reverse mutation test; therefore, 0.1% sodium carboxymethyl cellulose (CMC-Na) solution was applied for suspension in this test system. The maximum concentration of SWCNTs in 0.1% CMC-Na was 1 mg/mL without agglomeration. The study was conducted using duplicate plates for each dose

concentrations at 12.5, 25, 50, 100, 200 and 500 µg /plate. The Ames test was performed with a pre-incubation method in the presence and absence of metabolic activation, via S9 mix. S9 fraction, which was prepared from male Sprague–Dawley rats pretreated with the enzyme-inducing agents phenobarbital and 5,6-benzoflavone, was purchased from Kikkoman Corporation (Chiba, Japan) mixed with co-factor (Oriental Yeast Co., Ltd., Tokyo, Japan), give a final concentration of 10% (v/v) S9. Distilled water and 0.1% CMC-Na (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were used or the negative control and vehicle control, respectively. The positive controls used were 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide (AF-2; Wako Pure Chemical Industries, Ltd., Osaka, Japan), sodium azide (NaN₃; Wako Pure Chemical Industries, Ltd., Osaka, Japan), 9- aminoacridine hydrochloride (9AA; Aldrich Chemical Co. Inc., Wisconsin, USA), and 2-aminoanthracene (2AA; Wako Pure Chemical Industries, Ltd., Osaka, Japan). In the direct method (no metabolic activation), 0.5 mL of 100 mM sodium-phosphate buffer (pH 7.4) and 0.1 mL of each bacterial suspension were added to sterile tubes with distilled water (100 µL), 0.1% CMC-Na (100 and 500 µL), SWCNTs (100, 200, and 500 µL), or positive control (100 µL each). In the metabolic activation method, 0.5 mL of S9 mix and 0.1 mL of each bacterial suspension were added to sterile tubes with distilled water (100 µL), 0.1% CMC-Na (100 and 500 µL), SWCNTs (100, 200, and 500 µL), or positive controls (100 µL each). The mixtures were incubated at 37 °C with shaking (at a rate of 85 times per minute) for 20 min. Two milliliters of top agar, maintained at 45 °C, was then added. The mixtures were poured into plates and incubated at 37 °C for 48 h. The number of revertant colonies was counted using a colony counter.

Evaluation criteria

The test substance was considered to be mutagenic if either (a) a twofold or greater increase in the mean number of revertants per plate was observed compared with the mean number of revertants per plate in the appropriate negative control, in at least 1 of the tested strains, in the absence of cytotoxicity or (b) a dose-related increase in the mean number of revertants per plate, compared with that of the appropriate negative control, was observed at least 2-3 concentrations of the test substance and in at least 1 bacterial strain, in the absence of cytotoxicity.

Results and discussions

Test results

Species/strain	S. typhimurium TA 1535, TA 1537, TA 98, TA 100 and E. coli WP2
Metabolic activation	with and without
Test system	all strains/cell types tested
Genotoxicity	negative
Cytotoxicity	yes
Vehicle controls valid	yes
Positive controls valid	yes

Any other information on results incl. tables

In the preliminary cytotoxicity test, there was no growth inhibition at any concentration of SWCNTs, with or without metabolic activation. SWCNTs did not increase the mean number of revertants of any S. typhimurium strain or the E. coli strain per plate at any concentration, with or without metabolic activation, in comparison to the spontaneous reversion rate in the negative control. Precipitation of the SWCNTs was observed at concentrations between 50 and 500 µg/plate. There was no evidence of cytotoxicity under any of the test conditions. Positive controls induced increases in the number of revertant colonies, confirming the sensitivity of the test system. SWCNTs were not mutagenic in the bacterial system (See Table).

Overall remarks, attachments

Remarks on results including tables and figures

No mutation induction was observed under a metabolism activation existence and non-existence in all the tested concentration and in all the strain.

Though no strains showed the growth inhibition under a metabolism activation non-existence, it was observed under a metabolism activation existence for TA100 and *E. coli* at 100 µg/plate, for TA1535 and TA1537 at 50 and 100 µg/plate and for TA98 at 12.5 and 25 µg/plate.

Attached background material

Illustration (picture/graph)

Table 2

Results of bacterial reverse mutation test on SWCNT.

Dose (µg/plate)	S9 mix	No. of revertant colonies/plate (Mean)				
		Base substitution type			Flameshift type	
		TA100	TA1535	WP2 <i>uvrA</i> /pKM101	TA98	TA97
Distilled water	–	140	12	89	15	168
0.1% CMC-Na		123	8	95	19	155
0.1% CMC-Na ^a		154	9	105	13	156
12.5		130	7	97	20	156
25		134	9	103	21	167
50 ^a		132	12	74	20	159
100 ^a		123	9	88	13	150
200 ^a		124	9	93	14	147
500 ^a		114	6	92	17	168
AF-2 0.005				1131		
AF-2 0.01		612				
AF-2 0.1					402	
NaN ₃ 0.5			378			
9-AA 80						1527
Distilled water	+	125	7	145	18	197
0.1% CMC-Na		129	6	149	31	203
0.1% CMC-Na ^a		125	7	160	24	216
12.5		109	9	176	27	214
25		130	8	174	21	211
50 ^a		115	9	134	23	180
100 ^a		133	9	143	15	202
200 ^a		121	7	139	22	189
500 ^a		106	6	132	16	143
2AA 0.5					297	
2AA 1		717				
2AA 2			162	611		688

AF-2: 2-(2-furyl)-3-(5-nitro-2-furyl) acrylamide.

NaN₃: sodium azide.

9AA: 9-aminoacridine hydrochloride.

2AA: 2-aminoanthracene.

^a 500 µL of 0.1% sodium carboxymethyl cellulose (CMC-Na) were added.

^a Precipitation.

Applicant's summary and conclusion**Interpretation of results**

negative

Endpoint study record: Super Growth SWCNT Chromosomal Aberration Test; Genetic toxicity in vitro.002**Administrative Data**

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Super Growth SWCNT: In vitro Mammalian Chromosome Aberration Test	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) http://www.aist-riss.jp/main/modules/product/nano_rad.html	Institute of Environmental Toxicology (Ibaraki, Japan)	IET 07-0096			
publication	M. Naya et al.	2011	Evaluation of the genotoxic potential of single-walled carbon nanotubes by using a battery of in vitro and in vivo genotoxicity assays	Regulatory Toxicology and Pharmacology,61(2011),192-198					

Materials and methods**Type of genotoxicity**

chromosome aberration

Type of study

in vitro mammalian chromosome aberration test

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 473 (In vitro Mammalian Chromosome Aberration Test)	
according to	JAPAN: Guidelines for Screening Mutagenicity Testing Of Chemicals	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Super Growth SWCNT

Method**Test concentrations**

390, 500 and 1000µg/plate.

Vehicle

CNTs were suspended in the distilled water with 0.1% CMC-Na and the solution was used for the negative control.

Controls

Negative controls yes

Solvent / vehicle controls yes

True negative controls no

Positive controls yes

Positive control substance other: -S9:Benzo(a) pyrene +S9:Mitomycin C

Details on test system and conditions

The main study consisted of a short-term exposure (6h, with and without metabolic activation) and a continuous exposure (24h, with metabolic activation). 1%CMC-na was used as a negative control. The

positive control substance used were mitomycin and benzo(a)pyrene. In both exposure method, 1.5×10^5 cells were seeded on each tissue culture plastic plate (diameter 60 mm) and incubated at 37 °C in a volume of 5 mL culture medium for 2 days. In the short-term exposure method, 2.7 mL of culture medium with or without S9 mix was changed after 2 days, and 300 µL of SWCNT suspension was added. After 6-h exposure, the medium was discarded and the cells were washed with phosphate buffer. Fresh medium was then added and the cells were cultured for an additional 18 h. In the continuous exposure method, 4.5 mL of culture medium without S9 mix was changed after 2 days, and 500 µL of SWCNT suspension was added, and the cells were cultured for 24 h. In both the short-term and continuous exposure methods, colcemid (Wako Pure Chemical Industries, Ltd., Osaka, Japan; 0.2 µg/mL final concentration) was added 2 h before cell harvesting. The cells were then harvested, treated with 0.075 M KCl solution, fixed in fixative (methanol:acetic acid = 3:1), placed onto clean glass slides, and stained with 2% Giemsa solution (Merck Ltd., Germany). One hundred metaphases per slide (200 metaphases per dose) were examined blindly for structural aberrations and polyploidy.

Evaluation criteria

Pearson's chi-square test was used to determine significant differences between the negative control and the other group.

Results and discussions

Test results

Species/strain other: Chinese hamster CHL/IU

Metabolic activation with and without

Genotoxicity negative

Cytotoxicity no

Vehicle controls valid yes

Negative controls valid yes

Positive controls valid yes

Overall remarks, attachments

Remarks on results including tables and figures

In both short term tests (6 hours) under the metabolism activation existence and non-existence and long term tests (24 hours) under the metabolism activation non-existence, the expressions of structural chromosomal aberration and polyploidy were below 5%.

The numerical aberration was judged to be negative.

Illustration (picture/graph)**Table 4**

Results of chromosomal aberration test on SWCNT in Chinese hamster lung cell line.

Dose (µg/mL)	S9 mix	No. of cells	% Structure aberrations		% Polyploid cells	% Relative cell growth
			+gap	-gap		
0 ^a	-6 to 18 ^c	200	0	0	0.5	100
300 ^b		200	0.5	0.5	0	99
500 ^b		200	2.0	0.5	0.5	96
1000 ^b		200	1.5	1.0	0	94
MMC 0.1	+6 to 18 ^c	21.0	19.0 ^{**}	0	99	
0 ^a		200	0.5	0	1.0	100
300 ^b		200	4.0	2.5	0	100
500 ^b		200	2.0	0.5	0	97
1000 ^b		200	1.5	0	0	97
BaP 40	-24 to 0 ^c	46.5	43.5 ^{**}	0	82	
0 ^a		200	1.5	0.5	0	100
300 ^b		200	1.5	1.0	0	112
500 ^b		200	2.0	0.5	0.5	109
1000 ^b		200	4.0	2.0	0	93
MMC 0.1		200	38.0	37.0 ^{**}	0	118

MMC: Mitomycin C.

BaP: Benzo (a) pyrene.

^a 1% sodium carboxymethyl cellulose (CMC-Na).^b Precipitation.^c Exposure time - recovery time (h).^{**} Significantly different from negative control at $p < 0.01$ (Pearson's chi-squared test).**Applicant's summary and conclusion****Interpretation of results**

negative

Conclusions

The expressions of structural chromosomal aberration and polyploidy were not increased.

Endpoint study record: CNI SWCNT Ames test.Genetic toxicity in vitro.001**Administrative Data****Purpose flag** key study**Study result type** experimental result**Reliability** 2 (reliable with restrictions)**Rationale for reliability incl. deficiencies** Meets generally accepted scientific standards, well documented and acceptable for assessment**Data source****Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Kisin et al.	2007	Single-walled Carbon Nanotubes: Genotoxic and Cytotoxic Effects in Lung Fibroblast V79 Cells	Journal of Toxicology and Environmental Health, Part A, 70: 2071–2079, 2007					

Materials and methods**Type of genotoxicity**

gene mutation

Type of study

bacterial reverse mutation assay (e.g. Ames test)

Test materials**Test material identity**

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNT (CNI, Inc., Houston, TX) produced by the highpressure CO disproportionation process (HiPco) technique, employing CO in a continuous-flow gas phase as the carbon feedstock and Fe(CO)₅ as the iron-containing catalyst precursor (Bronikowski et al., 2001), and purified by acid treatment to remove metal contaminants (Gorelik et al., 2000), were used. The SWCNT were comprised of 99.7 wt% elemental carbon, and iron levels were approximately 2.3 mg Fe/g sample (0.23 wt%). The nanotube diameters in the samples ranged from 0.4 to 1.2 nm. The length of the individual SWCNT is approximately 1–3 µm. The surface area of SWCNT was 1040 m²/g.

Method

Species/strain

Species/strain *S. typhimurium*, other: YG1029, YG1024

Test concentrations

0,60,120,240 µg/plate

Controls

Solvent / vehicle controls yes (SWCNT 0 µg/plate)

Positive controls yes

Positive control substance other: 1-Nitropyrene

Details on test system and conditions

The microsuspension preincubation method was used, with *Salmonella typhimurium* strains YG1024 and YG1029 obtained from Dr. Takehiko Nohmi at the National Institute of Hygiene Sciences, Tokyo. Tester strains YG1024 and YG1029 were used to detect frame-shift and base-pair substitution mutations. Assays were done without S9 mix. 1-Nitropyrene (1-NP) was used as a positive control. Oxoid number 2 nutrient media (50 ml) containing 0.2 ml ampicillin solution (6.25 mg/ml), 0.2 ml tetracycline solution (1.56 mg/ml), and 0.1 ml thawed tester strains (YG1024 or YG1029) was added into each of two 125-ml culture flasks. The cultures were placed in a 37°C gyrating incubator and shaken at approximately 120 rpm for 16 h. The overnight cultures were then transferred to 50 ml centrifuge tubes and centrifuged at 3000 rpm for 20 min at 4°C. The supernatant liquid was discarded, and the cultures were resuspended in physiological saline to a concentration of approximately 4×10^9 cells/ml. SWCNT samples (0, 60, 120, or 240 µg/plate) or 1-NP (0.1 µg/plate), along with 65 µl physiological saline and 25 µl of the overnight cultures, were preincubated at 37°C for 30 min on a rotary shaker. Following preincubation, 2.5 ml molten top agar (at 45°C) was added to each sample tube; the contents were mixed, immediately poured onto a Vogel–Bonner minimal glucose plate, and incubated at 37°C for 48 h (YG1029) or 66 h (YG1024). Three readings of revertant colonies on each plate were scored by an automatic colony counter (Accucount 1000, Biologics, Inc., Gainesville, VA), and the average of the readings was recorded. The average number of revertant colonies and standard deviation per plate for at least two plates were calculated. Each sample was tested twice in separate experiments. The background bacterial lawn was checked regularly by light microscopy.

Results and discussions

Test results

Species/strain *S. typhimurium*, other: YG1029, YG1024

Metabolic activation without

Genotoxicity negative

Cytotoxicity yes

Positive controls valid yes

Any other information on results incl. tables

Samples were considered positive in this assay if there was a positive concentration-response relationship, and at least one concentration of sample yielded a result that was twice the reversion rate for the

respective negative control (Ames et al., 1975).

No increases in mutation frequencies in either YG1024 or YG1029 were found at all concentrations of SWCNT employed in the present study. The reproducibility of the test results was confirmed. The positive control used in the assay showed clear positive responses by the respective test strains, as evidenced by the number of revertant colonies being at least two-fold greater than the respective control value.

Table: Revertants in Two Strains of *Salmonella Typhimurium* Treated with Different Concentrations of SWCNT in the Absence of a Metabolic-Activating Enzyme (S9)

SWCNT, $\mu\text{g}/\text{plate}$ 1-NP, $\mu\text{g}/\text{plate}$

Salmonella typhimurium strains 0 60 120 240 0.1

YG1029 85.0 ± 1.8 92.8 ± 2.8 90.8 ± 1.0 82 ± 1.3 518 ± 66

YG1024 34.8 ± 0.6 27.8 ± 1.1 22.3 ± 0.3 20.8 ± 0.6 932 ± 14

SWCNT samples (0, 60, 120 or 240 $\mu\text{g}/\text{plate}$) or 1-NP (0.1 $\mu\text{g}/\text{plate}$), as a positive control, were incubated at 37°C with two *Salmonella typhimurium* strains: YG1029 (48 hr) or YG1024 (66 hr). Data represent mean values ($\pm\text{SEM}$) of the average number of revertant colonies per sample. Each sample was tested twice in two separate experiments.

Overall remarks, attachments

Illustration (picture/graph)

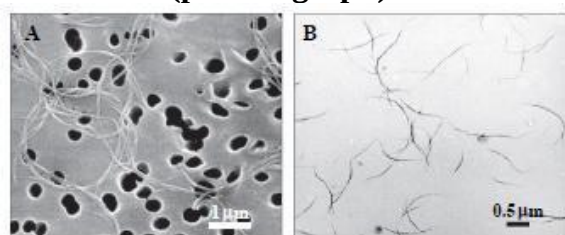


FIG. 2. (A) Scanning electron microscopy and (B) transmission electron microscopy images of dispersed SWCNT.

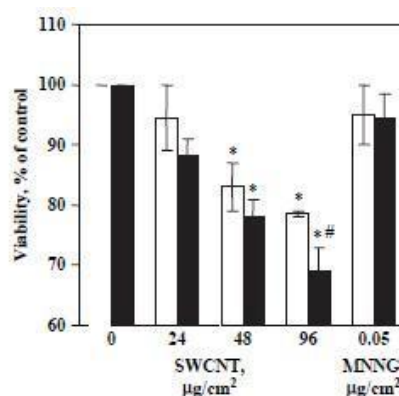


FIG. 3. Viability of V79 cells after 3 or 24 h of exposure with SWCNT: □, 3-h exposure with SWCNT; ■, 24-h exposure with SWCNT. Data are means $\pm$ SEM ($n=3$ experiments); asterisk indicates significant difference at $p < .05$ versus control cells; #, $p < .05$ versus V79 cells treated with SWCNT for 3 h. *N*-Methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) was used as a positive control for DNA damage.

TABLE 1
Revertants in Two Strains of *Salmonella Typhimurium* Treated with Different Concentrations of SWCNT in the Absence of a Metabolic-Activating Enzyme (S9)

	SWCNT, $\mu\text{g}/\text{plate}$				1-NP, $\mu\text{g}/\text{plate}$
<i>Salmonella typhimurium</i> strains	0	60	120	240	0.1
YG1029	85.0 ± 1.8	92.8 ± 2.8	90.8 ± 1.0	82 ± 1.3	518 ± 66
YG1024	34.8 ± 0.6	27.8 ± 1.1	22.3 ± 0.3	20.8 ± 0.6	932 ± 14

SWCNT samples (0, 60, 120 or 240 $\mu\text{g}/\text{plate}$) or 1-NP (0.1 $\mu\text{g}/\text{plate}$), as a positive control, were incubated at 37°C with two *Salmonella typhimurium* strains: YG1029 (48 hr) or YG1024 (66 hr). Data represent mean values ($\pm\text{SEM}$) of the average number of revertant colonies per sample. Each sample was tested twice in two separate experiments.

Applicant's summary and conclusion**Conclusions**

A reverse gene mutation assay for CNI(HiPco) SWCNT was negative in *S. typhimurium* YG1024 and YG1029 without S9 mix at 0-240 µg/plate.

Endpoint study record: CNI SWCNT Chromosomal aberration test.Genetic toxicity in vitro.002

Administrative Data

Purpose flag supporting study
Study result type experimental result
Reliability 2 (reliable with restrictions)
Rationale for reliability incl. deficiencies Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Kisin et al.	2007	Single-walled Carbon Nanotubes: Geno- and Cytotoxic Effects in Lung Fibroblast V79 Cells	Journal of Toxicology and Environmental Health, Part A, 70: 2071–2079, 2007					

Cross-reference to same study

7.6.1 CNI SWCNT AMES test, 7.6.1 CNI Comet assay

Materials and methods**Type of genotoxicity**

chromosome aberration

Type of study

in vitro mammalian chromosome aberration test

Test materials**Test material identity**

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNT (CNI, Inc., Houston, TX) produced by the highpressure CO disproportionation process (HiPco) technique, employing CO in a continuous-flow gas phase as the carbon feedstock and Fe(CO)₅ as the iron-containing catalyst precursor (Bronikowski et al., 2001), and purified by acid treatment to remove metal contaminants (Gorelik et al., 2000), were used. The SWCNT were comprised of 99.7 wt% elemental carbon, and iron levels were approximately 2.3 mg Fe/g sample (0.23 wt%). The nanotube diameters in the samples ranged from 0.4 to 1.2 nm. The length of the individual SWCNT is approximately 1–3 µm. The surface area of SWCNT was 1040 m²/g.

Method

Species/strain

Species/strain Chinese hamster lung fibroblasts (V79)

Details on mammalian cell lines (if applicable) Chinese hamster lung fibroblast (V79) cells were seeded in MEM with Earle's salts and L-glutamine and supplemented with pen-strep antibiotics (2%) and 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Test concentrations

0, 12, 24, 48, or 96 µg/cm²

Controls

Positive control substance other: N-methyl-N-nitro-N-nitrosoguanidine

Details on test system and conditions

Cells were seeded into 25-cm² flasks (1.0×10⁶/flask) with 5 ml complete medium (MEM medium supplemented with 10% FBS and 2% pen-strep) and cultured overnight. The medium was removed and replaced with suspensions of SWCNT in medium at concentrations of 12, 24, 48, or 96 µg/cm² for 24 h. Cells were rinsed with phosphate buffered saline (PBS), and incubated in 5 ml of complete medium for 24 h. Trypsinization was used for harvesting cells. The cell suspensions were centrifuged (10 min at 300×g), the supernatant was discarded, and 5 ml of 0.075 M KCl hypotonic solution at 37°C was added to the cells for 5 min. The hypotonic treatment was terminated by adding 5 ml of freshly prepared fixative solution (methanol: glacial acetic acid, 3:1), and the cells were centrifuged (10 min at 300×g). After removal of the supernatant, 5 ml of fixative solution was added to the cells at 37°C for 20 min, followed by centrifugation. This fixation step was repeated one more time. The cells were resuspended in 1–2 ml of fixative solution, and the suspensions were dropped on cold, clean microscope slides. Cells were stained with Diff Quik solution (Dade-Behring, Miami, FL). The slides were coded, and 3000 cells from each treatment were scored for micronucleus formation. All of the experiments were repeated five times. A standard positive control was used in each MN test: MNNG, N-methyl-N-nitro-N-nitrosoguanidine.

Results and discussions

Test results

Species/strain Chinese hamster lung fibroblasts (V79)

Genotoxicity other: limited but not statistically significant micronucleus induction

Cytotoxicity yes

Additional information on results

Micronuclei are a result of chromosomal breakage and/or mitotic spindle damage evaluated in interphase cells. The Chinese hamster lung fibroblast (V79) cells were exposed to SWCNT (0, 12, 24, 48, or 96 $\mu\text{g}/\text{cm}^2$) for 24 h. To allow damaged cell to form micronuclei in the next interphase, cells were removed from the SWCNT containing medium and grown for additional 24 h. The results of five replicate assays indicate limited but not statistically significant micronucleus induction by SWCNT in V79 cell line at the highest concentrations tested (Figure 4A). Figure 4B presents the V79 cell with micronucleus after challenge with SWCNT (96 $\mu\text{g}/\text{cm}^2$).

Overall remarks, attachments

Illustration (picture/graph)

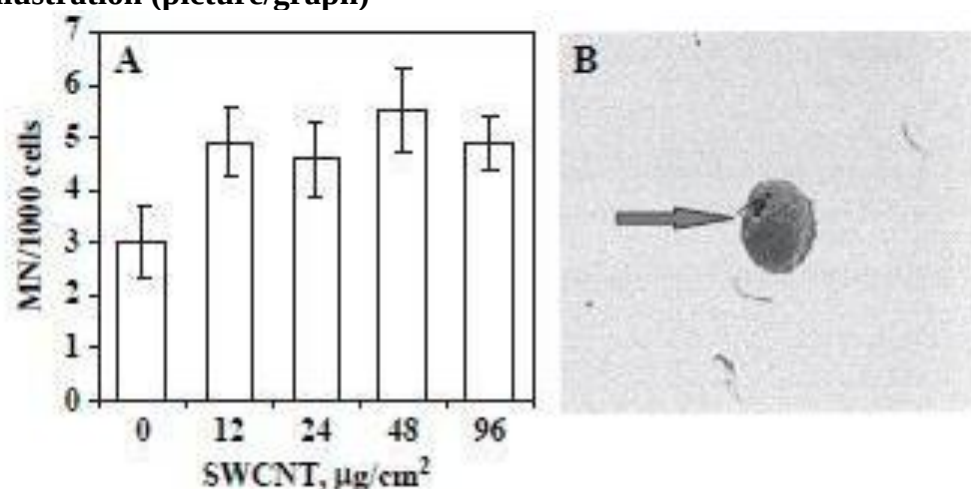


FIG. 4. (A) Micronucleated cell induction in 1000 scored cells after challenge with SWCNT in vitro. (B) V79 cell with micronucleus (arrow) after challenge with SWCNT, 1000 $\times$.

Applicant's summary and conclusion

Conclusions

No indications of chromosomal breakage and/or mitotic spindle damage were found in a in vitro mammalian cell micronucleus test for CNI(HiPco)SWCNT.

Endpoint study record: CNI SWCNT Comet assay.Genetic toxicity in vitro.003

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Kisin et al.	2007	Single-walled Carbon Nanotubes: Genotoxic and Cytotoxic Effects in Lung Fibroblast V79 Cells	Journal of Toxicology and Environmental Health, Part A, 70: 2071–2079, 2007					

Cross-reference to same study

7.6.1 CNI SWCNT MN test, 7.6.1 CNI SWCNT AMES test

Materials and methods**Type of genotoxicity**

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test materials**Test material identity**

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNT (CNI, Inc., Houston, TX) produced by the highpressure CO disproportionation process (HiPco) technique, employing CO in a continuous-flow gas phase as the carbon feedstock and Fe(CO)₅ as the iron-containing catalyst precursor (Bronikowski et al., 2001), and purified by acid treatment to remove metal contaminants (Gorelik et al., 2000), were used. The SWCNT were comprised of 99.7 wt% elemental carbon, and iron levels were approximately 2.3 mg Fe/g sample (0.23 wt%). The nanotube diameters in the samples ranged from 0.4 to 1.2 nm. The length of the individual SWCNT is approximately 1–3 µm. The surface area of SWCNT was 1040 m²/g.

Method**Species/strain****Species/strain** Chinese hamster lung fibroblasts (V79)

Details on mammalian cell lines (if applicable) Chinese hamster lung fibroblast (V79) cells were seeded in MEM with Earle's salts and L-glutamine and supplemented with pen-strep antibiotics (2%) and 10% fetal bovine serum. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Test concentrations

0,12, 24, 48, or 96 µg/cm²

Controls

Positive control substance other: N-methyl-N-nitro-N-nitrosoguanidine

Details on test system and conditions

V79 cells were seeded into 25-cm² flasks (2.0×10⁶/flask) with 5 ml MEM supplemented with 10% FBS and 2% pen-strep antibiotics. After cells were attached, medium was removed and replaced with suspensions of SWCNT in medium at concentrations of 0, 24, 48 or 96 µg/cm² or N-methyl-N-nitro-N-nitrosoguanidine(MNNG) as a positive control. After 3 or 24h of exposure, cells were washed with Mg²⁺- and Ca²⁺-freebalanced salt solution (Hanks balanced salt solution, HBSS). A cell scraper was used to remove cells from the flask. Each sample was adjusted to a concentration of 1×10⁶ cells/ml, frozen in liquid nitrogen, and stored at -70 ± 10°C until shipping to HELIX3, Inc. (Morrisville, NC), for comet assay analysis(Ostling And Johanson, 1984; Collins, 2001). In total, 50 randomly captured cells from each sample/slide were examined. All of the experiments were repeated three times.

Results and discussions**Test results**

Species/strain Chinese hamster lung fibroblasts (V79)

Genotoxicity positive (The induction of DNA damage after only 3 h of incubation with 96 mg/cm² of SWCNT)

Cytotoxicity yes

Additional information on results

The positive group (cells treated with MNNG) showed an increased level of DNA damage in all the parameters at both times of exposure. A short-term SWCNT treatment for 3 h led to significant DNA damage (3.1-,1.6-, and 4.2-fold increase of DNA migration, tail length, andolive tail moment, respectively, in comparison with vehicle-treated cells) only at the highest SWCNT dose of 96 µg/cm². More prolonged treatment with SWCNT for 24 h increased all parameters of DNA damage in a concentration-dependent way. A 24-h exposure to 48 µg/cm² of SWCNT significantly increased the level of migrated DNA, tail length and olive tail moment by 2.25-, 1.76-, and 2.8-fold, respectively, while treatment with 96 µg/cm² SWCNT produced elevation in these parameters by 2.5-, 1.94-, and 3.4-fold, respectively.

Overall remarks, attachments

Illustration (picture/graph)

DNA Damage in the Chinese Hamster Lung Fibroblast V79 Cells Treated with SWCNT

Treatment groups			Comet assay parameters		
Compounds	Concentration, mg/cm ²	Exposure Time, h	% Migrated DNA	Tail Length, microns	Olive Tail Moment
SWCNT	0	3	25.8 ± 2.1	53.5 ± 1.84	7.6 ± 0.9
	24		33.0 ± 2.6	51.0 ± 2.3	10.2 ± 1.1
	48		28.6 ± 2.1	55.9 ± 1.9	8.4 ± 0.8
	96		79.5 ± 2.1*	84.9 ± 2.5*	32.0 ± 1.3*
MNNG	0.05	24	73.6 ± 2.5*	97.2 ± 3.4*	34.5 ± 1.8*
SWCNT	0		23.1 ± 2.8	37.6 ± 2.5	7.0 ± 1.1
	24		28.3 ± 2.8	48.1 ± 2.7	9.2 ± 1.3
	48		51.9 ± 3.2*	66.3 ± 2.8*	19.7 ± 1.6*
	96		58.1 ± 3.5*	72.9 ± 3.2*	23.7 ± 1.8*
MNNG	0.05		30.2 ± 3.1*	47.2 ± 2.9*	10.5 ± 1.4*

MNNG was used as a positive control. Data represent mean values (+SEM) of % Migrated DNA, Olive Tail moment and Tail Length of the alkaline comet assay based on 50 cells scored per sample (results of one experiment showed as representation of three experiments). * $p < 0.05$ vs control group (cells treated with vehicle).

Applicant's summary and conclusion**Conclusions**

CNI(HiPco) SWCNT treatment led to DNA damage in CHL(V9) cells in a comet assay.

Endpoint study record: *EliCarb SWCNT DNAdamage and/or repair test.Genetic toxicity in vitro.001*

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment
Data source	

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Jacobsen et al.	2008	Genotoxicity, Cytotoxicity, and Reactive Oxygen Species Induced by Single-Walled Carbon Nanotubes and C60 Fullerenes in the FEI-Muta TM Mouse Lung Epithelial Cells	Environmental and Molecular Mutagenesis 49:476-487 (2008)					

Materials and methods

Type of genotoxicity

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test materials

Test material identity

Identifier	Identity
other:	EliCarb SWCNT

Details on test material

The EliCarb SWCNT was purchased as a dry powder from Thomas Swan (Consett, UK). They declared a primary particle cross-sectional diameter was 0.9-1.7 nm and an average length was <1 µm. Particle characteristics; BET surface area (m²/g) 731±2, Average pore size (nm) 15, Chemical composition; 95% C, 2% Fe, <0.001% Co, Ni, Mn

Method

Species/strain

Species/strain mammalian cell line, other: FEI-MML epithelial cell line

Details on mammalian cell lines (if applicable) The development and characterization of the FEI-MML epithelial cell line established from the transgenic MutaTMMouse [Myhr, 1991] have been described previously [White et al., 2003]. The cells were cultured in DMEM Fl2 (1:1) medium (Invitrogen, Taastrup, Denmark) supplemented with 2% (v/v) heat inactivated fetal bovine serum (Gibco, Invitrogen, Taastrup, Denmark), 100 U/ml Penicillin G, 100 flg/ml streptomycin (Gibco, Invitrogen), 2 mM L-glutamine (Merck, Sigma-Aldrich, Brøndby, Denmark), and 1 ng/ml murine epidermal growth factor (Roche, Hvidovre, Denmark).

Test concentrations

100 µg/ml or 15 µg/cm²

Vehicle

Approximately 11 mg of dry autoclaved SWCNT was sonicated for three rounds of 8 min in a 10-ml glass beaker on an ice-bath after the addition of 2 ml medium before each of three sonication steps. The sonicator (Branson Sonifier S-4500, Branson Ultrasonics, Danbury, CT) was equipped with a disruptor horn (model no.: 101-147-037) and operated under the following settings: each sonication round was 8 min, 10% amplitude, and alternating pulses of 10 sec on and off. The sonicator was on for a total of 12 min. The sonicated suspensions were diluted to a final concentration of 100 µg/ml and were added to the cells immediately thereafter. Suspensions were prepared freshly before every exposure.

Controls

Positive controls yes

Positive control substance other: Photosensitizer Ro19-8022

Results and discussions

Test results

Species/strain mammalian cell line, other: FEI-MML epithelial cell line

Genotoxicity positive

Cytotoxicity no (did not detect increased cell death)

Any other information on results incl. tables

Cell Viability and Proliferation; The viability of SWCNT-treated FEI-MML cells was assessed following 24hr of incubation at seven concentrations ranging from 0 to 200 µg/ml. There were, on average, 45% fewer cells (2.7million cells harvested cells) compared with controls (4.9 millioncells).

Butthe SWCNT effect on cell proliferation seems to be reversible.

Comet Assay; SWCNT induced strand breaks. However, resulted inoxidation of purines as detected by generation of FPG sensitive sites.

Overall remarks, attachments

Remarks on results including tables and figures

SWCNT were unable to increase~~cell~~ mutantfrequency or the level of strand breaks, but did increasethe level of FPG labile sites by 1.56-fold compared withcontrols.

Illustration (picture/graph)

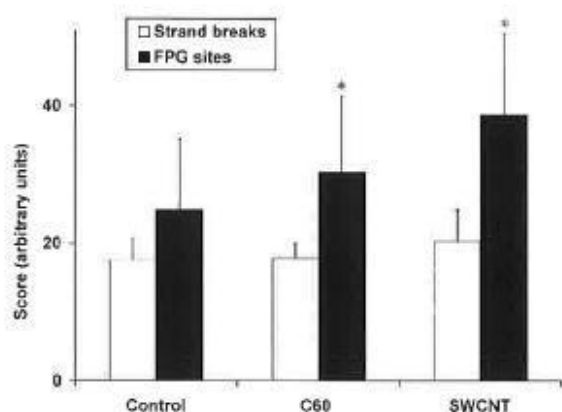


Fig. 5. DNA strand breaks and FPG-sensitive sites in FE1 MutaTM Mouse lung epithelial cells following 3 hr of incubation with either 100 µg C₆₀ or SWCNT /ml. Values are shown as mean ± SEM for 20 experiments conducted over 4 days. *N* = 4. (*) indicates statistical significance using paired t-tests and Bonferonni adjusted significance *P* < 0.025. The level of DNA damage in positive control samples (Ro19-8022) were 11.5 ± 1.8 and 178.5 ± 16.9 arbitrary units (mean ± SEM) for the strand breaks and FPG-sensitive sites, respectively.

TABLE III. The Effect of C₆₀ or SWCNT Exposure on the *cH* Mutant Frequency in FE1 MutaTM Mouse cells

		<i>cH</i>		
	Sample	Pfu screened	MF × 10 ⁻⁵	Avg. MF ± SD
Positive control	1	486,000	151.2	151.2
	Negative control	1	1,477,500	17.7
SWCNT	2	1,702,500	14.5	14.2 ± 2.8
	3	1,075,000	13.8	
	4	2,100,000	11.8	
	5	1,232,500	10.2	
	6	1,500,000	11.5	
	7	2,025,000	14.5	
	8	1,581,250	14.0	
	9	1,335,000	18.5	
	10	1,050,000	13.1	
	11	1,029,375	19.2	
	12	970,000	12.0	
	13	671,250	13.9	
	1	1,048,750	15.8	13.5 ± 4.0 <i>P</i> = 0.64
	2	2,297,500	12.8	
	3	1,737,500	13.2	
	4	1,180,000	20.2	
	5	1,955,000	14.6	
	6	1,056,250	8.5	
	7	1,456,250	13.5	
	8	1,247,500	8.1	
	9	1,503,750	18.7	
	10	1,222,500	9.9	

MF: mutant frequency; Pfu: total number of plaque-forming units screened.

Cells for positive control were exposed to benzo[*a*]pyrene as described in the "Methods and Materials" section. Values shown are individual mutant frequencies as well as mean ± SD.

Applicant's summary and conclusion

Conclusions

SWCNT did not increase the level of strand breaks, but significantly increased the level of FPG sensitive sites/oxidized purines determined by the comet assay.

Endpoint study record: Arc method SWCNT Chromosomal aberration;Genetic toxicity in vitro.001

Administrative Data

Purpose flag

supporting study

Study result type

experimental result

Reliability

2 (reliable with restrictions)

Rationale for reliability incl. deficiencies

Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Cveticanin et al.	2010	Using carbon nanotubes to induce micronuclei and double strand breaks of the DNA in human cells	Nanotechnology 21 (2010) 015102					

Cross-reference to same study

7.6.1 Genetic toxicity in vitro; Arc method SWCNT DNA damage test; Genetic toxicity 002

Materials and methods**Type of genotoxicity**

chromosome aberration

Type of study

in vitro mammalian cell micronucleus test

Test materials**Test material identity**

Identifier	Identity
other: unknown identification	

Details on test material

SWCNTs produced by the arc method were used with purification. The purity of SWCNTs was about 70%. Functionalized SWCNTs were obtained by oxidation of SWCNTs and functionalization with amides.

Method**Species/strain**

Species/strain lymphocytes: human lymphocytes

Test concentrations

25,50,100,150µl/ml

Vehicle

Into 4.5 ml of PBmax-karyotyping medium (Invitrogen-Gibco, Paisley, UK) 0.5 ml of whole blood was added. SWCNTs and amide functionalized purified SWCNTs) were suspended in dimethylsulfoxide (DMSO) and ultrasonically dispersed for 30 min. 1 h after the stimulation of cells, cultures were treated

with four different doses of carbon nanotubes (25-150 µl per 5 ml of total cell culture volume).

Any other information on materials and methods incl. tables

For micronuclei preparation, the cytokinesis block method was followed with some modifications

Cytochalasin B (SigmaAldrich) at a final concentration of 4 µg/ml was added to the samples after 44h of culture and the lymphocyte cultures were incubated for a further 24h. After culture initiation, cells were washed with 0.9% NaCl, collected by centrifugation and treated with hypotonic solution at 37°C. Hypotonic solution consisted of 0.56% KCl + 0.9% NaCl (mixed in equal volumes) and 0.1 ml of trypsin (final concentration 0.001 mg/ml). Cell suspensions were prefixed in a 3:1 methanol/acetic acid ratio, washed three times with fixative and placed onto a clean slide. Slides were air-dried and stained with 2% alkaline Giemsa (Sigma-Aldrich). Slide scoring: at least 1000 binuclear (BN) cells per sample were scored. All slides were analysed with an Aximager-1 microscope (Zeiss, Germany) using magnification 400x or 1000x when necessary. A minimum of 1000 binucleated cells were scored to evaluate the percentage of cells with one, two, three, four, or more than four micronuclei. A cytokinesis block proliferation index (CBPI) was calculated as follows: $CBPI = \frac{MI + 2MII + 3(MIII + MIV)}{N}$, where MI-MIV represents the number of cells with one to four nuclei, respectively, and N is the number of cells scored.

Results and discussions

Additional information on results

The results of micronucleus analysis (mean values for micronucleus yield, mean values of CBPI as well as for mono-, bi-, and polynucleated cells, and binucleated cells with anaphase bridges) in samples treated with different doses of SWCNT suspension, functionalized SWCNT suspension and MWCNT suspension are presented in figure 2. Treatment of human lymphocytes with SWCNTs (doses of 25 µL/ml, and 50 µL/ml, respectively) enhance the incidence of micronuclei (MN) by 45.58% compared to the control ($t = 23.4273$, $p = 0.0018$) and decrease the proliferation potential (CBPI) of cells. The statistical analysis demonstrated statistically significant inverse correlation between MN and CBPI ($r = -0.9976$, $p = 0.0438$). In human fibroblasts the same dose of SWCNTs markedly increased the number of DSBs ($t = 9.5547$, $p = 0.0108$). Functionalized SWCNTs induce micronuclei formation in a dose-dependent manner, statistically significant at all employed concentrations ($t = 238.24$, $p < 0.0001$), which is not accompanied by alterations in cell proliferation potential.

Overall remarks, attachments

Remarks on results including tables and figures

SWCNTs induce micronuclei (MN) formation in lymphocytes and decrease the proliferation potential (CBPI) of cells.

Amide functionalized purified SWCNTs behave differently: they do not disturb the cell proliferation potential of harvested lymphocytes, but induce micronuclei to a higher extent than SWCNTs.

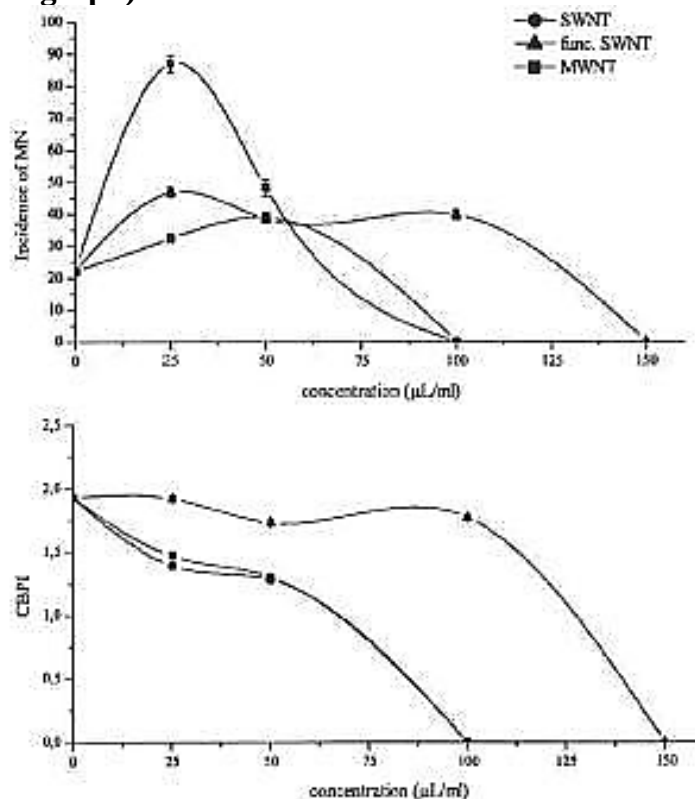
Illustration (picture/graph)

Figure 2. The incidence of micronuclei (MN) and proliferation potential (CBPI) of harvested lymphocytes treated with different doses of SWCNTs, functionalized SWCNTs, and MWCNT.

Applicant's summary and conclusion**Conclusions**

SWCNTs induce micronuclei (MN) formation in lymphocytes and decrease the proliferation potential (CBPI) of cells. In a fibroblast cell line the same dose of SWCNTs induces γH2AX foci 2.7-fold higher than in a control.

Endpoint study record: Arc method SWCNT DNA damage test; Genetic toxicity in vitro.002

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Cveticanin et al.	2010	Using carbon nanotubes to induce micronuclei and double strand breaks of the DNA in human cells	Nanotechnology 21 (2010) 015102					

Cross-reference to same study

7.6.1 Genetic toxicity in vitro; Arc method SWCNT Chromosomal aberration; Genetic toxicity 001

Materials and methods**Type of genotoxicity**

DNA damage and/or repair

Type of study

other: Double strand breaks(DSB) assay

Test materials**Test material identity**

Identifier	Identity
other: unknown identification	

Details on test material

SWCNTs produced by the arc method were used with purification. The purity of SWCNTs was about 70%. Functionalized SWCNTs were obtained by oxidation of SWCNTs and functionalization with amides.

Method**Species/strain**

Species/strain mammalian cell line, other: normal human dermal fibroblasts HDMEC(PromoCell)

Test concentrations

25,50,100,150µl/ml

Vehicle

Into 4.5 ml of PBmax-karyotyping medium (Invitrogen-Gibco, Paisley, UK) 0.5 ml of whole blood was added. SWCNTs and amide functionalized purified SWCNTs) were suspended in dimethylsulfoxide

(DMSO) and ultrasonically dispersed for 30 min. 1 h after the stimulation of cells, cultures were treated with four different doses of carbon nanotubes (25-150 µl per 5 ml of total cell culture volume).

Any other information on materials and methods incl. tables

For the human fibroblast cell culture, normal humandermal fibroblasts HDMEC (PromoCell) were grown under standard tissue culture conditions in DMEM supplemented with 10% offetal bovine serum at 37°C and 10% of C02. Exponentially growing cells were seeded on polylysine glass-slides (SigmaAldrich) and were further harvested after 24h. After that time cells were treated with four different doses of carbon nanotubes (0.5-30µl /ml). The repair kinetics of induced DSBs was measured after 24h.

Cells were fixed with 2% paraformaldehyde, permeabilized with 0.2% triton X-100 and stained with aphosphospecific primary antibody and an FITC-labeled secondary antibody. Green shows γ-H2AX foci, red nucleistained with PI. Foci were counted using an epifluorescent microscope AxiomagerAI (Zeiss) and the program ImageJ.

Results and discussions

Any other information on results incl. tables

SWCNTs induce micronuclei formation in harvested human lymphocytes (50% above the control value) which is followed with a significant decline of cell proliferation potential (CBPI is 30% lowered when compared with the corresponding control).

When applied on monolayer fibroblast cells SWCNTs induce DSBs in a dose-dependent manner. The concentrations of SWCNTs, which enhance micronuclei incidence up to 50% above the control, induce a 2.7-fold higher incidence of γH2A foci when compared to the control.

Since the incidence of induced γH2AX foci is much higher than the incidence of micronuclei, it appears that a portion of induced DSBs are successfully repaired. Moreover, observed apoptotic bodies in fibroblasts a monolayerof cell cultures treated with SWCNTs indicate that a portion of cells in the G1/S phase of the cell cycle die via apoptosis and, consequently, they cannot reach the second division and display micronuclei. SWCNTs also induce micronuclei formation and anaphase bridges among nuclei, which is a consequence of the mis repair of multiple inducedDSBs.

Overall remarks, attachments

Remarks on results including tables and figures

In a fibroblast cell line the same dose ofSWCNTs induces γH2AX foci2.7-fold higher than in a control. Amide functionalized SWCNTsalso induce γH2AX foci,3.18-fold higher than the control.

Endpoint study record: Sigma-Aldrich CNT Chromosomal aberration; Genetic toxicity in vitro.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Lindberg et al.	2009	Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells in vitro	Toxicology Letters 186 (2009) 166–173					

Cross-reference to same study

Sigma-Aldrich CNT damage test ;Genetic toxicity 002

Materials and methods**Type of genotoxicity**

chromosome aberration

Type of study

in vitro mammalian cell micronucleus test

Test materials**Test material identity**

Identifier	Identity
other: Sigma-Aldrich	

Details on test material

CNTs; >50% single-walled; ~40% other nanotubes; 1.1 nm×0.5–100µm; manufactured by chemical vapour deposition; Sigma–Aldrich product no. 636797, catalyst metals; <5 wt.% Co and Mo, Specific surface; 567m²/g

Method**Species/strain**

Species/strain mammalian cell line, other: BEAS 2B

Details on mammalian cell lines (if applicable) BEAS 2B, a transformed human bronchial epithelial cell line exhibiting an epithelial phenotype (Reddel et al., 1988) was obtained from American Type Culture Collection through LGC Promochem AB (Borås, Sweden). The BEAS 2B cells were grown in serum-free BEGM medium (Clonetics, Walkersville, MD, USA) at 37 °C in a humidified atmosphere of 5% CO₂. Log-phase BEAS 2B cells were plated on 24-well plates (cell viability and comet assays), and 4-well chamber slides (micronucleus assay) (Nunc, Roskilde, Denmark) 2 days prior to exposure.

Test concentrations

3.6, 18, 36, 72, 144, 216, 288, and 360 µg/ml

Vehicle

The materials were dispersed in BEGM cell culture medium (Clonetics, Walkersville, MD) and subjected to ultrasonication (Elmasonic, Singen, Germany) for 20 min at 37 kHz prior to addition to the cell cultures. Measurements from light microscopic images indicated that the average size of the agglomerates in dispersion was 8.5 µm (S.D. 11.6). Transmission electron microscopy verified that the BEGM dispersions also contained nano-scale (<100 nm) material in addition to the m-scale agglomerates seen in the optical microscope.

Controls

Solvent / vehicle controls yes

Positive controls yes

Positive control substance mitomycin C Sigma–Aldrich, Steinheim, Germany; 500 ng/ml

Details on test system and conditions

The micronucleus assay was applied to study chromosomal damage in BEAS 2B cells after exposure to the carbon nanomaterials. The cells were plated on 4-well chamber slides (culture area 1.8 cm²/well, culture medium volume 0.5 ml/well; Nalge Nunc International, Rochester, USA) at a density of 20,000 cells per well and incubated for 48 h prior to the treatment. Based on the viability assay, the cells were exposed to eight doses of each nanomaterial type: 1, 5, 10, 20, 40, 60, 80, and 100 µg/cm² (in µg/ml culture medium, the corresponding doses were: 3.6, 18, 36, 72, 144, 216, 288, and 360 µg/ml). Treatment time was 24 h, 48 h or 72 h. Cytochalasin B (9 µg/ml; Sigma–Aldrich Chemie, Steinheim, Germany) was added to the cell cultures simultaneously with the particles to induce binucleation of dividing cells. Untreated controls and positive controls treated with mitomycin C (Sigma–Aldrich, Steinheim, Germany; 500 ng/ml) were also included for the 24-h and 48-h time points. After the end of the exposure, the medium and chambers were removed from the slides, and the cells were briefly rinsed in phosphate-buffered saline (PBS). The cells were stained with ethidium bromide (10 µg/ml) and acridine orange (3 µg/ml) for 5 min after which the slides were rinsed twice in PBS and fixed in absolute methanol for 5 min. Finally, the DNA of the cells was stained with 4,6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml) for 5 min, after which the slides were rinsed in tap water and allowed to dry. The stained and fixed slides were kept at 4 °C and were protected from light until analysis. The slides were coded and the frequency of micronucleated cells in 2000 binucleate cells (1000 cells/scorer) per culture were scored by two microscopists using an Axioplan 2E Universal microscope (Zeiss, Jena, Germany). Binucleate cells were identified using a 63× objective lens together with a DAPI filter and a green/red (FITC/TRITC) double filter. Micronuclei in the cells were verified by a 100× objective lens using DAPI, and FITC/TRITC double filters. Cytokinesis-block proliferation index (CBPI) was calculated from 100 cells per sample (Surrallés et al., 1995) as follows: $CBPI = [(no. mononucleate cells) + 2(no. binucleate cells) + 3(no. multinucleate cells)] / (total no. cells)$.

Results and discussions**Test results**

Species/strain mammalian cell line, other: BEAS 2B

Genotoxicity positive

Cytotoxicity yes

Vehicle controls valid no

Positive controls valid yes

Additional information on results

CNTs did not increase the frequency of micronucleated cells at any of the tested doses after the 24-h or 72-h treatments. In fact, the frequency of micronucleated cells was significantly lower as compared with the controls at two doses after the 24-h treatment (40 and 60 µg/cm²) and the 72-h treatment (1 and 80 µg/cm²). The variation in micronucleus frequencies appeared to be due to the presence of CNTs on the microscopic slides, which disturbed the analysis. However, the 48-h treatment induced a significant increase in micronucleated cells at three different doses, 10, 60, and 100 µg/cm² ($P < 0.001$, $P = 0.002$, $P < 0.001$, respectively; Fisher's exact test, 2-tailed), but a clear dose-dependency was not seen. CBPI differed significantly as compared with the controls at two doses after the 24-h (5 and 20 µg/cm², $P < 0.05$; one-way ANOVA) and 48-h (10 and 20 µg/cm², $P < 0.05$) CNT treatments, and at one dose (10 µg/cm², $P < 0.05$) after the 72-h treatment. As there were several statistically significant data points for CNTs, the results of the micronucleus assay were considered positive, despite the lacking dose-responses.

Overall remarks, attachments

Illustration (picture/graph)

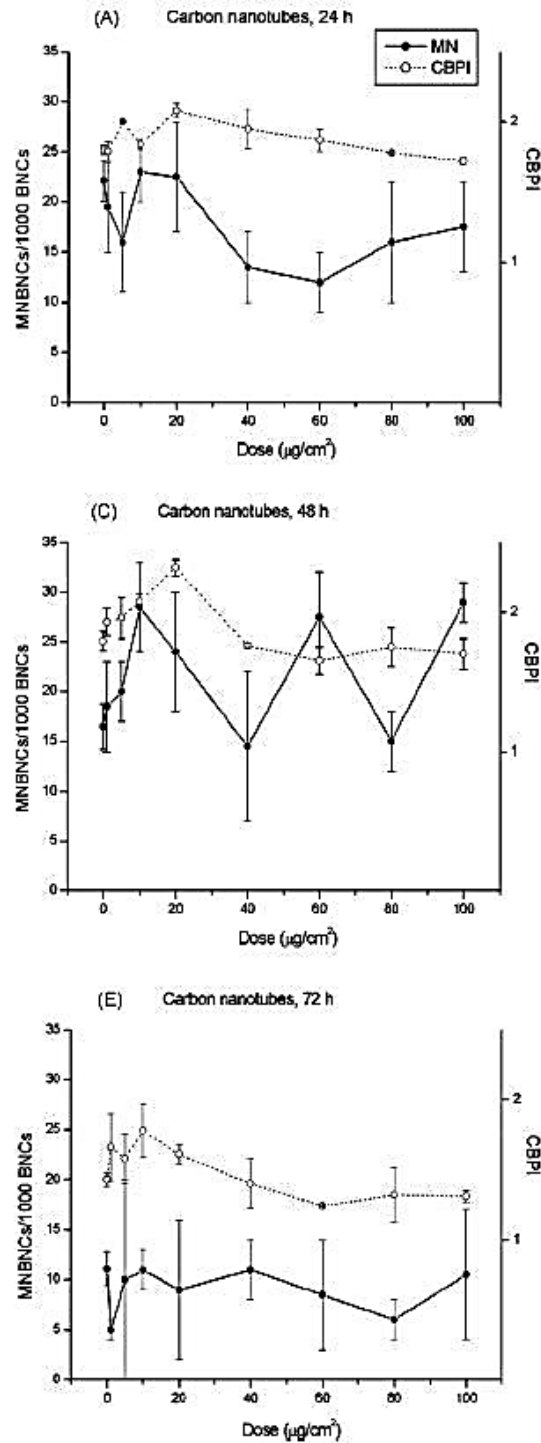


Fig. 4. Frequency (mean (±S.E.) of duplicate cultures) of micronucleated binucleate cells (MNBNcs) per 1000 binucleate cells (BNCs) and cytokinesis-block proliferation index (CBPI) in cultured human bronchial epithelial BEAS 2B cells after exposure to carbon nanotubes (A, C, and E).

Applicant's summary and conclusion

Conclusions

The CNTs are genotoxic to human bronchial epithelial BEAS 2B cells in vitro at doses that are not overly cytotoxic. This activity may be due to the fibrous nature of the material with a possibly contribution by catalyst metals (Co, Mo, and Fe) present in the carbon nanomaterials.

Endpoint study record: Sigma-Aldrich CNT Chromosomal aberration; Genetic toxicity in vitro.002

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Migliore et al.	2010	Carbon Nanotubes Induce Oxidative DNA Damage in RAW 264.7 Cells	Environmental and Molecular Mutagenesis 51:294-303 (2010)					

Cross-reference to same study

Sigma-Aldrich CNT damage test ;Genetic toxicity 004

Materials and methods

Type of genotoxicity

chromosome aberration

Type of study

in vitro mammalian cell micronucleus test

Test materials

Test material identity

Identifier	Identity
other: Sigma-Aldrich	

Details on test material

CNTs; >50% single-walled; ~40% other nanotubes; outer diameter 1.1 nm(0.7-1.2nm), length 0.5–100µm; manufactured by chemical vapour deposition; Sigma–Aldrich product no. 636797, elemental composition; 96.7% Carbon, catalyst metals; 1.5 wt.% Co , 1.0% Mo, 0.08% Al , specific surface; 400 m²/g, density 1.7 g/cm³ at 25°C

Method

Species/strain

Species/strain mammalian cell line, other: RAW 264.7

Details on mammalian cell lines (if applicable) RAW 264.7 cells were kindly provided by Prof. O. Bussolati (University of Parma). The cells were cultured in minimal essential medium (CELBIO, Milan, Italy) supplemented with 10%, heat inactivated, fetal bovine serum (CELBIO, Milan, Italy), 1% penicillin/streptomycin (CELBIO), and 1% L-glutamine (CELBIO). The cell cultures were maintained in a humidified atmosphere (5% CO₂) at 37°C. After 24 hr of incubation, the cell cultures were treated with different mass concentrations of CNT (0.01-100 µg/ml) or for the MN test.

Test concentrations

0.01, 0.1, 1, 10 and 100 µg/ ml

Controls

Solvent / vehicle controls yes

Positive controls yes

Positive control substance mitomycin C About 0.17µg/ml mitomycin C (MMC, Kyowa Hakko Kogyo Co., Tokyo)

Details on test system and conditions

To assess the genotoxic effects of CNT in RAW 264.7 macrophages, the micronucleus (MN) assay was performed as follows. Briefly, making the appropriate dilutions, a suitable quantity of cells (2 X 10⁴ cells/cm²) was seeded into six-well plates, and two paired, independent cultures for each concentration were prepared. After 24 h, the macrophages were treated with SWCNT as described above. About 0.17 µg/ml mitomycin C (MMC, Kyowa Hakko Kogyo Co., Tokyo) was used as a positive control. Forty-four hours after the initial preparation of the cultures, cytochalasin B was added to each test well to a final concentration of 4 µg/ ml to block cytokinesis of dividing cells [Fenech, 2007]. Macrophage cultures were then harvested after 28 hr: cells were treated with a 0.075 M KCl hypotonic solution for 2 min, pretreated in 3:5 methanol:acetic acid, and subsequently washed twice with a 6:1 methanol:acetic acid fixative solution. Finally, the cell solution was dropped onto cold glass slides. The air-dried slides were stained with a 2% Giemsa solution. Two thousand binucleated cells were examined for each experimental point in a blind mode (1,000 from each independent culture replicate) using an optical microscope (final

magnification of 40X). The scoring criteria adopted by the HUMAN MicroNucleus Project [Bonassi et al., 2001] were followed. We evaluated the binucleate micronucleated macrophages frequency as number of binucleate cells containing one or more micronuclei/1,000 binucleated cells. Moreover, 500 macrophages were scored to evaluate the percentage of mono-, bi-, tri-, and tetra-nucleated cells, and the CBPI was calculated [Surrall et al., 1995] as an index of cytotoxicity. Finally, other damage events were scored in once-divided binucleate cells, namely: (a) NBP, a biomarker of DNA misrepair and/or telomere end-fusions and (b) NBUD, a biomarker of elimination of amplified DNA and/or DNA repair complexes [Fenech, 2007]. The following endpoints were considered: the number of binucleated cells with NBP on 1,000 binucleated cells (BNNPB/1000BN), the number of NBP in 1,000 binucleated cells (NPBBN/1000BN), the number of binucleated cells with NBUD on 1,000 binucleated cells (BNBUD/1000BN), and the number of NBUD in 1,000 binucleated cells (BUDBN/1000BN).

Results and discussions

Test results

Species/strain	mammalian cell line, other: RAW 264.7
Genotoxicity	positive
Cytotoxicity	yes
Vehicle controls valid	no
Positive controls valid	yes

Any other information on results incl. tables

In SWCNT-treated cells, the number of micronucleated cells was significantly increased above the control at a concentration of 0.1 µg/ ml. Linear regression showed a statistically significant dose-dependent increase of micronucleated binucleate cells for SWCNT ($r = 0.76, P = 0.002$). SWCNT did not affect the CBPI over the whole dose range, with the exception of a weak, statistically insignificant decrease at the highest dose.

In binucleate cells, neither the NBP nor the NBUD endpoints were appreciably modulated by either treatment.

To confirm the cytotoxic potential of SWCNT and to avoid any possible interference with the cytokinesis-block protocol, the trypan blue exclusion test was carried out [Monteiro-Riviere et al., 2009]. The result shows a concentration-dependent decrease in the percentage of viable cells in the SWCNT-treated cultures. The number of viable cells was obtained by taking into consideration the total number of cells counted for each well. A statistically significant decrease in the cell number of cells was observed when the mean of three experiments was considered.

MN Assay in RAW 264.7 Cells with Different Mass Concentrations of SWCNT

Treatment	Conc. µg/ml	CBPI	BNMN /1000	BNNPB /1000	NPBBN /1000	BNBUD /1000	BUDBN /1000
Negative control		2.04	9.67 ±0.92	2.7 ±0.15	2.7 ± 0.15	05 ± 0	0.5 ± 0
C+ (MMC)	0,17	1.58	76.83± 24.3l ***	8.7 ± 1.21	8.8 ± 1.16	0.8± 0.48	1.2± 0.48
SWCNT	0.01	2.08	13.67 ±1.36	3.6 ± 0.37	3.3 ± 0.43	0.7± 0.15	0.6± 0.12
	0.1	2.05	18.00 ±0.76*	3.4 ± 1.00	2.7 ± 0.94	0.4± 0.31	0.4± 0.31
	10	1.98	20.00 ±0.57*	3.1 ±0.47	3.1;±; 0.47	1.0±: 0,28	1.1±: 0.24
	100	1.48	18.66 ±1.20*	2.7± 0.43	2.8± 0.38	0.6;±:0.24	0.5±: 0.28

Asterisk denote a significant difference from the control. Fisher's exact test.

* p< 0.05, **p< 0.01, ***p< 0.001

Applicant's summary and conclusion**Conclusions**

Micronuclei was increased at dose above 0.1 µg/ml in a cytokinesis-block micronucleus (CBMN) assay.

Endpoint study record: Sigma-Aldrich CNT DNA damage test; Genetic toxicity in vitro.003**Administrative Data**

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Lindberg et al.	2009	Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells in vitro	Toxicology Letters 186 (2009) 166–173					

Cross-reference to same study

Sigma-Aldrich CNT Chromosomal aberration ;Genetic toxicity 001

Materials and methods**Type of genotoxicity**

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test materials**Test material identity**

Identifier	Identity
other: Sigma-Aldrich	

Details on test material

CNTs; >50% single-walled; ~40% other nanotubes; 1.1 nm×0.5–100µm; manufactured by chemical vapour deposition; Sigma–Aldrich product no. 636797), catalyst metals;<5 wt.% Co and Mo, Specific surface; 567m²/g

Method**Species/strain**

Species/strain mammalian cell line, other: BEAS 2B

Details on mammalian cell lines (if applicable) BEAS 2B, a transformed human bronchial epithelial cell line exhibiting an epithelial phenotype (Reddel et al., 1988) was obtained from American Type Culture Collection through LGC Promochem AB (Borås, Sweden). The BEAS 2B cells were grown in serum-free BEGM medium (Clonetics, Walkerville, MD, USA) at 37 °C in a humidified atmosphere of 5% CO₂. Log-phase BEAS 2B cells were plated on 24-well plates (cell viability and comet assays), and 4-well chamber slides (micronucleus assay) (Nunc, Roskilde, Denmark) 2 days prior to exposure.

Test concentrations

3.8, 19, 38, 76, 114, 228, 304, and 380 µg/ml

Vehicle

The materials were dispersed in BEGM cell culture medium (Clonetics, Walkerville, MD) and subjected to ultrasonication (Elmasonic, Singen, Germany) for 20 min at 37 kHz prior to addition to the cell cultures. Measurements from light microscopic images indicated that the average size of the agglomerates in dispersion was 8.5 µm (S.D. 11.6). Transmission electron microscopy verified that the BEGM dispersions also contained nano-scale (<100 nm) material in addition to the m-scale agglomerates seen in the optical microscope.

Controls

Solvent / vehicle controls yes

Positive controls yes

Positive control substance other: hydrogen peroxide (20mM, Riedel-de Haen, Seelze, Germany)

Details on test system and conditions

The comet (single cell gel electrophoresis) assay was used to study DNA strand breaks and alkaline labile sites in BEAS 2B cells after the nanomaterial exposures. BEAS 2B cells in log-phase were plated in 24-well plates (culture area 1.9 cm²/well, culture medium volume 0.5 ml/well; Nunc, Roskilde, Denmark) 2 days prior to exposure. Semiconfluent cultures were exposed for 24 h, 48 h or 72 h to eight doses of each nanomaterial: 1, 5, 10, 20, 40, 60, 80, and 100 µg/cm² (in µg/ml culture medium, the corresponding doses were: 3.8, 19, 38, 76, 114, 228, 304, and 380 µg/ml). The doses were chosen according to the viability assay. Untreated controls and positive controls treated with hydrogen peroxide (20mM, Riedel-de Haen, Seelze, Germany) were included in all series. The comet assay was performed in alkaline conditions (pH > 13) as described previously (Nygren et al., 2004). Briefly, after the exposure the cells were trypsinised and centrifuged at 1100 rpm for 5 min. 1–3 × 10⁴ cells were resuspended in 75 µl molten (37 °C) 0.5% low-melting-point agarose (LMPA; Bio-Rad Laboratories, Hercules, CA). The resuspended cells in agarose were put onto dry microscope slides (Assistant, Sondheim/Röhn, Germany) pre-coated with 1% normal-melting agarose (International Biotechnologies, New Haven, CT, USA), and the agar was allowed to solidify for 10 min. The slides were thereafter immersed in cold lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 1% Triton X-100) for at least 1 h at 4 °C, after which they were transferred to an electrophoresis tank containing freshly made electrophoresis buffer (1mM EDTA, 300mM NaOH; pH > 13), where they were kept for 20 min at room temperature to allow DNA unwinding. Electrophoresis was performed in the same buffer at room temperature for 15 min at 24V and 300mA (0.8 V/cm). The slides were then neutralized three times with 0.4M Tris buffer (pH 7.5), air-dried, and fixed in methanol. DNA was stained with ethidium bromide (2 µg/ml) in water for 5 min. The slides were coded, and one scorer performed the comet analysis using a fluorescence microscope (Axioplan 2, Zeiss, Jena, Germany) and an interactive automated comet counter (Komet 4.0, Kinetic Imaging Ltd., Liverpool, UK). The percentage of DNA in the comet tail from 100 cells per sample (two replicates, each with 50 cells/slide) was used as a measure of the amount of DNA damage.

Results and discussions**Test results**

Species/strain mammalian cell line, other: BEAS 2B

Genotoxicity positive

Cytotoxicity yes

Vehicle no
controls valid

Positive yes
controls valid

Additional information on results

CNTs induced a dose-dependent increase in DNA damage ($r = 0.73$, $P = 0.03$) after the 24-h treatment, and a significant effect was seen at 1, 60, 80, and 100 $\mu\text{g}/\text{cm}^2$ ($P = 0.003$, $P = 0.004$, $P < 0.001$, $P < 0.001$, respectively; t-test, 2-tailed). After the 48-h and 72-h treatments with CNTs, the effect was also very clear; a statistically significant increase in DNA damage was observed at all tested doses, with a significant dependence on dose ($r = 0.87$, $P = 0.003$; $r = 0.80$, $P = 0.01$, respectively).

Overall remarks, attachments

Remarks on results including tables and figures

Genotoxicity was assessed by the single cell gel electrophoresis (comet) assay in human bronchial epithelial BEAS 2B cells cultured for 24 h, 48 h, or 72 h with various doses (1–100 $\mu\text{g}/\text{cm}^2$, corresponding to 3.8–380 $\mu\text{g}/\text{ml}$) of the carbon nanomaterials. CNTs induced a dose-dependent increase in DNA damage at all treatment times, with a statistically significant effect starting at the lowest dose tested.

Illustration (picture/graph)

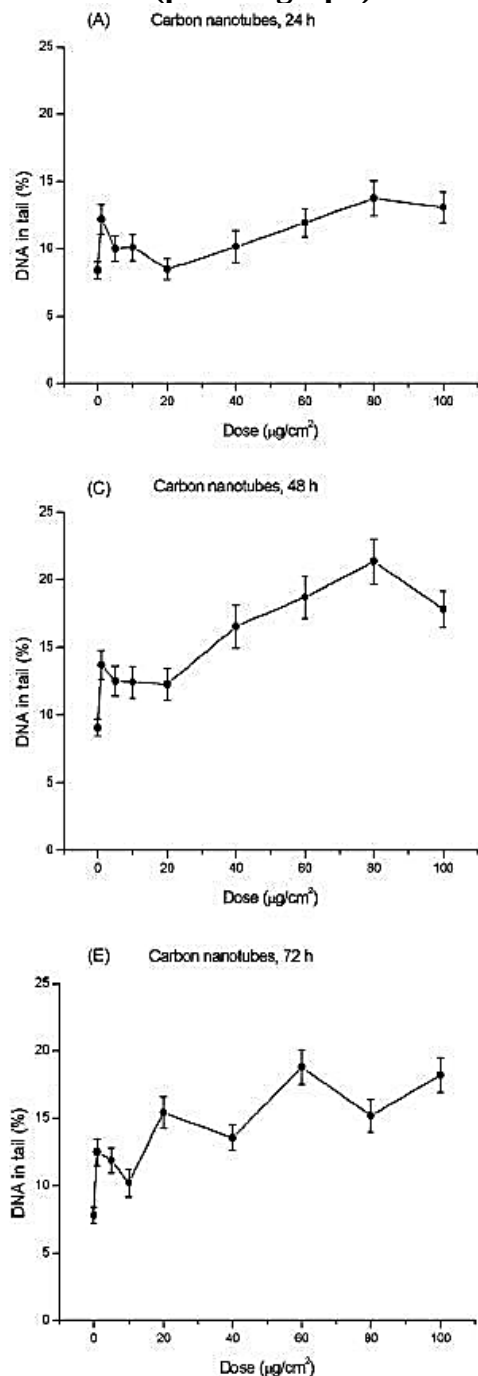


Fig. 3. DNA damage as measured by the comet assay (percentage of DNA in tail) in cultured human bronchial epithelial BEAS 2B cells after exposure to carbon nanotubes

Applicant's summary and conclusion

Conclusions

In a cytokinesis-block micronucleus (CBMN) assay, dose independent increases of micronucleated cells at three concentrations in the 48 h treatment. (The authors' conclusion is positive.)

Endpoint study record: Sigma-Aldrich CNT DNA damage test;Genetic toxicity in vitro.004**Administrative Data**

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Migliore et al.	2010	Carbon Nanotubes Induce Oxidative DNA Damage in RAW 264.7 Cells	Environmental and Molecular Mutagenesis 51:294-303 (2010)					

Cross-reference to same study

Sigma-Aldrich CNT damage test ;Genetic toxicity 004

Materials and methods**Type of genotoxicity**

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test materials**Test material identity**

Identifier	Identity
other: Sigma-Aldrich	

Details on test material

CNTs; >50% single-walled; ~40% other nanotubes; outer diameter 1.1 nm(0.7-1.2nm), length 0.5–100µm; manufactured by chemical vapour deposition; Sigma–Aldrich product no. 636797, elemental composition; 96.7% Carbon, catalyst metals; 1.5 wt.% Co , 1.0% Mo, 0.08% Al , specific surface; 400 m²/g, density 1.7 g/cm³ at 25°C

Method

Species/strain

Species/strain mammalian cell line, other: RAW 264.7

Details on mammalian cell lines (if applicable) RAW 264.7 cells were kindly provided by Prof. O. Bussolati (University of Parma). The cells were cultured in minimal essential medium (CELBIO, Milan, Italy) supplemented with 10% heat inactivated fetal bovine serum (CELBIO, Milan, Italy), 1% penicillin/streptomycin (CELBIO), and 1% L-glutamine (CELBIO). The cell cultures were maintained in a humidified atmosphere (5% CO₂) at 37°C. After 24 hr of incubation, the cell cultures were treated with different mass concentrations of CNT (0.01-100 µg/ml) or for the MN test.

Test concentrations

0.01, 0.1, 1, 10 and 100 µg/ml

Controls

Solvent / vehicle controls yes

Positive controls yes

Positive control substance other: Hydrogen peroxide (H₂O₂) (10 µM)

Details on test system and conditions

The modified alkaline comet assay [Collins et al., 1996] was carried out on RAW 264.7 cultures seeded at a concentration of 2×10^4 cells/cm² in six well plates: at least two independent experiments were performed for each concentration tested. After attachment, cells were treated with CNT at different concentrations (see above) by dispersing SWCNT in culture medium. Incubation was performed for 2 or 24 hr (three and two experiments, respectively) to verify the protocol procedure, Hydrogen peroxide (H₂O₂) (10 µM) was used as positive control. After treatment, the cell pellet was used for the comet assay. Briefly, the slides were spread with 1% normal melting agarose (Sigma-Aldrich) in PBS (Sigma-Aldrich) and left to solidify at 4°C. Cell pellets were suspended in 85 µl of 0.5% low-melting agarose (Agarose wide range, Sigma-Aldrich) in PBS at 37°C, and two slides per culture were prepared. The cell suspension was dropped on top of the first agarose layer, covered with a coverslip, and allowed to solidify at 4°C. A final layer of 0.5% low-melting agarose was then added to the slide. The lysis procedure was performed in cold lysis buffer (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, 10% DMSO, and 1% Triton X-100, pH 10.0) for 1 hr at 4°C. The slides were then placed for 20 min in alkaline buffer (300 mM NaOH, 1 mM Na₂EDTA, and pH > 13.0), and electrophoresed for 20 min at 25 V adjusted at 300 mA. The slides were neutralized in 0.4 M Tris buffer, pH 7.5, for 5 min, twice. Coded slides were scored after staining with ethidium bromide (20 µg/ml) using a fluorescence microscope (Nikon Eclipse E800) at 20X magnification. The percentage of DNA in the comet tail in a total of 100 randomly selected cells per sample (two replicates, each with 50 cells/slide) was used as a measure of the amount of DNA damage. Analysis was carried out by using a Comet Image Analysis System, version 5.5 (Kinetic Imaging, Nottingham, UK). Results were reported as % Tail DNA.

Results and discussions

Test results

Species/strain mammalian cell line, other: RAW 264.7

Genotoxicity positive

Cytotoxicity yes

Vehicle controls valid no

Positive controls valid yes

Any other information on results incl. tables

Cells were exposed for 2 and 24 hr, with the same range of CNT concentrations used for the MN assay. No increase in DNA migration

(i.e., primary DNA damage) was detected after a 2-hr treatment. Conversely for the 24-hr treatment, the mean percentage of DNA in the tail [tail DNA (%)] was slightly, but significantly increased compared to the control values for those cells treated with 10 and 100 tg/ml SWCNT. Overall, a significant dose effect was observed for SWCNT ($r = 0.80$, $P = 0.015$). The 24-hr treatment was then used to investigate oxidative DNA damage, using the repair enzymes Endo-III and Fpg. The effects of the treatments with SWCNT were detectable at all concentrations tested (1-100µg/ml): oxidized pyrimidines increased significantly as compared to baseline values, whereas oxidized pyrimidines showed a significant increase ($p < 0.001$) at the highest mass concentration tested. The comet assay was performed using H₂O₂ as positive control, which markedly increased DNA damage.

Applicant's summary and conclusion**Conclusions**

%Tail DNA was increased at 10.0 and 100.0 µg/ml in a comet assay.

Endpoint study record: NIST SWCNT DNA damage test; Genetic toxicity001**Administrative Data**

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Pacurari et al.	2008	Raw Single-Wall Carbon Nanotubes Induce Oxidative Stress and Activate MAPKs, AP-1, NF-κB, and Akt in Normal and Malignant Human Mesothelial Cells	Environmental Health Perspectives, Vol. 116, No. 9 (2008)					

Materials and methods

Type of genotoxicity

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test materials

Test material identity

Identifier	Identity
other: NIST SWCNT	

Details on test material

National Institute of Standards and Technology (Gaithersburg, MD); Reported impurities include nickel and yttrium, which are encapsulated in carbon shells. Detailed high-resolution transmission electron microscopy revealed that the diameters ranged from 0.8 to 2.0 nm

Method

Species/strain

Species/strain mammalian cell line, other: normal human (NM) and malignant mesothelial (MM) cells

Test concentrations

25 or 50 µg/cm²

Vehicle

RPMI-1640 medium containing 0.1% fetal bovine serum(FBS)

Details on test system and conditions

1. DNA damage by comet assay. The NM and MM cells (105) were seeded overnight and treated them with vehicle or 25 or 50 µg/cm² SWCNTs for 24 hr. We assessed DNA damage using a commercially available comet assay according to the manufacturer's instructions (Trevigen, Gaithersburg, MD)
2. Histone H2AX phosphorylation of DNA double-strand breaks. NM and MM cells cultured in black-wall/clear-bottom microplates to 25 or 50 µg/cm² SWCNTs or crocidolite for 24 hr were exposed. H2AX phosphorylation according to the manufacturer's protocol (Millipore, Billerica, MA) was detected.

Results and discussions

Test results

Species/strain mammalian cell line, other: normal human (NM) and malignant mesothelial (MM) cells

Genotoxicity positive

Cytotoxicity yes

Vehicle yes
controls valid

Overall remarks, attachments

Remarks on results including tables and figures

1. DNA damage induced by SWCNTs, because SWCNT exposure caused generation of ROS, we investigated whether raw SWCNT-induced oxidative stress resulted in DNA damage. DNA damage, investigated using a comet assay in NM and MM cells exposed to 25 or 50 µg/cm² SWCNTs or vehicle for 24 hr, showed that the SWCNTs induced DNA damage in both cell types (Figure 2A). Figure 2B shows semiquantitative measurements of SWCNT-induced dose-dependent DNA tail migration, demonstrating significant DNA damage at both doses in NM and MM cells (Figure 2B). Exposure of NM cells to 25 or 50 µg/cm² SWCNTs for 24 hr resulted in a 5.2- and 6.6-fold increase in DNA tail length migration, respectively. In contrast, exposure of MM cells to the same mass concentrations of crocidolite for 24 hr caused an increased DNA tail migration of 7.9- and 11.1-fold, respectively. Coincubation of MM cells with SWCNTs (25 µg/cm²) and catalase (100 U/mL), SOD (100 U/mL), or deferoxamine (1 mM; an iron chelator) for 24 hr resulted in a 35%, 30%, and 32% decrease in DNA damage, respectively.
2. H2AX phosphorylation by SWCNTs and crocidolite Exposure of NM and MM cells to 25 or 50 µg/cm² SWCNTs resulted in a nominal increase in phosphorylation of H2AX on Ser139, which was moderately higher in MM cells. The same concentrations of crocidolite induced a significantly greater phosphorylation in both cell types (Figure 3).

Illustration (picture/graph)

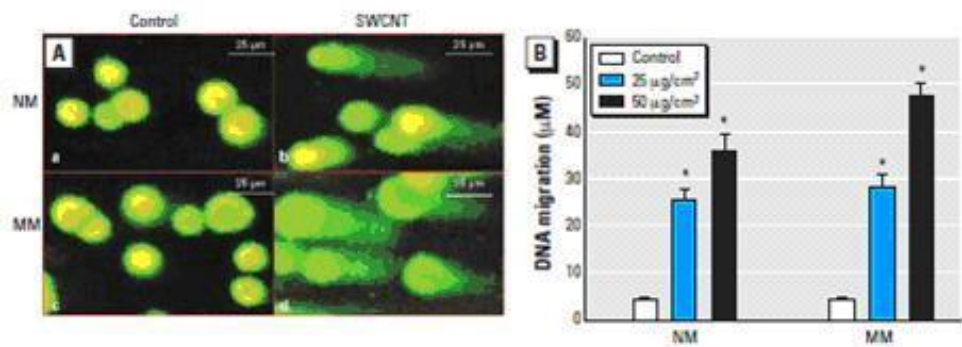


Figure 2. Effect of SWCNTs (25 or 50 µg/cm²) on DNA migration in NM and MM cells using the comet assay. (A) Micrographs of NM (a,b) and MM (c,d) cells treated with vehicle (control; a,c) or 50 µg/cm² SWCNTs (b,d) for 24 hr. (B) Semiquantitative analysis of concentration-dependent effects of SWCNTs on DNA migration in NM and MM cells using the comet assay. Data are presented as the mean ± SE of three experiments. Bars = 25 µm. *Significant increase from control (p < 0.05).

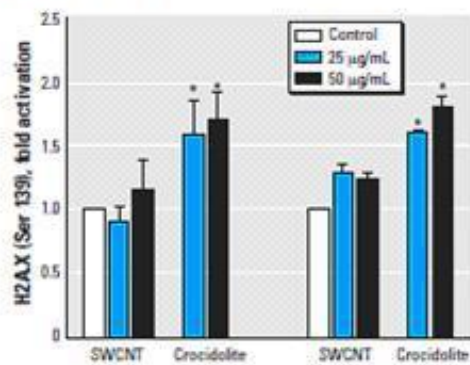


Figure 3. Effect of 24-hr exposure to SWCNTs or crocidolite (25 or 50 µg/mL) on the activation of γ-H2AX (Ser139) in NM and MM cells. Absorbance values were normalized to control values; data are presented as mean ± SE of three experiments. *Significantly different from control (p ≤ 0.05).

Applicant's summary and conclusion

Conclusions

SWCNTs (supplied by NIST) can cause potentially adverse cellular responses in mesothelial cells through activation of molecular signaling associated with oxidative stress.

Endpoint study record: Heji SWCNT DNA damage test;Genetic toxicity in vitro.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Zeni et al.	2008	Cytotoxicity Investigation on Cultured Human Blood Cells Treated with Single-Wall Carbon Nanotubes	Sensors 2008, 8, 488-499					

Materials and methods**Type of genotoxicity**

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test materials**Test material identity**

Identifier	Identity
other: Heji, Inc.	

Details on test material

SWCNTs were obtained in highly purified form (>90%) from HeJi, Inc. (Hong Kong, China). According to the certificate of analysis reported by the manufacturer, they have an average outside diameter of 1.1 nm, an average length of 50 µm and the following components content in percentage: C 96.30; Al 0.08; Cl 0.41; Co 2.91; S 0.29.

Method**Species/strain**

Species/strain mammalian cell line, other: human peripheral blood lymphocytes (HPBL)

Details on mammalian cell lines (if applicable) The experiments were approved by the Ethical Committee of ASL Na 1. HPBLs were obtained with informed consent from anonymized buffy coats of healthy donors, provided by the “San Paolo” Hospital (Naples, Italy). They were isolated through lymphoprep density gradient centrifugation, in accordance with the manufacturer’s instructions. Mononuclear cells were washed twice with phosphate-buffered saline (PBS) were seeded in RPMI 1640 medium supplemented with 15% heatinactivated foetal bovine serum and 1% L-glutamine. PHA (1%) was added as mitogen to stimulate T-lymphocytes to enter the cell cycle. HPBLs from at least 3 healthy donors were employed for each of the biological targets examined.

Test concentrations

0 (control), 1, 5 and 10 µg/ml

Vehicle

SWCNTs are water insoluble and were suspended at a concentration of 0.5 mg/ml in RPMI medium, sterilized by autoclavation and dispersed by 3 h treatment in an ultrasound bath prior to being administered to the cells.

Controls

Negative controls	no
Solvent / vehicle controls	yes
Positive controls	yes
Positive control substance	methylmethanesulfonate

Any other information on materials and methods incl. tables

For the evaluation of primary DNA damage, the alkaline comet assay was applied. Un-stimulated leukocytes were seeded at a density of 5×10^5 cells/ml, and treated for 6 h with SWCNTs at final concentrations of 0 (control), 1, 5 and 10 µg/ml. Positive controls were also included by treating cells with MMS for 2 h at final concentration of 150 µM. For each donor/treatment, cultures were set up in duplicate. The alkaline Comet assay was performed according to the method developed by Singh and co-workers (Singh 1988) with minor modifications, and for each sample two replicate slides were set up. The method has been described in details in a previous paper (Zeni 2007). Briefly, cells were resuspended in low melting point agarose (LMA; 0.7% w/v; 37°C) and sandwiched between a lower layer of 1% normal melting agarose (NMA; 1% w/v) and an upper layer of LMA (0.7% w/v) on microscope slides. Following an overnight immersion in cold lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 10 mM Tris, pH 10) with 1% Triton X-100 and 10% DMSO, DNA was unwound for 25 min at 4°C in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na₂EDTA, pH >13) and electroforesed at 4°C for 25 min at 30 V and 300 mA. Then slides were rinsed with sodium acetate (300 mM)-ethanol absolute solution for 30 min, dehydrated (absolute ethanol for 2 h) and re-hydrated (70% ethanol for 5 min), and were stained, just before analysis, with ethidium bromide (12 µg/ml). Images of 300 randomly selected cells (150 from each of two replicate slides) were analysed by a computerized Image Analysis System (Delta Sistemi, Rome, Italy) fitted with a Leica DM BL fluorescence microscope at 250 X magnification. DNA damage was evaluated by calculating the tail length (in µm from the estimated leading edge of the head region to leading edge of the tail), the percentage of migrated DNA (calculated as the integrated intensity of DNA in the tail divided by the integrated intensity of DNA for the total image and multiplied by 100) and the tail moment (the fraction of DNA in the tail multiplied by tail length).

Results and discussions**Test results**

Species/strain	mammalian cell line, other: human peripheral blood lymphocytes (HPBL)
Genotoxicity	negative
Cytotoxicity	no
Vehicle controls valid	yes
Positive controls valid	yes

Overall remarks, attachments

Remarks on results including tables and figures

No increase in DNA migration, evaluated in terms of migrated DNA percentage, tail length and tail moment, was found in human leukocytes following 6 h treatment with SWCNT concentrations of 0.5, 1, 5 and 10 µg/ml. On the contrary, a statistically significant increase in all the parameters investigated was found when MMS treated samples were compared to control ones ($P < 0.0001$).

Illustration (picture/graph)

Table 1. Migrated DNA, tail length and tail moment in human leukocytes treated for 6 h with SWCNTs. Data related to positive control (MMS, 150 µM for 2 h) are also reported. Each data point represents the mean $\pm$ SD of 1200 nuclei scored (300 from each of the 4 donors tested).

Treatment	Migrated DNA (%)	Tail length (microns)	Tail moment (arbitrary units)
Control	1.62 $\pm$ 0.76	3.49 $\pm$ 1.72	0.45 $\pm$ 0.30
CNTs- 1 µg/ml	1.52 $\pm$ 1.20	3.20 $\pm$ 1.60	0.46 $\pm$ 0.44
CNTs- 5 µg/ml	1.94 $\pm$ 1.07	3.69 $\pm$ 1.63	0.67 $\pm$ 0.41
CNTs - 10 µg/ml	1.60 $\pm$ 0.68	3.34 $\pm$ 1.14	0.45 $\pm$ 0.23
MMS - 150 µM	14.92 $\pm$ 1.16*	19.28 $\pm$ 1.61*	5.44 $\pm$ 0.61*

* significant ($P < 0.0001$) difference from the control: two tailed paired Student's t test.

Applicant's summary and conclusion

Conclusions

Treatments of 6 h with SWCNTs (obtained from Heji Inc.) concentrations of 1, 5 and 10 µg/ml failed to induce primary DNA damage on the entire human leukocytes population.

Endpoint study record: COCC SWCNT DNA damage test; Genetic toxicity in vitro.001**Administrative Data**

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Yang et al.	2009	Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition	J. Appl. Toxicol. 2009;29:69-78					

Materials and methods**Type of genotoxicity**

DNA damage and/or repair

Type of study

single cell gel/comet assay in mammalian cells for detection of DNA damage

Test materials**Test material identity**

Identifier	Identity
other: COCC SWCNT	

Details on test material

SWCNTs were obtained from COCC, Chinese Academy of Science, Chengdu, rope-shaped with lengths less than 5 μ m and diameters of approximate 8 nm, and carbon composition was more than 99.99%.

Method**Species/strain**

Species/strain mammalian cell line, other: Primary mouse embryo fibroblasts

Test concentrations

5 and 10 µg/ml

Vehicle

Cell culture medium; Dulbecco's modified Eagle's low glucose medium (DMEM/Iow, Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS), plus 2 mM L-glutamine, 1 %(v/v) penicillin-streptomycin (10 U/ml penicillin and 0.1 mg/ml streptomycin)

Controls

Solvent / vehicle controls yes

Positive controls no data

Details on test system and conditions

After exposure to nanoparticles at 5 and 10 µg/ml for 24h, cells in six-well plates were washed twice with pre-chilled PBS, centri futed at 78g for 5min and resuspended in PBS. As assessed by Trypan blue dye-exclusion staining, cell viability was over 95% under the tested doses in this study. The alkaline comet assay for assessment of DNA damage was performed according to the method described by Singh et al. (1991) with some modifications. Electrophoresis was conducted in the refrigerator at 4°C for 30min at 23V and 300mA. After electrophoresis, the slides were rinsed with deionized water and immersed in 70% ethanol for 40min, then drained and 50 µl of propidium iodide, PI (5 µg/ml) was added. All steps after lysis were carried out under yellow light in the cold room to prevent any induction of additional DNA damage. Slides were scored at 200 magnification using a fluorescence microscope (Olympus BX-50, UK) with an excitation filter of 515-560 nm and barrier filter of 590 nm and photographed with a high-resolution CCD camera (CoolSNAP, Olympus). At least 50 randomly selected images were analyzed from each sample with the CASP software package (Konca et al., 2003). In our study, DNA damage was evaluated by comet length, Olive tail moment and the percentage of DNA in the tail (%Tail DNA), which are considered the most informative and reliable measurements (Olive and Durand, 2005; Kumaravel and Jha, 2006).

Results and discussions**Test results**

Species/strain mammalian cell line, other: Primary mouse embryo fibroblasts

Genotoxicity positive

Cytotoxicity yes

Vehicle controls valid yes

Positive controls valid no data

Additional information on results

In this assay, there were significant increases in tail length, percentage of DNA in tail, tail moment and

Olive tail moment after PMEF cells were treated with four types of nanoparticles at both examined concentrations. SWCNTs and ZnO caused more DNA damage than CB and SiO₂ nanoparticles; however, there was no significant difference between them at 5 µg/ml dosage level. As for 10 µg/ml, their genotoxicity can be classified from most to least toxic as follows: SWCNTs > ZnO > CB > SiO₂. Take tail DNA% as example, 38.2, 18.8, 12.8 and 6.8%, respectively, for the group of CNTs, ZnO, CB and SiO₂, vs 3.26% for the untreated control.

Applicant's summary and conclusion

Conclusions

24 h Exposure of SWCNTs (COCC) to Primary mouse embryo fibroblast (BALB/c mouse) at 5 or 10 µg/ml resulted in increase of %Tail DNA, Tail length and Tail moment.

7.6.2 Genetic toxicity in vivo

Endpoint study record: Nikkiso SWCNT Micronucleus test; Genetic toxicity in vivo.001

Administrative Data

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies

The test was conducted under GLP according to OECD TG

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Micronucleus test of Nikkiso SWCNT in male mice	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) at http://www.aist-riss.jp/main/modules/product/nano_rad.html	Ina Research Corp.	ZT10112			

Cross-reference to same study

7.2.1 Nikkiso SWCNT Acute toxicity: oral.001

Materials and methods

Type of study

micronucleus assay

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 474 (Mammalian Erythrocyte Micronucleus Test)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals**Species**

mouse

Strain

other: Crlj:CDI(ICR)

Sex

male

Details on test animals and environmental conditions

Mice were purchased from Charles River Japan Laboratory Inc. and individually housed in polycarbonate cages and reared on a basal diet (CRF-1; Oriental Yeast Co., Ltd., Chiba, Japan) and tap water ad libitum. Mice were maintained at room temperature (21-25°C, a humidity of 40-70%, 14-19 air changes per hour and a 12 hr dark/12 hr light cycle. Body weights of male were 25.0-35.6g and 8 week old at the beginning of administration.

Administration / exposure**Route of administration**

oral: gavage

Vehicle(s)

CNTs were suspended in injection water and diluted with 0.3% CMC

Details on exposure

20mL/kg/day; 5,10 and 20 mg/kg/day

Duration of treatment / exposure

administrated twice orally in the interval of 24 hours

Frequency of treatment

twice

Post exposure period

In 24 hours, the bone-marrow cell was harvested from the femur and micronucleus assay was performed

No. of animals per sex per dose

6 male mice/dose

Control animals

yes, concurrent vehicle

Positive control(s)

MMC(mitomycin C); 5mg/kg/day

Examinations

Tissues and cell types examined

bone marrow cells

Details of tissue and slide preparation

The bone marrow cells were flushed into tubes with fatal bovine serum to prepare bone marrow smear specimens. The specimens were air-dried, fixed with methanol and stained with acridine orange. When significant increase was observed, the result was judged to be positive.

Evaluation criteria

Appearance rate (%) of MNIE in the IE (Immature erythrocyte) of 2000 were calculated and simultaneously IE% in the erythrocytes of 1000 were counted.

Statistics

MNIN% ; Fisher method, IE % ; Dunnett method

Results and discussions

Test results

Sex	male
Genotoxicity	negative
Vehicle controls valid	yes
Positive controls valid	yes

Overall remarks, attachments

Remarks on results including tables and figures

No abnormality was seen in all the group of mice and no micronucleus formation was observed in each dose group of CNTs.

It was judged that there was no micronucleus formation.

Illustration (picture/graph)

Table 1 Micronucleus test of Carbon nanotube A in male mice - Main test -

Dose (mg/kg)	Number of doses	Number of animals	% MNIE ^{a)}	% IE ^{b)}
			Mean±S.D. [Min.-Max.]	Mean±S.D. [Min.-Max.]
0 ^{c)}	2	6	0.11 ± 0.08 [0.05 - 0.25]	58.7 ± 7.5 [50.3 - 70.0]
5	2	6	0.05 ± 0.05 [0.00 - 0.10]	61.5 ± 8.2 [46.8 - 71.9]
10	2	6	0.13 ± 0.07 [0.05 - 0.20]	61.8 ± 9.2 [52.8 - 71.4]
20	2	6	0.13 ± 0.12 [0.00 - 0.30]	64.7 ± 5.1 [56.6 - 70.3]
MMC ^{d)} 5	1	6	3.28 ± 1.71 ** [1.45 - 6.35]	56.1 ± 5.6 [51.4 - 64.3]

^{a)} Micronucleated immature erythrocyte

^{b)} Immature erythrocyte

^{c)} Negative control, 0.3% CMC

^{d)} Positive control, Mitomycin C

** P<0.01, significantly different from negative control (Fisher's exact test).

Applicant's summary and conclusion

Interpretation of results

negative

Conclusions

The test substance did not produce micronuclei in the immature erythrocytes of the test species.

Endpoint study record: Super Growth SWCNT Micronucleus test; Genetic toxicity in vivo.001**Administrative Data**

other:OECD/WPMN

Purpose flag

key study

Study result type

experimental result

Reliability

1 (reliable without restriction)

Rationale for reliability incl. deficiencies The test was conducted under GLP according to OECD TG

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	Micronucleus test of Super Growth SWCNT in mice	Risk Assessments of Manufactured Nanomaterials-Carbon Nanotubes (CNT)- (August 17, 2011) (in Japanese). http://www.aist-riss.jp/main/modules/product/nano_rad.html	Institute of Environmental Toxicology (Ibaraki, Japan)	IET07-0097			
publication	Naya et al	2011	Evaluation of the genotoxic potential of single-walled carbon nanotubes by using a battery of in vitro and in vivo genotoxicity assays	Regulatory Toxicology and Pharmacology,61(2011),192-198					

Cross-reference to same study

7.2.1 Super Growth SWCNT Acute toxicity: oral.001

Materials and methods**Type of study**

micronucleus assay

Test guideline

Qualifier	Guideline	Deviations
according to	OECD Guideline 474 (Mammalian Erythrocyte Micronucleus Test)	

GLP compliance

yes

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

yes

Test material identity

Identifier	Identity
other:	Super Growth SWCNT

Test animals**Species**

mouse

Strain

other: Crlj:CDI(ICR)

Sex

male

Details on test animals and environmental conditions

Mice were purchased from Charles River Japan Laboratory Inc. and individually housed in polycarbonate cages and reared on a basal diet (MF solid type; Oriental Yeast Co., Ltd., Chiba, Japan) and tap water ad libitum. Mice were maintained at room temperature (19-25°C, a humidity of 30-70%, 14-19 air changes per hour and a 12 hr dark/12 hr light cycle. Body weights of male were 28.7-35.2g and 7 week old at the beginning of administration.

Administration / exposure**Route of administration**

oral: gavage

Vehicle(s)

CNTs were suspended in RBS with 1% Tween 80

Details on exposure

60 and 200 mg/kg/day

Duration of treatment / exposure

administered twice orally in the interval of 24 hours

Frequency of treatment

twice

Post exposure period

In 24 hours, the bone-marrow cell was harvested from the femur and micronucleus assay was performed

No. of animals per sex per dose

5 male mice/dose

Control animals

yes, concurrent vehicle

Positive control(s)

MMC(mitomycin C); 10mg/kg/day

Examinations

Tissues and cell types examined

bone marrow cells

Details of tissue and slide preparation

The bone marrow cells were flushed into tubes with fetal bovine serum to prepare bone marrow smear specimens. The specimens were air-dried, fixed with methanol and stained with 3% Giemsa solution for 30 minutes at room temperature.

Evaluation criteria

Appearance rate (%) of MNIE in the IE (Immature erythrocyte) of 2000 were calculated and simultaneously IE% in the erythrocytes of 1000 were counted. When significant increase was observed, the result was judged to be positive.

Statistics

MNIN% ; Kastenbaum-Bowman's test, IE % ; Wilcoxon's Rank Sum test

Results and discussions

Test results

Sex	male
Genotoxicity	negative
Vehicle controls valid	yes
Positive controls valid	yes

Overall remarks, attachments

Remarks on results including tables and figures

No abnormality was seen in all the group of mice and no micronucleus formation was observed in each

dose group of CNTs.

It was judged that there was no micronucleus formation.

Illustration (picture/graph)

Table 1 Summary of results - micronucleus test

Sampling time (hr)	Substance	Dose (mg/kg)	No. of mice	MNPCE / PCE (%)		PCE / (PCE+NCE) (%)	
				Mean $\pm$ SD (range)	S ^{RC}	Mean $\pm$ SD (range)	S ^W
24 ^{A)}	Vehicle (1% Tween 80)	0 x 2	5	0.28 $\pm$ 0.10 (0.15 ~ 0.40)	—	54.7 $\pm$ 2.2 (52.1 ~ 57.8)	—
	SG-SWCNT	60 x 2	5	0.28 $\pm$ 0.17 (0.05 ~ 0.50)	N.S.	57.4 $\pm$ 4.9 (50.0 ~ 63.3)	N.S.
		200 x 2	5	0.27 $\pm$ 0.14 (0.10 ~ 0.45)	N.S.	50.1 $\pm$ 8.4 (41.2 ~ 61.1)	N.S.
	Mitomycin C	10 x 1	5	4.95 $\pm$ 1.68 (2.65 ~ 6.95)	***	40.5 $\pm$ 10.0 (29.7 ~ 53.6)	*

MNPCE : Micronucleated polychromatic erythrocytes.

PCE : Polychromatic erythrocytes.

NCE : Normochromatic erythrocytes.

SD : Standard deviation.

S^{RC} : Statistical analysis using the tables of Kastenbaum-Bowman or a chi-square test.

S^W : Statistical analysis using Wilcoxon's sum of ranks test.

N.S. : Not significantly different from the concurrent vehicle control ($p > 0.05$).

*, *** : Significantly different from the concurrent vehicle control at $p \leq 0.05$ and $p \leq 0.001$, respectively.

A) : 24 hours after the final administration.

Applicant's summary and conclusion

Interpretation of results

negative

Conclusions

The test substance did not produce micronuclei in the immature erythrocytes of the test species.

Endpoint study record: Elicarb SWCNT DNA damage test; Genetic toxicity in vivo.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Folkmann et al.	2009	Oxidatively Damaged DNA in Rats Exposed by Oral Gavage to C60 Fullerenes and Single-Walled Carbon Nanotubes	Environmental Health Perspectives volume 117, number 5, pp703-708					

Materials and methods**Type of genotoxicity**

DNA damage and/or repair

Type of study

mouse spot test

Test materials**Test material identity**

Identifier	Identity
other: Thomas Swan and Co Ltd.	Elicarb

Details on test material

SWCNT was described by the manufacturer to have a primary particle size of 0.9–1.7 nm and a fiber length < 1 µm (Thomas Swan and Co Ltd, Consett, UK).

Test animals**Species**

rat

Strain

Fischer 344

Sex

female

Details on test animals and environmental conditions

84 female Fisher 344 rats were obtained from Taconic (Ry, Denmark). Animals were acclimatized for at least 1 week before entering the experiments. The rats were housed in a temperature-controlled (22–24°C) and moisture-controlled (40–70%) room with a 12-hr light/12-hr dark cycle. All animals had free access to tap water and Standard Altromin no. 1314 rat chow (Altromin, Lage, Germany) during the

acclimation and housing/treatment periods. Rats were sacrificed at 9 weeks of age. Animals were treated humanely and with regard for alleviation of suffering. All animal procedures followed the guidelines for the care and handling of laboratory animals established by the Danish government, and the Animal Experiment Inspectorate, Ministry of Justice, approved the study (no. 2006/561-1161).

Administration / exposure

Route of administration

oral: gavage

Vehicle(s)

saline or corn oil

Details on exposure

The rats received a single intragastric dose of the particle preparations by oral gavage (0.064 and 0.64 mg/kg body weight suspended in saline or corn oil; n = 8), saline solution (control; n = 10), or corn oil (control; n = 10). Each rat received 200 µL fluid. The rats were sacrificed by cervical dislocation 24 hr after intragastric administration.

Post exposure period

24 hr after intragastric administration

Doses / concentrations

0.064 and 0.64 mg/kg body weight

No. of animals per sex per dose

dose of particles in saline or corn oil; n = 8, saline solution ;control; n = 10, or corn oil ;control; n = 10

Examinations

Tissues and cell types examined

liver, lung, and colon tissues

Details of tissue and slide preparation

The liver, lung, and colon tissues were snap-frozen in liquid nitrogen and stored at –80°C. We investigated effects in these organs because they are along the likely local and systemic exposure route and we have data from similar experiments on DEP as Standard Reference Material 2975 (SRM2975; National Institute of Standards and Technology, Gaithersburg, MD, USA), which was previously reported to be associated with increased levels of DNA damage 24 hr after a single intragastric application (Danielsen et al. 2008b).

Overall remarks, attachments

Remarks on results including tables and figures

ROS-generating ability; Exposure to SWCNT increased the level of 8-oxodG in the liver by 22% (95%

CI, 8–38) and 20% (95% CI, 7–36) in the rats given the low dose and high dose, respectively. There were increased levels of 8-oxodG in the lung after exposure to SWCNT at both the low [21% (95% CI, 9–33)] and high doses [23% (95% CI, 11–35)].

mRNA expression of HO1, MUTYH, NEIL1, NUDT1, and OGG1; Effect of SWCNT did not reach statistical significance.

OGG1 repair activity; Neither the exposure to particles nor the vehicle were associated with a significantly altered level of OGG1 repair activity.

Illustration (picture/graph)

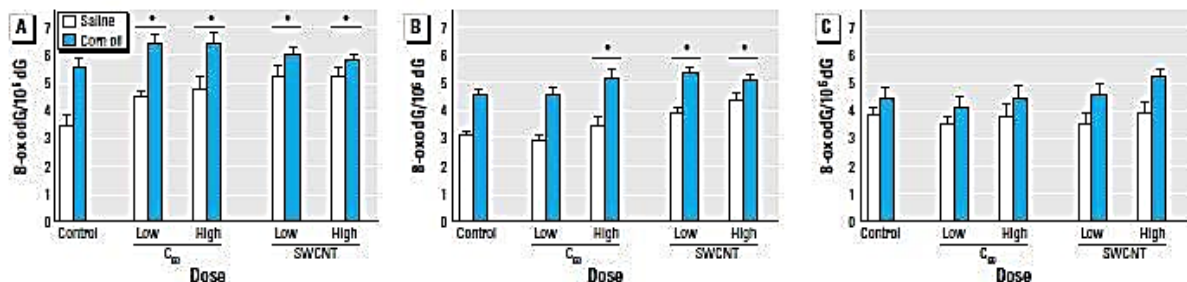


Figure 2. Level of 8-oxodG in liver (A), lung (B), and colon (C) tissue 24 hr after oral exposure to a single dose of C₆₀ fullerenes or SWCNT suspended in saline or corn oil. Values shown are mean $\pm$ SE of 8 treated animals and 10 control animals.

* $p < 0.05$ compared with the control group (single-factor effect of particle exposure, ANOVA).

Table 1. mRNA expression levels of genes involved in the removal of oxidized DNA base lesions in the liver and lung 24 hr after oral administration of C₆₀ fullerenes or SWCNT suspended in either saline or corn oil.

Tissue	Particle dose in saline (mg/kg body weight)			Particle dose in corn oil (mg/kg body weight)		
	0	0.064	0.64	0	0.064	0.64
C₆₀ fullerenes						
Lung						
HO1	10.4 $\pm$ 1.70	6.30 $\pm$ 0.76	6.26 $\pm$ 0.96	5.79 $\pm$ 0.58	8.63 $\pm$ 2.41	7.66 $\pm$ 1.50
MUTYH	6.26 $\pm$ 1.23	2.97 $\pm$ 0.56	4.75 $\pm$ 0.95	5.65 $\pm$ 1.50	7.71 $\pm$ 1.67	8.94 $\pm$ 3.46
NEIL1	3.16 $\pm$ 0.49	2.26 $\pm$ 0.51	2.01 $\pm$ 0.33	2.94 $\pm$ 0.862	2.97 $\pm$ 0.87	3.53 $\pm$ 1.01
NUDT1	0.34 $\pm$ 0.04	0.27 $\pm$ 0.02	0.40 $\pm$ 0.09	1.26 $\pm$ 0.75	1.37 $\pm$ 0.49	1.29 $\pm$ 0.97
OGG1	0.55 $\pm$ 0.07	0.34 $\pm$ 0.06	0.38 $\pm$ 0.06	0.49 $\pm$ 0.07	0.57 $\pm$ 0.11	0.57 $\pm$ 0.10
Liver						
HO1	1.05 $\pm$ 0.12	1.55 $\pm$ 0.28	1.32 $\pm$ 0.18	1.47 $\pm$ 0.28	1.49 $\pm$ 0.37	1.27 $\pm$ 0.22
MUTYH	0.36 $\pm$ 0.07	0.45 $\pm$ 0.09	0.69 $\pm$ 0.30	0.35 $\pm$ 0.04	0.34 $\pm$ 0.05	0.40 $\pm$ 0.06
NEIL1	0.86 $\pm$ 0.16	1.08 $\pm$ 0.25	1.57 $\pm$ 0.74	0.72 $\pm$ 0.10	0.58 $\pm$ 0.13	0.70 $\pm$ 0.08
NUDT1	0.25 $\pm$ 0.07	0.35 $\pm$ 0.1	0.47 $\pm$ 0.18	0.26 $\pm$ 0.09	0.18 $\pm$ 0.03	0.21 $\pm$ 0.07
OGG1	0.12 $\pm$ 0.02	0.14 $\pm$ 0.02	0.25 $\pm$ 0.07*	0.10 $\pm$ 0.02	0.14 $\pm$ 0.03	0.16 $\pm$ 0.03*
SWCNT						
Lung						
HO1	10.4 $\pm$ 1.70	5.03 $\pm$ 1.04	14.1 $\pm$ 7.81	5.79 $\pm$ 0.58	7.15 $\pm$ 1.73	8.14 $\pm$ 2.79
MUTYH	6.26 $\pm$ 1.23	3.48 $\pm$ 1.07	9.80 $\pm$ 3.54	5.65 $\pm$ 1.50	8.65 $\pm$ 3.23	3.63 $\pm$ 0.68
NEIL1	3.16 $\pm$ 0.49	2.03 $\pm$ 0.56	2.80 $\pm$ 0.75	2.94 $\pm$ 0.862	4.88 $\pm$ 1.45	2.24 $\pm$ 0.53
NUDT1	0.34 $\pm$ 0.04	0.32 $\pm$ 0.08	1.78 $\pm$ 0.81	1.26 $\pm$ 0.75	2.29 $\pm$ 1.15	0.47 $\pm$ 0.12
OGG1	0.55 $\pm$ 0.07	0.34 $\pm$ 0.03	1.17 $\pm$ 0.59	0.49 $\pm$ 0.07	0.54 $\pm$ 0.10	0.45 $\pm$ 0.06
Liver						
HO1	1.05 $\pm$ 0.12	1.56 $\pm$ 0.23	1.65 $\pm$ 0.19	1.47 $\pm$ 0.28	1.05 $\pm$ 0.14	1.30 $\pm$ 0.22
MUTYH	0.36 $\pm$ 0.07	0.36 $\pm$ 0.05	0.51 $\pm$ 0.09	0.35 $\pm$ 0.04	0.37 $\pm$ 0.05	0.30 $\pm$ 0.06
NEIL1	0.86 $\pm$ 0.16	0.88 $\pm$ 0.15	0.83 $\pm$ 0.17	0.72 $\pm$ 0.10	0.69 $\pm$ 0.11	0.57 $\pm$ 0.09
NUDT1	0.25 $\pm$ 0.07	0.22 $\pm$ 0.03	0.48 $\pm$ 0.20	0.26 $\pm$ 0.09	0.37 $\pm$ 0.12	0.26 $\pm$ 0.05
OGG1	0.12 $\pm$ 0.02	0.15 $\pm$ 0.03	0.16 $\pm$ 0.04	0.10 $\pm$ 0.02	0.15 $\pm$ 0.04	0.17 $\pm$ 0.04

The mRNA expression is relative to the expression of 18S per 10⁶. Values are mean $\pm$ SE.

* $p < 0.05$ (single-factor effect of the particle exposure, ANOVA).

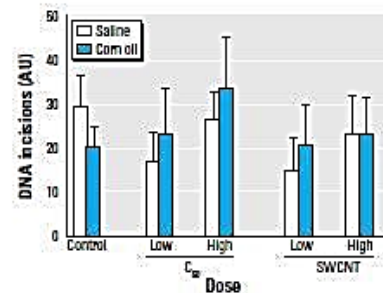


Figure 3. OGG1 repair activity in the liver 24 hr after exposure to a single dose of C₆₀ fullerenes or SWCNT dissolved in saline or corn oil. The activity is indicated as the number of repair incisions in arbitrary units (AU) of substrate nuclei treated with Ro19-8022 and white light, which generate oxidative damage to the DNA. Values shown are mean $\pm$ SE for 8 treated animals and 10 control animals.

Applicant's summary and conclusion

Conclusions

The data obtained from the present study indicate that C60 fullerenes and SWCNT generated oxidatively damaged DNA in liver and lung cells by a gastrointestinal route. The genotoxic effect resulting from exposure to C60 fullerenes and SWCNT was smaller than that from DEP exposure; however, it may be a

cause for concern in humans.

Endpoint study record: Elicarb SWCNT Comet assay; Genetic toxicity in vivo.002

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Jacobsen et al.	2009	Lung inflammation and genotoxicity following pulmonary exposure to nanoparticles in ApoE-/- mice	Particle and Fibre Toxicology 2009, 6:2					

Materials and methods

Type of genotoxicity

DNA damage and/or repair

Type of study

mouse spot test

Test materials

Test material identity

Identifier	Identity
other: Thomas Swan and Co Ltd.	Elicarb

Details on test material

The EliCarb®SWCNT was purchased as a dry powder from Thomas Swan and Co. Ltd. (Consett, UK). Declared primary particle size was 0.9–1.7 nm as diameter and $\leq 1 \mu\text{m}$ as length.

Test animals

Species

mouse

Strain

other: C57BL/6-Apoetm1 (ApoE-/-)

Sex

female

Details on test animals and environmental conditions

Mice aged 4–6 weeks were obtained from Taconic (Ry, Denmark). The mice were randomly divided into groups of 10 housed in polypropylene cages (425mm × 266 mm × 150 mm) with pinewood sawdust bedding and enrichment as sticks of aspen wood and rodent tunnels (Brogaarden, Denmark). The cages were stored in rooms with a 12 h light period from 6 a.m. to 6 p.m., and the temperature and relative humidity in the animal room were $21 \pm 2^{\circ}\text{C}$ and $50 \pm 5\%$, respectively. The cages were sanitized twice weekly. All mice were given free access to tap water and standard mouse chow diet (Altromin no.1324, Christian Petersen, Denmark). The mice were kept under pathogen-limited conditions and were allowed to acclimatize for 2–4 weeks before they entered the experimental protocol. All mice were 8 weeks old at the time of the experiment.

Administration / exposure

Route of administration

intratracheal

Vehicle(s)

0.9% NaCl MilliQ water containing 10% v/v BAL from ApoE-/- mice.

Details on exposure

The mice were anesthetized using Hypnorm® (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml from Janssen Pharma) and Dormicum® (Midazolam 5 mg/mL from Roche). Both were mixed with equal vol. sterile water. A volume of 0.2 ml was injected subcutaneously in the neck of each mouse. The sedated mice were kept on 37°C heating plates. During instillation the mice were placed on their backs on a 40 degree slope. A diode light was placed touching the larynx. The tongue was pressed towards the lower jaw by a small spatula. The trachea was intubated using a 24 gauge BD Insyte catheter (Ref: 381212, BectonDickinson, Denmark) with a shortened needle. The correct location of each intubation was tested by a small but highly sensitive pressure transducer developed by our laboratory in collaboration with John Frederiksen (FFE/P, Copenhagen, Denmark). The particle suspensions were rigorously mixed by pipetting immediately before instillation. A 50 μl suspension was instilled followed by 150 μl air with a 250 μl SGE glass syringe (250F-LT-GT, Micro-Lab, Aarhus, Denmark). The intubation catheter was removed and the mouse transferred to a vertical hanging position with the head up. This ensures that the delivered material is maintained in the lung and does not block the airways. After 5 to 10 min the mice were transferred to the 37°C heating plate until they recovered from anaesthesia.

Post exposure period

3 and 24 hr after instillation

Doses / concentrations

54µg/rat

No. of animals per sex per dose

7 mice per sex per dose

Results and discussions**Any other information on results incl. tables**

Lung tissue: Expression of cytokine mRNA; There were highly significant increases of Mip-2, Mip-1 and Il-6 mRNAs in response to SWCNT instillation at both time points. SWCNT elicited the highest response at 3 h after instillation (52 – 195-fold). At twenty-four hours following instillation CB instilled mice showed increased cytokine levels between 26- and 40-fold whereas SWCNT instilled mice showed increased cytokine levels of 7- to 30-fold.

BAL fluid: Cell differentiation, genotoxicity, protein and LDH; Increased levels of neutrophils and decreased levels of macrophages were detected at both time points following exposure for SWCNT. However, the altered cell composition was only significant following 24 h.

The comet assay was used for determining DNA damage in BAL cells. BAL cells obtained 3 h after SWCNT instillation had elevated level of DNA damage measured as % DNA in the tail. When was analysed the data by tail length, SWCNT particles induced significant DNA damage ($p < 0.001$). SWCNT exposure significantly increased the amount of protein in BAL fluid at both 3 h (1.7 and 1.4-fold, respectively) and 24 h (2.6 and 1.6-fold, respectively).

Overall remarks, attachments**Illustration (picture/graph)**

Table 3: Expression (mRNA) of *Mip-2*, *Mcp-1* and *Il-6* in lung tissue and cell distribution, DNA damage by the comet assay and protein in BAL fluid 3 and 24 h after instillation of Au and carbonaceous nanoparticles in ApoE^{-/-} mice.

		Control	Au 0.54 µg	C ₆₀ 54 µg	SWCNT 54 µg	CB 54 µg	significant dose-related differences ^b
3 h lung tissue	<i>Mip-2</i>	10.2 ± 3.2	34.0 ± 17.1*	10.9 ± 2.0	1170.9 ± 530.1***	108.1 ± 15.1***	SWCNT ≈ CB > Au > C ₆₀
	<i>Mcp-1</i>	10.1 ± 1.2	20.4 ± 3.4***	20.4 ± 2.1***	526.7 ± 214.8***	265.5 ± 163.9***	SWCNT ≈ CB >>> C ₆₀ ≈ Au
	<i>Il-6</i>	2.1 ± 0.4	2.7 ± 0.7	1.7 ± 0.2	411.5 ± 180.6***	31.4 ± 4.8***	SWCNT >>> CB >>> Au ≈ C ₆₀
BAL	Neutrophils % ^a	3.7 ± 1.2	5.5 ± 2.6	2.8 ± 0.7	19.1 ± 8.9	13.8 ± 10.9	SWCNT ≈ CB ≈ Au ≈ C ₆₀
	Macrophages % ^a	93.9 ± 2.0	93.8 ± 2.5	96.9 ± 0.7	78.8 ± 9.0	83.1 ± 10.7	SWCNT ≈ CB ≈ Au ≈ C ₆₀
	Total BAL cells	83262 ± 4819	65081 ± 8276	68929 ± 3849	55426 ± 16930	49417 ± 7700	
	Comet %T	9.6 ± 0.6	11.6 ± 0.9	12.1 ± 0.9	13.4 ± 1.3**	14.4 ± 1.6**	CB ≈ SWCNT >> C ₆₀ ≈ Au
	Protein µg/ml	102.5 ± 5.2	105.2 ± 6.2	77.4 ± 2.9**	171.8 ± 22.2***	139.3 ± 17.3**	SWCNT ≈ CB > Au >> C ₆₀
24 h lung tissue	<i>Mip-2</i>	5.1 ± 0.5	8.3 ± 1.7	31.0 ± 12.8***	34.4 ± 9.0***	134.8 ± 33.2***	CB > SWCNT ≈ C ₆₀ >> Au
	<i>Mcp-1</i>	28.3 ± 2.7	34.6 ± 8.5	116.0 ± 22.8***	372.7 ± 110.2***	1087.0 ± 310.6***	CB ≈ SWCNT >> C ₆₀ >>> Au
	<i>Il-6</i>	1.1 ± 0.1	2.0 ± 0.5	5.6 ± 1.1***	32.5 ± 9.9***	44.0 ± 13.0***	CB ≈ SWCNT >> C ₆₀ >> Au
BAL	Neutrophils % ^a	5.3 ± 1.6	4.2 ± 3.1	6.4 ± 4.2	64.7 ± 7.1***	75.8 ± 3.4***	CB ≈ SWCNT >>> C ₆₀ ≈ Au
	Macrophages % ^a	93.6 ± 1.5	94.7 ± 3.6	93.1 ± 4.2	28.6 ± 4.9***	22.1 ± 3.7***	CB ≈ SWCNT >>> C ₆₀ ≈ Au
	Total BAL cells	65290 ± 5246	67881 ± 6667	76008 ± 7420	61643 ± 19999	78596 ± 21414	
	Protein µg/ml	110.6 ± 5.7	109.5 ± 5.2	83.6 ± 7.8***	288.8 ± 21.3***	182.4 ± 7.1***	SWCNT >>> CB >>> Au >>> C ₆₀

Results are given as mean ± SEM. Expression of mRNA is normalized to 18S rRNA and is multiplied by 10⁷. The statistical analysis was carried out with one-factor ANOVA tests and the type of particles as categorical variable. ^aDue to uneven variance, we determined 95; 99 and 99.9% confidence interval for means. ^b≈, >, >>, >>> indicate statistical significance $p > 0.05$, $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. *, **, *** refer to statistical significance $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Applicant's summary and conclusion

Executive summary

ApoE^{-/-} mice is a sensitive model for comparing inflammatory potential of (nano) particle instillation. CB and SWCNT caused more inflammation and DNA and cell damage than C60 did. This inflammatory signalling appeared shorter for SWCNT than for CB and C60.

Endpoint study record: CNI SWCNT K-ras mutation test; Genetic toxicity in vivo.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Shvedova et al.	2008	Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis	Am J Physiol Lung Cell Mol Physiol 295: L552–L565, 2008.					

Cross-reference to same study

7.9.3 Specific investigations: other studies CNI SWCNT Acute toxicity; Pharyngeal aspiration.003

Materials and methods**Type of genotoxicity**

gene mutation

Type of study

other: K-ras mutation

Test materials**Test material identity**

Identifier	Identity
other: CNI SWCNT	

Details on test material

SWCNT were purchased from Carbon Nanotechnology (CNI, Houston, TX). The nanotubes were manufactured using the high-pressure CO disproportionation process (HiPco) and were used in the inhalation and pharyngeal aspiration studies as produced; i.e., without being purified or otherwise treated after the initial production process. Analysis indicates that these SWCNT contained elemental carbon (82% wt), Fe (17.7%). Trace elements present included Cu (0.16%), Cr (0.049%), and Ni (0.046%). The diameter of SWCNT measured by transmission electron microscopy (TEM) was 0.8 –1.2 nm. The length of SWCNT was 100–1,000 nm measured by Carbon Nanotechnology using atomic force microscopy. The surface area of SWCNT measured by the nitrogen absorption-desorption technique (Brunauer-Emmett-Teller method, BET) was 508 m²/g.

Test animals**Species**

mouse

Strain

C57BL

Sex

female

Details on test animals and environmental conditions

Specific pathogen-free adult female C57BL/6 mice (8–10 wk) were supplied by The Jackson Laboratory (Bar Harbor, ME) and weighed 20.0 ± 1.9 g when used. Animals were housed one mouse per cage receiving HEPA filtered air in Association for Assessment and Accreditation of Laboratory Animal Care-approved National Institute for Occupational Safety and Health (NIOSH) animal facilities. All animals were acclimated in the animal facility under controlled temperature and humidity for 1 wk before use. Animals were supplied with water and certified chow 5020 (Purina Mills, Richmond, IN) ad libitum in accordance with guidelines and policy set forth by the Institute of Laboratory Animals Resources, National Research Council. All experimental procedures were conducted in accordance with a protocol approved by the NIOSH Institutional Animal Care and Use Committee.

Administration / exposure**Route of administration**

other: inhalation and pharyngeal aspiration

Vehicle(s)

PBS

Details on exposure

Inhalation; The generation system used to deliver respirable SWCNT structures (5 mg/m³) at a flow rate of 10 l/min to 2 animal exposure chambers each containing 12 mice. Exposure was at a target mass concentration of 5.0 mg/m³ in the chamber, 5 h/day for 4 days. The mean SWCNT concentration determined by the gravimetric samples was 5.52 ± 1.37 mg/m³ (mean SD). SWCNT mass mode aerodynamic diameter was 4.2 µm. The deposited dose in the pulmonary region of the mouse respiratory tract was assumed to be ~5 µg. Particulate instillation; Mouse pharyngeal aspiration was used for particulate administration. Briefly, after anesthetization with a mixture of ketamine and xylazine (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), a suspension (50 l) of SWCNT prepared in PBS (5, 10, and 20 µg per mouse) was placed posterior on the throat and the tongue, which was held until the suspension was aspirated into the lungs. Control mice were administered sterile Ca²⁺ Mg²⁺-free PBS vehicle. Mice were euthanized on days 1, 7, and 28 following the exposures.

Post exposure period

1, 7 and 28 days following the exposure.

Doses / concentrations

5, 10, and 20 µg per mouse

Overall remarks, attachments

Remarks on results including tables and figures

To assess genotoxicity of SWCNT, accumulation of K-ras mutations after the inhalation and aspiration exposure was evaluated. An example of K-ras mutation analysis by DGGE is shown in Fig. 12. The DGGE reveals the patterns of three different K-ras mutant alleles detected in this study, the first two consisting of a change of the wild-type codon 12 (GGT, glycine) to an AGT (serine, lane 2) and a GAT (aspartate, lane 3). The third mutant allele (lane 4) contains a double mutation, including a GGT to GAT (aspartate) at codon 12 and a GTG to ATG at codon 8 (valine to methionine). K-ras mutations identified in tissue sections prepared from lungs of C57BL/6 mice in this study are summarized in Table 4. These mutations were found in mice treated with SWCNT by inhalation, whereas aspiration caused a low frequency of mutations that did not differ from untreated controls. Taken together, the results of these studies indicate a trend that a difference in the proportion of mutations exists among some of the various treatment/time groups. Particularly, the mutation proportion is higher among the mice treated with SWCNT by inhalation route and euthanized at both 7 and 28 days postexposure, compared with that in mice treated with SWCNT by aspiration. Interestingly, one of the mutations consists of a double mutation in codons 12 and 8 of the K-ras gene in mice at day 28 postinhalation. These mutations have not been reported previously and may be specific for the SWCNT-exposure.

Illustration (picture/graph)

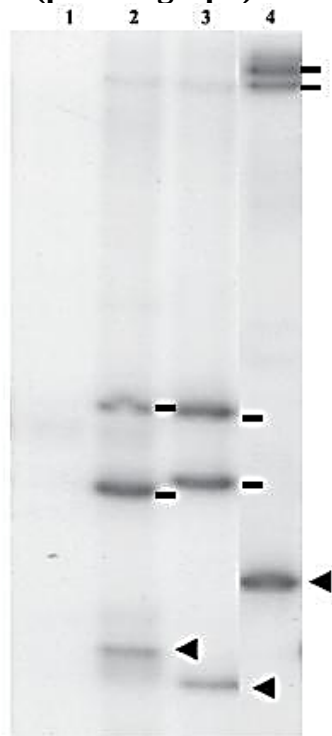


Fig. 12. An example of *K-ras* mutation analysis by denaturing gradient gel electrophoresis (DGGE). The gel shows 4 lanes (lanes 1–4) corresponding each to a specific pattern of *K-ras* mutant alleles from 4 different mouse lungs as separated by DGGE. Lung DNA sample 1 (lane 1) shows no mutation. Lung DNA samples 2, 3, and 4 each show a different mutation as revealed by the difference in their mutant band pattern, consisting of 1 mutant homoduplex (indicated by an arrowhead) at the bottom of the gel and the 2 respective mutant/wild-type heteroduplexes (each indicated by a line) in the middle (lanes 2 and 3) and at the top of the gel (lane 4). The 1st 2 mutations corresponded to a change at the wild-type *K-ras* gene codon 12 (GGT, glycine) to AGT (lane 2, serine) and GAT (lane 3, aspartate). The 3rd mutation is a double mutation consisting of a GGT to GAT (glycine to aspartate at codon 12) and a GTG to ATG (valine to methionine at codon 8). The type of mutation in each lane was determined by sequencing analysis of the mutant homoduplex band isolated from the gel.

Table 4. *K-ras* mutations among the various groups of mice

Type of Exposure	Recovery, days	Exposure	Mutation Incidence	Mutation Type, wild-type <i>K-ras</i> codon 12: GGT	Total
Inhalation	1	Air	0/5 (0.0%)		Air: 4/15 (26.7%)
		SWCNT	2/6 (33.3%)	1 GAT, 1 AGT	
	7	Air	2/5 (40.0%)	1 GAT, 1 AGT	SWCNT: 10/16 (62.5%)
		SWCNT	4/5 (80.0%)	2 GAT, 2 AGT	
	28	Air	2/5 (40.0%)	1 GAT, 1 AGT	
		SWCNT	4/5 (80.0%)	2 GAT, 1 AGT, 1 double mutation*	
Aspiration	7	PBS	0/3 (0.0%)		PBS: 1/8 (12.5%)
		SWCNT	0/3 (0.0%)		
	28	PBS	1/5 (20.0%)	1 GAT	SWCNT: 1/8 (12.5%)
		SWCNT	1/5 (20.0%)	1 AGT	

*The double mutation contains a GGT > GAT at codon 12 and a GTG > ATG at codon 8 of the *K-ras* gene. The χ^2 analysis of the proportion of mutations in the 6 treatment/time groups: 7 days/SWCNT/Aspiration (Asp), 7 days/SWCNT/Inhalation (Inh), 7 days/Sham, 28 days/SWCNT/Asp, 28 days/SWCNT/Inh, 28 days/Sham, $P = 0.045$, implying statistical significance. Note that there are 11 cells with expected counts <5. The χ^2 analysis of the proportion of mutations in the 3 treatment/time groups (7 days postexposure) with $P = 0.045$, implying statistical significance. Note that there are 5 cells with expected counts <5. The χ^2 analysis of the proportion of mutations in the 3 treatment/time groups (28 days postexposure) with $P = 0.075$. Note that there are 5 cells with expected counts <5. The χ^2 analysis of the proportion of mutations in the 3 treatment/time groups: 7 days/SWCNT/Inh, 28 days/SWCNT/Inh, 28 days/Sham, $P = 0.08$. Note that there are 5 cells with expected counts <5.

Applicant's summary and conclusion

Conclusions

The detected mutations frequency of 62.5% (10/16) in mice after SWCNT inhalation is significantly higher than that in the untreated group of mice 26.7% (4/5). Further studies involving a bigger sample size may help in determining the usefulness of this gene mutation as a biomarker of exposure to SWCNT. Furthermore, the potential role in lung carcinogenesis requires further investigation by comparing C57BL/6 mice with other mouse strains susceptible to lung tumor formation.

Endpoint study record: CNI SWCNT DNA damage test; Genetic toxicity in vivo.002

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Li et al.	2007	Cardiovascular Effects of Pulmonary Exposure to Single-Wall Carbon Nanotubes	Environmental Health Perspectives, Vol. 115, No.3, 377-382(2007)					

Cross-reference to same study

7.9.3 Specific investigations: other studies CNI SWCNT Acute toxicity; Pharyngeal aspiration.002

Materials and methods

Type of genotoxicity

DNA damage and/or repair

Type of study

mouse spot test

Test materials**Test material identity**

Identifier	Identity
other: CNI SWCNT	

Details on test material

SWCNT (CNI, Houston, TX) were produced by the high pressure carbon monoxide proportionation process (HiPco) technique (Scott et al. 2003; Scott and Smalley 2003) and purified by acid treatment to remove metal contaminants (Gorelik et al. 2000). Their physicochemical characteristics, including chemical content, mean diameter, and surface area, were reported previously (Shvedova et al. 2005).

Test animals**Species**

mouse

Strain

C57BL

Sex

male

Details on test animals and environmental conditions

Male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were between 2 and 3 months of age when exposures began, and were fed an irradiated commercial mouse chow diet (LM-485; Harlan, Indianapolis, IN). All mice were provided tap water ad libitum and were housed in ventilated cages on autoclaved hardwood chip bedding. Sentinel mice were free of endogenous pathogens. Animal room conditions included HEPA-filtered air, controlled temperature and humidity, and a 12-hr light/dark cycle. Animal care and use procedures, including sacrifice by carbon dioxide asphyxiation, were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare 2002) and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996); these procedures were approved by the National Institute for Occupational Safety and Health institutional animal care and use committee. All mice used in these studies were treated humanely and with regard for alleviation for suffering.

Administration / exposure**Route of administration**

other: Pharyngeal aspiration

Vehicle(s)

PBS

Details on exposure

Mice were anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL) and placed on a slant board, and the tongue was gently held in full extension while a 60- μ L suspension of particles was pipetted onto the base of the tongue. This technique provides for a wide dissemination of particles in a peribronchiolar pattern within the alveolar region (Rao et al. 2003).

Post exposure period

1, 7, 28, and 56 days after exposure

Doses / concentrations

0,10,40 μ g/mouse

No. of animals per sex per dose

4 mice per sex per dose

Examinations**Any other information on materials and methods incl. tables**

Genomic DNA was isolated using the Qiagen genomic tip 20G kit (Qiagen Inc., Valencia, CA). The DNA concentration was measured using the PicoGreen dsDNA quantitation kit (Molecular Probes, Eugene, OR). QPCR of extra long amplification product was carried out using the Expand 20 kb PCRplus System (Roche, Mannheim, Germany). A mouse 12-kb mtDNA fragment was amplified using the following primers: F5'-AGAGATTCTCTACACCTTCGAATTTGC-3' and R5'-ATTTAATTTGAGGGTGACGGGCGG-3'. The PCR reaction system contained 100 ng DNA as a template. Amplification consisted of an initial cycle of denaturation (92°C, 2 min) followed by 25 cycles of denaturation (92°C, 30 sec), annealing (65°C, 30 sec), and extension (68°C, 15 min), with a final extension (68°C, 10 min) cycle. A short (180 bp) fragment from the D-loop part of mtDNA was amplified using the following primers: F5'-TAATCAGCCCATGACCAACA-3' and R5'-GATTGGGTTTTGCGGACTAA-3'. Ten microliters of PCR products were applied to gel electrophoresis (0.8% agarose gel, 75 V, 1 hr). Gels were stained by ethidium bromide, imaged using the Alphaimager System (Alpha Innotech Corp., San Leandro, CA), and quantified by scanning laser densitometry.

Overall remarks, attachments**Remarks on results including tables and figures**

Significant reduction in mtDNA amplification, reflecting an increase in mtDNA damage, was observed in the aortic tissue of C57BL/6 mice exposed to SWCNT (10 and 40 μ g/mouse) at days 7, 28, and 60 after a single exposure, compared with age-matched, vehicle-treated control mice ($p < 0.01$) (Figure 2A). Maximum mtDNA damage occurred 7 days after exposure in both treatment groups and persisted in the high-dose treatment group 60 days after exposure. Amplification of a small fragment of the D-loop of the mitochondrial genome (Figure 2A), as well as nuclear 18S rRNA (data not shown), was not affected by the exposure. Accordingly, the amplification of the large mtDNA fragment was normalized to the amplification of the small fragment. In contrast to SWCNT, UfCB pharyngeal instillation did not result in mtDNA damage in the aortic tissue at comparable doses of exposure (Figure 2B).

Changes in mitochondrial GSH levels are also manifestations of oxidative stress (Suliman et al. 2004). The aortic mitochondrial GSH/GSSG ratios were significantly reduced 7 days after pulmonary exposure to a single high dose of SWCNT (Figure 3A). Oxidative stress also may lead to oxidation of amino acid

residues on proteins, forming protein carbonyls (Dalle-Donne et al. 2003). Consistently, significant accumulation of aortic mitochondrial protein carbonyl groups occurred in SWCNTexposed mice compared with the age-matched vehicle-exposed mice at both exposure levels ($p < 0.05$) (Figure 3B).

Illustration (picture/graph)

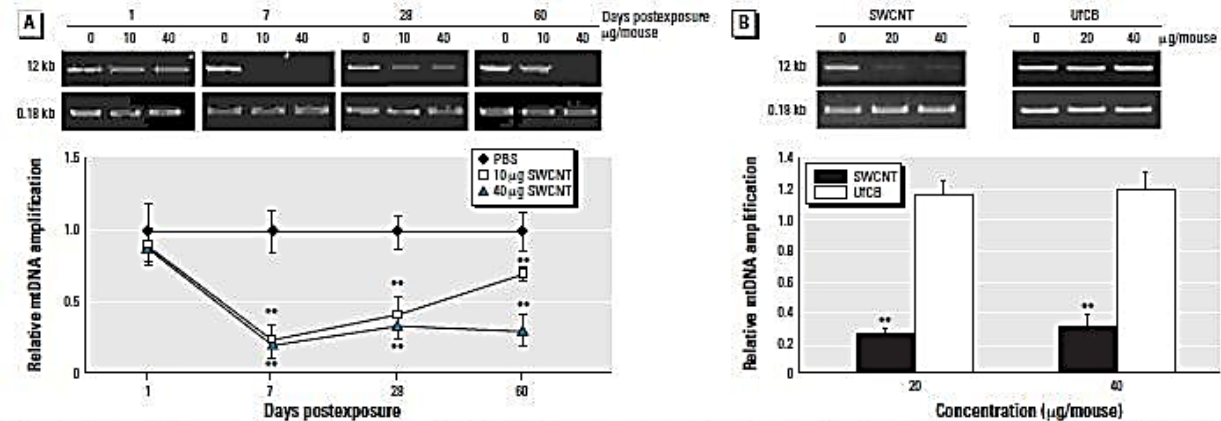


Figure 2. Aortic mtDNA damage in C57BL/6 mice exposed to SWCNTs shown as representative gel images of the long (12 kb) and short fragment (0.18 kb) mtDNA amplification products and quantitative analyses of the mtDNA damage (a reduction in the amplification) presented as a fold difference between each SWCNT-treated group and the vehicle-treated group. (A) Mice were treated with SWCNTs (0, 10, or 40 µg) and aortas were collected at 7, 28, and 60 days postexposure. (B) Mice were treated with SWCNTs (0, 20, or 40 µg) or UICBs (0, 20, or 40 µg) and aortas were collected 60 days postexposure. The 12 kb mtDNA expression was normalized to 0.18 kb mtDNA expression. Each value represents the mean $\pm$ SE of four mice. ** $p < 0.01$.

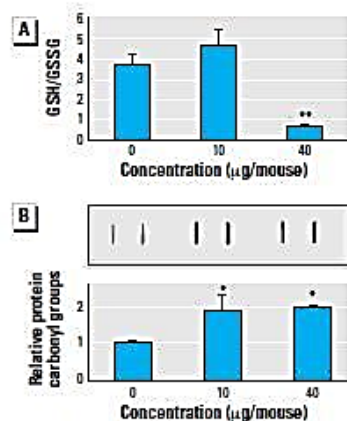


Figure 3. Oxidative modifications in aortic mitochondria freshly isolated from C57BL/6 mice treated with SWCNTs (0, 10, or 40 µg) 7 days postexposure. (A) GSH/GSSG ratio of aortic mitochondria. (B) Protein carbonyl groups in the aortic mitochondrial proteins (10 µg) shown by representative dot blot images and densitometric analysis. The results are presented as a fold change in the aortic protein carbonyl levels of SWCNT-exposed mice compared with PBS-exposed mice. Each value represents the mean $\pm$ SE of four mice. * $p < 0.05$. ** $p < 0.01$.

Applicant's summary and conclusion

Executive summary

C57BL/6 mice, exposed to SWCNT (10 and 40 µg/mouse), developed aortic mtDNA damage at 7, 28, and 60 days after exposure. mtDNA damage was accompanied by changes in aortic mitochondrial glutathione and protein carbonyl levels.

7.7 Carcinogenicity

7.8 Toxicity to reproduction

7.8.1 Toxicity to reproduction

7.8.2 Developmental toxicity / teratogenicity

Endpoint study record: Cheap Tubes SWCNT Developmental toxicity / teratogenicity.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Pietrojusti et al.	2011	Low Doses of Pristine and Oxidized Single-Wa II Carbon Nanotubes Affect Mammalian Embryonic Development	ACS Nano, vol.5, No.6, 4624-4633					

Materials and methods

Test materials

Test material identity

Identifier	Identity
other: Cheap Tubes Inc.	Cheap Tubes SWCNT

Details on test material

p-SWCNT and o-SWCNTs were purchased from Cheap Tubes Inc.(Brattleboro, VT).

Test animals

Species

mouse

Strain

CD-1

Details on test animals and environmental conditions

All animal procedure were approved by the Institutional Animal Care and Use Committee and comply with European rules (116/92). Animals used in the Present study were six to eight week old females of the CDI outbred strain (Charles River. Calko. Italy), considered as a general multipurpose model for in vivo studies. All animals were housed and mated under standard laboratory conditions. Their weight was between 30 and 35 g. Females at day 5.5 of gestation were injected in the retrobulbar plexus with n-CB, p,o, u-SWCNTS (stock solutions of 1 mg/mL in DMEM containing 10 mg/mL BSA), or control medium (DMEM-BSA) saline solution was used to adjust the final injection volume to 100µL.

Administration / exposure

Route of administration

intravenous

Vehicle

other: DMEM-BSA

Details on exposure

The mouse was immobilized on absorbent paper, keeping it motionless. Using a 1 cc syringe and a 27 gauge needle, a volume of 100µL was gently injected in the center of the retroorbital sinus of the right eye. No local complications related to the procedure were observed. Ten days later, control and treated females were euthanized by carbon dioxide, and fetuses and their placentas collected and analyzed for the presence of malformations under a stereomicroscope. Fetuses that presented evident morphological abnormalities were photographed and then fixed with their placentas in 4% paraformaldehyde together with a morphologically normal sibling for subsequent histochemical and immuno-histochemical analysis. In parallel, fetuses and placentas from control mothers were analyzed.

Duration of treatment / exposure

SWCNT was intravenously (iv) injected in the retrobulbar venous plexus of pregnant females at postcoital day 5.5 (5.5 dpd. At 15.5 dpd, animals were sacrificed, and fetuses and placentas were analyzed.

Doses / concentrations

Basis analytical conc. (0.01,0.1,0.3,3,30µg/mouse)

No. of animals per sex per dose

18~22 females per dose

Results and discussions***Maternal toxic effects***

no effects

Details on maternal toxic effects

Injected mice were daily observed for possible acute or delayed side effects related to the injection of nanomaterials. No deaths, clinically relevant disorders, or evident behavioral changes were recorded.

Details on embryotoxic / teratogenic effects

At 30µg/mouse dose, all types of SWCNTs but not nCB, were able to induce gross fetal morphological abnormalities (Table 2). In addition, a substantial percentage of SWCNTs exposed mice (ranging from 19% to 31%) presented swollen uteri (at least twice the diameter of a nonpregnant normal uterus) with no developed embryos (Table 2), a finding never observed in control females and which we interpreted as evidence of early miscarriages. With the aim to identify the concentration of SWCNTs showing no teratogenic or abortive effects, decreasing doses of the materials were used in subsequent experiments. Interestingly, at 3µg/mouse the percentage of females with early miscarriages greatly decreased, but percentages of mothers with malformed fetuses increased (Table 2). At further lower dosages (0.3 and 0.1µg/mouse), no miscarriages were observed, but mothers carrying fetuses with gross malformations were still present, although in much lower percentages (Table 2). Finally, at the concentration of 0.01µg/mouse no miscarriages or fetal malformations were observed (Table 2). A wide variety of malformations was found (Figure 2A-H), but no differences in their type and severity were observed among the three groups of CNTs and among different concentrations within each group. In some cases the fetuses appeared morphologically normal, but significantly growth retarded (Figure 2E); more often, fetuses with abdominal wall or head deformities or limb hypoplasia (Figure 2D and F) were observed. In more abnormal fetuses, a severe retardation in the development of several organs and tissues was associated with an abnormal torsion of the trunk (Figure 2G), or the body plan was profoundly affected and the outlines of the fetus could be barely identified (Figure 2H). In a small set of experiments, females were treated with low doses of SWCNTs (0.3 and 0.1µg/mouse) at the peri-implantation stage between day 4.5 and 5 of gestation. Interestingly, in p- and uo-SWCNTs treated females, besides the presence of malformations, a few fetuses were found to share the same placenta (Figure 2I and L).

Overall remarks, attachments

Illustration (picture/graph)

TABLE 1. Physico-chemical Properties of SWCNTs

	p-SWCNTs	o-SWCNTs	uo-SWCNTs
elemental analysis ^a (% w/w)	Si 0.24 ± 0.06	Si 0.28 ± 0.08	Si 0.12 ± 0.03
	S 0.45 ± 0.04	S 0.48 ± 0.02	S 0.44 ± 0.10
	Ca 1.22 ± 0.31	Ca 0.20 ± 0.004	Ca 0.11 ± 0.02
	Cr 1.12 ± 0.26	Cr 0.63 ± 0.03	Cr 0.20 ± 0.07
	Fe 1.10 ± 0.25	Fe 0.26 ± 0.02	Fe 0.14 ± 0.04
diameter (nm) ^b	2.37 ± 0.40	1.58 ± 0.20	1.80 ± 0.40
	length (µm) ^b	0.85 ± 0.42	0.76 ± 0.70
	weight loss % (TGA) ^c	0.50 ± 0.50	1.90 ± 0.50
acidic sites (nmol/mg) ^d	24.20 ± 1.10	42.90 ± 6.70	56.50 ± 8.40
extent of defect (1/µg) ^e	0.90	0.10	0.10

^a Evaluated by XRF spectroscopy (mean ± SE). ^b Evaluated by TEM analysis (mean ± SD). ^c Mean ± SD. ^d Acidic sites evaluated as described (ref 4 in SI) (mean ± SD). ^e Evaluated by Raman spectroscopy

TABLE 2. Summary of the Percentage of Females Whose Pregnancy Was Affected by the Exposure to Different Concentrations of the Three Samples of SWCNTs^a

	30 µg/mouse				3 µg/mouse				0.3 µg/mouse				0.1 µg/mouse				0.01 µg/mouse			
	♀	A	B	A+B	♀	A	B	A+B	♀	A	B	A+B	♀	A	B	A+B	♀	A	B	
CTRL	20	0	0	0	21	0	0	0	21	0	0	0	21	0	0	0	20	0	0	
n-CTRL	23	0	2	0	18	0	0	0	18	0	0	0	20	0	0	0	20	0	0	
p-SWCNTs	21	19	4.7	23.7	22	4.5	27.3	31.8	19	0	5.3	5.3	20	0	5	5	18	0	0	
o-SWCNTs	20	20	20	40	21	4.8	9.5	14.3	20	0	5	5	18	0	5.5	5.5	18	0	0	
uo-SWCNTs	16	31	25	56	20	0	45	45	16	0	18.7	18.7	23	0	8.7	8.7	20	0	0	

^a ♀: number of females analyzed in each group. A: percentage of ♀ with swollen uteri and no fetuses. B: percentage of ♀ with at least one malformed fetus.

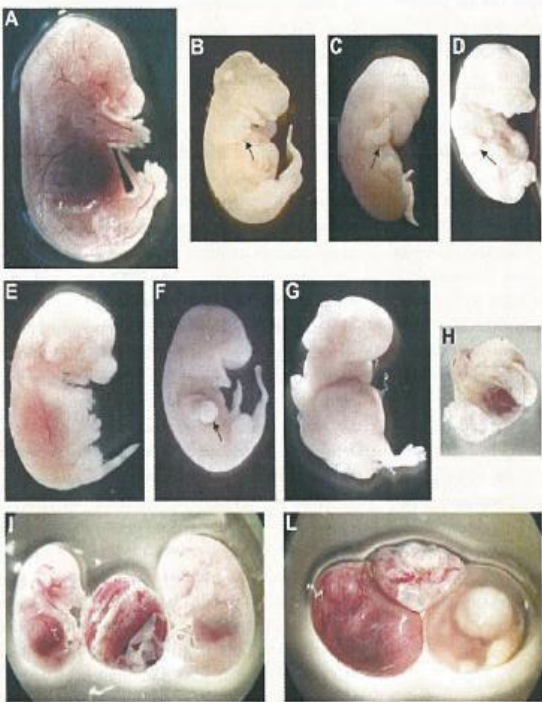


Figure 2. Representative images of 15.5 dpc malformed fetuses from SWCNT-treated mothers. Images refer to 0.3 µg/mouse exposed fetuses, but similar phenotypes were observed at all other effective doses tested. (A) Normal fetus. (B–D) Malformed fetuses recovered from (B) p-, (C) o-, and (D) uo-SWCNT-treated mothers. SWCNT-exposed fetuses appeared developmentally hindered compared to the control. Arrows indicate limbs that appear severely retarded in their development. (E–H) Independently from the type and dosage of SWCNT injected, fetuses with malformations of different severity were recovered. (E) Some fetuses, although smaller, appeared only slightly affected by the treatment, with an overall body plan comparable to that of normal fetuses. (F) Fetuses with a more severe retardation in the development of the limbs and snout were observed. (G) In some cases fetuses missed the crown-rump organization and appeared folded, with swollen abdomen. (H) Small abnormal placentas with a very early developmentally arrested embryo were also recovered. (I, L) Two cases of monozygotic, dizygotic twin fetuses. (I) Twins appear morphologically normal but the fetus on the left side is significantly smaller. (L) In this couple the twin on the right side appears severely developmentally retarded. All photographs are at the same

Applicant's summary and conclusion

Conclusions

Results suggest that exposure to SWCNTs may represent a potential risk for pregnant women, especially in the occupational setting, where the risk of accidental exposure may become real in light of the foreseen increased production of ENM in the near future.

7.8.3 Toxicity to reproduction: other studies

7.9 Specific investigations

7.9.1 Neurotoxicity

Endpoint study record: Neurotoxicity.German contribution to in vitro neurotoxicity 001

Administrative Data

other:OECD/WPMN

Purpose flag supporting study

Study result type experimental result

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
other: report to DDP	GER/INOS project								

Materials and methods

Test materials

Details on test material

SWCNTs cleaned with acid in order to remove contaminations of metal-catalysts (SWCNT (a.t.), SWCNTs cleaned with acid and functionalised with salmon sperm DNA (fSWCNT (a.t.) and SWCNTs functionalised with salmon sperm DNA but not acid treated (fSWCNT)).

Examinations

Any other information on materials and methods incl. tables

Several cell culture systems were used (oligodendrocyte cell line OLN 93, primary neuronal cultures and astrocytes obtained from fetal or newborn rat brain). fSWCNT (a.t.) featured a stable suspension with loose or small clustered tubes of a diameter of 1-2nm, while not-functionalised SWCNTs showed strong sedimentation and agglomeration (investigated by REM). Investigated Endpoints were: *in vitro* viability and cytotoxicity, cell proliferation, oxidative stress, measurement of mitochondrial membrane potential, adhesion of cells, gene expression of adhesion molecules, apoptosis, calcium signals (using the following assays: CCK 8 -> cell adhesion, Alamar Blue test -> metabolic activity, MTT assay -> cell viability, , BrdU assay -> cell proliferation, JC-1 assay -> mitochondrial membrane potential, annexin/propidium iodide staining-> apoptosis and necrosis, RT-PCR -> gene expression, calcium binding fluorescent dyes Fura-2/AM and Fluo-4/AM -> changes of intracellular calcium concentration)

Results and discussions

Further observations for developmental neurotoxicity study

Any other information on results incl. tables

Localisation of SWCNTs (TEM): SWCNTs could not be detected inside the cells. No SWCNTs were found in the nucleus. Adhesion: Upon 96h exposure of (directly with sowing of cells) fSWCNT (a.t.) reduced the normal adhesion of OLN-93 cells (starting at an exposure concentration of 5 µg/ml). PCR arrays revealed changes in gene expression of proteins of the extracellular matrix of proteins and involved in adhesion upon 72 h exposure of 5 µg/ml fSWCNT (a.t.) (upregulated genes: *mmp1a*, *mmp11*, *mmp15*, down regulated genes: *itga1*). Down regulation upon exposure of 10 µg/ml fSWCNT (a.t.) was found for *forcdh2* and *emilin*.

Cell viability: Upon 72 h exposure of SWCNT (a.t.) CCK-8 assays revealed significant decrease of cell viability in all tested cell types (OLN-93 cells: exposure of 10 µg/ml, astrocytes: exposure of 1 µg/ml, neurons: exposure of 5 µg/ml). Cytotoxicity via the MTT assay was found in astrocytes and neurons starting at 1 µg/ml SWCNTs (a.t.). Using functionalized SWCNTs (a.t.) (fSWCNT (a.t.)) the MTT assay also revealed significant increase of cytotoxicity in astrocytes and neurons starting with an exposure concentration of 1 µg/ml. DNS alone also induced cytotoxic effects in astrocytes, but only in slight extent. The CCK-8 assay showed a decrease of metabolic activity in astrocytes upon exposure of 5 µg/ml fSWCNT (a.t.), but not upon exposure of fSWCNT without acid treatment. The Alamar Blue assay showed no effect in all three cell types (OLN-93, astrocytes, neurons).

Cell proliferation: Proliferation of OLN-93 and astrocytes was determined using the BrdU assay. Upon 48 h exposure of fSWCNT (a.t.) cells were counted using flow through cytometry. OLN-93 cells exhibited a strong reduction of cell proliferation upon exposure of 5 µg/ml fSWCNT (a.t.). Significant reduction of cell proliferation in astrocytes was found upon 20 µg/ml fSWCNTs (a.t.). 72 h exposure of 5 µg/ml fSWCNTs (a.t.) and more significantly reduced the total cell numbers of astrocytes and OLN-93 cells. Upon exposure of fSWCNTs (a.t.) directly with the sowing of cells cell number of OLN_93 cells was even more reduced (down to less than 10% compared to control upon exposure of 10 µg/ml for 96h).

Changes of the mitochondrial membrane potential: Using the JC-1 assay a reduction of the mitochondrial membrane potential was found in all 3 tested cell types (OLN-93, astrocytes, neurons) upon 72 h exposure of fSWCNT (a.t.) (OLN-93 cells and astrocytes: reduction down to 45% and 70% respectively starting upon exposure of 10 µg/ml, neurons: reduction down to 65 % starting upon exposure of 20 µg/ml). Changes in the mitochondrial membrane potential were not found upon exposure of fSWCNTs which were not acid treated.

Apoptosis: Annexin-V-FITC staining combined with propidium iodide staining was used to identify apoptotic and necrotic cells in primary astrocytes and OLN-93 cells upon 72 h exposure of 20 µg/ml fSWCNT (a.t.) and fSWCNT. Astrocytes showed a not-significant increase of apoptotic and necrotic cells upon exposure to fSWCNT (a.t.). No changes compared to control were found upon exposure to fSWCNT. OLN-93 cells showed no increase of apoptotic and necrotic cells upon exposure to fSWCNT (a.t.) or fSWCNT.

Changes of the intracellular calcium concentration: OLN-93 cells were pre-exposed to fSWCNT and fSWCNT (a.t.) as well as DNS, then exposed for 96h and stimulated with 10 ng/ml rIL-3. Experiments were performed in calcium-containing buffer. Fluorescence of the introduced dyes was used as index for changes of the free intracellular calcium concentration. Upon 96 h of 10 and 20 µg/ml fSWCNT (a.t.) exposure cells revealed a significant increase of intracellular calcium concentration. Stimulation of calcium by rIL-3 strengthened the effect. No increase of calcium concentration was found for the not acid-treated fSWCNT of the identical exposure concentrations and for DNS alone. The decline of calcium concentration after 30-40 seconds was steeper in cells exposed to 5, 10 and 20 µg/ml fSWCNTs (a.t.) compared to controls, but these changes were not significant.

7.9.2 Immunotoxicity

7.9.3 Specific investigations: other studies

Endpoint study record: Nikkiso SWCNT Intratracheal Instillation.001

Administrative Data

Purpose flag	key study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	NEDO Project P06041, Risk Assessments of Manufactured Nanomaterials - Carbon Nanotubes (CNT)-	http://www.aist-riss.jp/main/modules/product/nano_rad.html?ml_lang=en	University of Occupational and Environmental Health, Japan (UOEH)				

Materials and methods

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Nikkiso SWCNT

Test animals

Species

rat

Strain

Wistar

Sex

male

Details on test animals and environmental conditions

9 weeks old, 300g

Administration / exposure**Route of administration**

intratracheal

No. of animals per sex per dose

20 males/dose

Control animals

no

Examinations**Any other information on materials and methods incl. tables**

0.2 mg/rat (low dose: 0.67 mg/kg) and 0.4 mg/rat (high dose: 1.33 mg/kg) SWCNTs were suspended in distilled water including 0.1% of Triton and single intratracheal instillations were performed to the rats. Histopathological observation of the lung and examination of inflammation biomarkers in BALF: 3 d, 1 w, 1 m, 3 m, 6 m, 1 y and 2y after instillation. Inflammation and fibrosis were examined up to six months and will be examined up to two years.

Results and discussions**Any other information on results incl. tables**

Both the high dose and the low dose showed inflammatory cellular infiltration and particularly it lasted for six months for high dose. Also, HO—1 gene expression increased continuously in both high and low doses.

Applicant's summary and conclusion**Conclusions**

Both the high dose and the low dose showed inflammatory cellular infiltration and particularly it lasted for six months for high dose. Also, HO—1 gene expression increased continuously in both high and low doses. Observation is ongoing up to two years. Further information will be added and conclusion will be made after last observation.

Endpoint study record: Super Growth SWCNT Intratracheal Instillation.001**Administrative Data****Purpose flag** key study**Study result type** experimental result**Reliability** 2 (reliable with restrictions)**Rationale for reliability incl. deficiencies** Meets generally accepted scientific standards, well documented and acceptable for assessment**Data source****Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
study report	AIST	2011	NEDO Project P06041, Risk Assessments of Manufactured Nanomaterials - Carbon Nanotubes (CNT)-	http://www.aist-riss.jp/main/modules/product/nano_rad.html?ml_lang=en					
publication	Kobayashi et al.	2011	Pulmonary and systemic responses of highly pure and well-dispersed single-wall carbon nanotubes after intratracheal instillation in rats	Inhalation Toxicology; Vol.23, No.13, 814-828	Mitsubishi Chemical Medicine Corp.				

Materials and methods**GLP compliance**

yes

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Super Growth SWCNT

Test animals**Species**

rat

Strain

Crj: CD(SD)

Sex

male

Details on test animals and environmental conditions

Animals; Seven week old male CrI:CD(SD)rats were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). The rats were kept in an animal facility and housed in positive-pressure air-conditioned units (19-25°C, 35-75% relative humidity) on a 12:12h light/dark cycle. Rats weighing 277-327g. Doses were 0.04, 0.2, 1 and 2 mg/kg. Two intratracheal instillation experiments were conducted in the present study. In the first experiment (experiment 1) pulmonary and systemic responses of SWCNTs were compared with those of crystalline silica up to 3 months after instillation. In the second experiment (experiment 2) were confirmed the reproducibility of the results of experiment 1. In addition, a detailed dose-response relationship and reversibility of the biological responses in rats intratracheally instilled with SWCNTs was examined up to 6 months after instillation. BALF inflammatory cell counts, BALF inflammatory cell counts (LDH, Total protein, Interleukins, GM-CSF, INF- γ , TNF- α were measured. Histopathological evaluation study and processing of lung tissue for TEM observation were performed.

Overall remarks, attachments**Remarks on results including tables and figures**

Intratracheal instillation of highly pure and well-dispersed SWCNTs in rats induced inflammatory responses in the lungs in a dose-dependent manner. However, this inflammatory response was not induced in other tissues (i.e. the liver, kidney, spleen and cerebrum).

Progressive lung tissue thickening responses were observed in the highest dose (2 mg/kg) of SWCNTs exposed group.

However, fibrosis, atypical lesion, or tumor-related findings were not observed in all groups up to 6 months after instillation. SWCNTs did not induce pulmonary inflammation at 0.04 mg/kg (corresponding to approximately 0.04 mg/m²/kg or 2.2 × 10¹² fiber/kg) of pulmonary

deposition. The present study is applicable to judge a NOAEL of highly pure and well-dispersed SWCNTs.

Illustration (picture/graph)

Table 3. Summary of the results in experiments 1 and 2.

Endpoint		SWCNT-exposed group				Silica-exposed group
Dose	Mass [mg/kg]	0.04	0.2	1.0	2.0	5.0
	Surface area [m ² /kg]	0.04	0.2	1.0	2.0	0.025
	Number [particle/kg]	1.8×10^{12}	8.8×10^{12}	4.4×10^{13}	8.8×10^{13}	7.3×10^4
Clinical sign		-	-	-	-	-
Body weight		-	-	-	-	-
Lung weight		-	+	++	+++	-
BALF	Inflammatory cells	-	+	++	++++	+++
	LDH/protein	-	+	++	++++	+++
	IL-1 β	-	+	++	+++	-
Histopathology	Pulmonary inflammation	-	+	++	++++	+++
	Inflammation (other tissues)	-	-	-	-	-

Note: Within the table, ++++ indicates the greatest change, where + indicates the least change. - indicates no significant change compared to the corresponding control.

Applicant's summary and conclusion

Conclusions

Highly pure and well-dispersed SWCNTs did not induce pulmonary inflammation at 0.04mg/kg corresponding to approximately 0.04m²/kg or 2.2×10^{12} fiber/kg of pulmonary deposition.

Endpoint study record: CNI SWCNT Acute toxicity: Pharyngeal aspiration.001

Administrative Data

Study result type experimental result

Reliability 2 (reliable with restrictions)

Rationale for reliability incl. deficiencies Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Shvedova et al.	2005	Unusual inflammatory and fibrogenic pulmonary responses to single-walled carbon nanotubes in mice	Am J Physiol: Lung Cell Mol. Physiol. 289(5): L698-708					

Materials and methods**Test materials**

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNT (CNI, Houston, TX) produced by the highpressure CO disproportionation process (HiPco) technique , employing CO in a continuous-flow gas phase as the carbon feedstock and Fe(CO)₅ as the iron-containing catalyst precursor and purified by acid treatment to remove metal contaminates , were used in the study. SWCNT comprises 99.7% (wt) elemental carbon and 0.23% (wt) iron. Comparative analytical data obtainedby TGA-DSC, TPO, NIR, and Raman spectroscopy revealed that 99% of carbon content in the SWCNT HiPco product was accountable in a carbon nanotube morphology.

Test animals**Species**

mouse

Strain

C57BL

Sex

female

Details on test animals and environmental conditions

Specific-pathogen-free adult female C57BL/6 mice (7–8 wk) were supplied by Jackson Lab (Bar Harbor, ME) and weighed 20.3 0.21 g at time of use. Animals were individually housed in American Association for Accreditation of Laboratory Animal Careapproved National Institute of Occupational Safety and Health (NIOSH) animal facilities in microisolator cages for 1 wk before use. Autoclaved Beta Chip bedding (Northeastern Products, Warrensburg, NY) was changed weekly. Animals were supplied with water and food (Harlan Teklad, 7913, NIH-31 Modified Mouse/Rat Diet, Irradiated; Harlan Teklad, Madison, WI) and housed under controlled light, temperature, and humidity conditions. Experiments were conducted under a protocol approved by the Animal Care and Use Committee of NIOSH.

Administration / exposure**Route of administration**

other: Pharyngeal aspiration

Vehicle

other: phosphate-buffered saline (PBS)

Details on exposure

After anesthetization with a mixture of ketamine (Phoenix, St. Joseph, MO) and xylazine (Phoenix) (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), the mouse was placed on a board in a near vertical position, and the animal's tongue was extended with lined forceps. A suspension (50 l) of particulates prepared in PBS (SWCNT at a dose of 0, 10, 20, 40g/ mouse, UfCB, or SiO₂ at a dose of 40 g/mouse) was placed posterior in the throat, and the tongue was held until the suspension was aspirated into the lungs.

Duration of treatment / exposure

Single pharyngeal aspiration: 0, 10, 20, and 40 µg per mouse

Post exposure period

Animals were killed 1, 3, 7, 28, and 60 days following exposures.

Doses / concentrations

Basis nominal conc.

Control animals

yes, concurrent vehicle

Further details on study design

Mice were weighed and killed with intraperitoneal injection of pentobarbital sodium (100 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). The trachea was cannulated with a blunted 22-gauge needle, and BAL was performed with cold sterile Ca² plus Mg²-free PBS at a volume of 0.9 ml for first lavage (kept separate) and 1.0 ml for subsequent lavages. Approximately 5 ml of BAL fluid per mouse were collected and pooled in sterile centrifuge tubes. Pooled BAL cells were washed in Ca² plus Mg²-free PBS by alternate centrifugation (800 g for 10 min at 4°C) and resuspension. Cell-free first-fraction BAL aliquots were frozen or kept until processed. Inflammation was evaluated by total cell counts, cell differentials, and accumulation of inflammatory cytokines [TNF-, IL-1, transforming growth factor (TGF)-1] in the bronchoalveolar lavage (BAL) fluid. Pulmonary toxicity was assessed by elevation of lactate dehydrogenase (LDH) and -glutamyl transferase (GGT) activities, and protein levels in acellular BAL fluid. Fibrogenic responses to exposed materials were assessed by histopathological evaluation of fibrous collagen in lung tissues and by morphometric measurement of alveolar wall thickening. Accumulation/reduction of biomarkers of oxidative stress [4-hydroxy-2-nonenal (4-HNE) and glutathione (GSH)] was determined in BAL fluid and lung tissue. Functional respiratory deficiency after SWCNT exposure was assessed by measurement of pulmonary function and by decreased bacterial clearance (*Listeria monocytogenes*) in lung of inoculated mice.

Examinations***Positive control***

Two reference materials [ultrafine carbon black (UfCB) or SiO₂, 40 g/mouse] were used.

Overall remarks, attachments

Remarks on results including tables and figures

Two distinct particle morphologies were observed: compact SWCNT aggregates (Fig. 1A) and dispersed SWCNT structures (Fig. 1B). Further investigation of dried suspension samples using the TEM and scanning electron microscope suggested that the compact aggregates may have been present as discrete particles within the suspension, but the data were not conclusive. Alternatively, it is feasible that the two morphologies observed were associated with suspension droplets drying before or after collection. Accordingly, two morphologically distinct responses were detected in the lung as early as 7 days postexposure. Morphological response varied depending upon the association with dense SWCNT aggregates. Foci of granulomatous inflammation, including discrete granulomas often surrounded by hypertrophied epithelial cells, were associated with dense SWCNT aggregates (Fig. 1D). The carbon nanotube material was clearly visualized with granulomatous inflammation (Fig. 1, E and F), and interfacing bundles of fibrous connective tissue were observed within discrete granulomas. In lung regions distant from observed SWCNT aggregates, morphological alterations were predominantly diffuse interstitial fibrosis with alveolar wall thickening. Importantly, deposition of collagen and elastin was also observed in both granulomatous regions as well as in the areas distant from granulomas. This was established by conventional light microscopy of lung sections specifically stained with Sirius red (Fig. 1D) as well as by TEM (Fig. 1H). The fibrotic response progressed with time, with alveolar wall thickening being significantly greater at 60 days than 28 days postexposure (Fig. 1G, inset). Morphometric analysis indicated a significant granulomatous response, a significant fibrotic response within granulomas, and significant alveolar septal fibrosis (Fig. 1G). It is observed that studied reference materials (UfCB and SiO₂), in contrast to SWCNT, 1) did not cause thickening of alveolar walls, 2) did not induce formation of granulomas, and 3) resulted in a significantly lower magnitudes of inflammatory responses (cell differentials, cell counts, and protein in BAL) when animals were exposed to comparable doses of these particles (Fig. 2). Increased numbers of alveolar type II (AT-II) cells, the progenitor cells that replicate following alveolar type I (AT-I) cell death, were noted in response to SWCNT. This was confirmed (Fig. 3B) by increased immunofluorescent detection of cytokeratins 8/18, markers of AT-II cells in the alveolus (11) and by increased numbers of cells expressing cytoplasmic lamellar bodies in TEM sections. This proliferation of AT-II cells indicates either AT-I cell injury, proliferation of AT-II cells to cover new basement membrane associated with the nodules, or both. To more accurately determine the level of cell injury, assays of protein content, LDH, and -glutamyl transferase (GGT) in the acellular BAL fluid obtained from the mice were performed.

A dose-dependent and early (1–3 days postexposure) increase in the level of protein and LDH activity in acellular BAL fluid was observed (Fig. 4, A and B). Cell damage (LDH activity) was dose dependently increased 1 day postexposure and remained elevated until 28 days postexposure, whereas damage to the alveolar air-blood barrier, measured as increased protein secretion in BAL fluid, peaked at 3 days and declined thereafter. Activity of GGT was dose dependently increased with significant elevations observed 3 days postexposure and increases being sustained through 60 days (Fig. 4C). Exposure to SWCNT also resulted in the accumulation of a characteristic biomarker of oxidative stress, 4-HNE, in BAL fluid as early as 1 day postexposure (Fig. 4D). There also was a dose- and time-dependent depletion of a major antioxidant, GSH, in the lung being most severe 1 day postexposure (Fig. 4E).

BAL cytology indicated a robust and early (day 1) accumulation of neutrophils, followed by a sequential appearance of lymphocytes (peak 3 days) and macrophages (peak 7 days) (Fig. 5). These cellular responses increased with exposure dose. In line with these findings, proinflammatory cytokines (TNF- α , IL-1) in the acellular BAL fluid were increased by 16- and 10-fold, respectively, on day 1 and rapidly returned to near control levels at 3 days after exposure (Fig. 6, A and B). In contrast, fibrogenic TGF- β 1 was not significantly increased until day 7 and returned toward control by 28 days postexposure (Fig. 6C). Thus the time course of TNF- α and IL-1 corresponded to PMN influx, whereas that for TGF- β 1 paralleled macrophage recruitment.

Attached background material

Applicant's summary and conclusion

Conclusions

Experimental results demonstrate that pharyngeal aspiration of SWCNT induces a robust acute inflammatory reaction with the very early onset of a fibrogenic response and the formation of granulomas in C57BL/6 mice. The formation of granulomas is mainly associated with deposition of dense micrometer-scale SWCNT aggregates. However, a unique fibrogenic response is apparent in alveolar septa distant from deposition sites of aggregates and in the absence of persistent inflammation. In addition, pulmonary exposure to SWCNT caused persistent changes in pulmonary functions and decreased bacterial clearance.

Endpoint study record: CNI SWCNT Acute toxicity: Pharyngeal aspiration.002

Administrative Data

Study result type experimental result

Reliability 2 (reliable with restrictions)

Rationale for reliability incl. deficiencies Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Li et al.	2007	Cardiovascular Effects of Pulmonary Exposure to Single-Wall Carbon Nanotubes	Environ mental Health Perspectives ; Vol.115, No.3, 377-382	US NIOSH				

Materials and methods

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNT (CNI, Houston, TX) produced by the highpressure CO disproportionation process (HiPco)

technique , employing CO in a continuous-flow gas phase as the carbon feedstock and Fe(CO)₅ as the iron-containing catalyst precursor and purified by acid treatment to remove metal contaminants , were used in the study. SWCNT comprises 99.7% (wt) elemental carbon and 0.23% (wt) iron. Comparative analytical data obtained by TGA-DSC, TPO, NIR, and Raman spectroscopy revealed that 99% of carbon content in the SWCNT HiPco product was accountable in a carbon nanotube morphology.

Test animals

Species

mouse

Strain

other: Male FVB/N-TgN (Ho1-luc) Xen mice , Male C57BL/6J and B6.129P2-Apoetm1Unc (ApoE^{-/-}) mice,

Sex

male

Details on test animals and environmental conditions

Male FVB/N-TgN (Ho1-luc) Xen mice were obtained from Xenogen Corp.(Alameda, CA). These mice carry a fusion of a 15-kb mouse HO-1 promoter with modified firefly luciferase cDNA (Promega pGL-3) and were used to assess HO-1 activation. Male C57BL/6J and B6.129P2-Apoetm1Unc (ApoE^{-/-}) mice were obtained from Jackson Laboratory (Bar Harbor, ME). The ApoE^{-/-} mice lack apolipoprotein E (a high-affinity ligand for lipoprotein receptors), consequently have elevated plasma levels of cholesterol and triglycerides, and develop atherosclerotic plaques in a fashion similar to humans (Reddick et al. 1994). Mice were between 2 and 3 months of age when exposures began, and were fed an irradiated commercial mouse chow diet (LM-485; Harlan, Indianapolis, IN). For some of the atherosclerosis studies, ApoE^{-/-} mice were fed a high fat diet (15.8% fat; TD 88051; Harlan), which exacerbates the atheroma formation. All mice were provided tap water ad libitum and were housed in ventilated cages on autoclaved hardwood chip bedding. Sentinel mice were free of endogenous pathogens. Animal room conditions included HEPA-filtered air, controlled temperature and humidity, and a 12-hr light/dark cycle. Animal care and use procedures, including sacrifice by carbon dioxide asphyxiation, were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals (Office of Laboratory Animal Welfare 2002) and the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources 1996); these procedures were approved by the National Institute for Occupational Safety and Health institutional animal care and use committee. All mice used in these studies were treated humanely and with regard for alleviation for suffering.

Administration / exposure

Route of administration

other: Pharyngeal aspiration

Vehicle

other: phosphate-buffered saline (PBS)

Details on exposure

After anesthetization with a mixture of ketamine (Phoenix, St. Joseph, MO) and xylazine (Phoenix) (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), the mouse was placed on a board in a near vertical position, and the animal's tongue was extended with lined forceps. A suspension (50 l) of particulates prepared in PBS (SWCNT at a dose of 0, 20, 40g/mouse, UfCB at a dose of 40 g/mouse) was placed posterior in the throat, and the tongue was held until the suspension was aspirated into the lungs. Suspensions of SWCNTs and UfCBs (positive controls) were prepared by sonification using a Branson Sonifier 450 (Branson Ultrasonics Corp., Danbury, CT) in PBS for 3 min (at 400 W) at room temperature before animal exposure.

Duration of treatment / exposure

Single pharyngeal aspiration: 0, 20, and 40 µg per mouse for Ho1-luc reporter mice or C57BL/6 mice. A medium dose of SWCNT (20 µg/mouse) via multiple exposures (once every other week for 8 weeks) for ApoE^{-/-} mice.

Post exposure period

Animals were killed 1, 7, 28, and 56 days following exposures.

Doses / concentrations

Basis nominal conc.

No. of animals per sex per dose

At least 4 mice per treatment for Ho1-luc reporter mice or C57BL/6 mice. 10/treatment for ApoE^{-/-} mice.

Control animals

yes, concurrent vehicle

Further details on study design

To screen for systemic oxidative effects of SWCNT exposure, Ho1-luc reporter mice or C57BL/6 mice were exposed. To evaluate SWCNT effects on atherosclerosis progression, a chronic process, ApoE^{-/-} mice were exposed.

Examinations***Positive control***

Ultrafine carbon black (UfCB) particles (Printex 90, Degussa, Germany), used as a control material, were a gift from G. Oberdörster (University of Rochester, Rochester, NY).

Any other information on materials and methods incl. tables

Experimental settings were selected to correspond to previous pulmonary toxicity studies (Shvedova et al. 2005).

Their physicochemical characteristics were reported previously (Shvedova et al. 2005).

Results and discussions

Details on results

HO-1 activation in cardiovascular tissue. Initial screening for extrapulmonary effects using Ho1-luc reporter transgenic mice were conducted. To evaluate HO-1 gene expression, a surrogate biomarker of oxidative stress (Prawan et al. 2005; Rao et al. 2003), the activation of tissue luciferase in Ho1-luc reporter transgenic mice were measured. HO-1 demonstrated a trend for activation in the lung, aorta, and heart tissue 7 days after SWCNT exposure; the activity declined approximately to control levels by 28 days after exposure. Significant reduction in mtDNA amplification, reflecting an increase in mtDNA damage, was observed in the aortic tissue of C57BL/6 mice exposed to SWCNT (10 and 40 µg/mouse) at days 7, 28, and 60 after a single exposure, compared with age-matched, vehicle-treated control mice ($p < 0.01$). Maximum mtDNA damage occurred 7 days after exposure in both treatment groups and persisted in the high-dose treatment group 60 days after exposure. In contrast to SWCNT, UfCB pharyngeal instillation did not result in mtDNA damage in the aortic tissue at comparable doses of exposure levels ($p < 0.05$). ApoE^{-/-} mice were exposed chronically to PBS or SWCNT (four instillations of SWCNT at 20 µg/mouse every other week for 8 weeks) and fed a regular chow diet (regimen 1) or a fat chow diet for 4 weeks, followed by regular chow for 4 weeks (regimen 2). Vehicle-exposed ApoE^{-/-} mice on regimen 1 had lower plasma cholesterol ($p \leq 0.01$) and minimal plaque occurrence in the aorta compared with the mice on regimen 2. The body weights, as well as plasma cholesterol, glucose, and lactate dehydrogenase (LDH) levels were comparable in SWCNT and vehicle-exposed mice under both diet regimens. Plaque formation, although appearing slightly increased after exposure to SWCNT, compared with the vehicle exposure in mice on regimen 1, was not quantified because the size of the plaques was not large enough for meaningful analysis or comparisons to be made. However, in mice on regimen 2, the aortic morphometric en face analysis demonstrated a significant increase in the plaque areas in SWCNT-exposed mice compared with the vehicle-treated mice ($p < 0.05$). Histologic assessment of the lesion size in the BCA (brachiocephalic artery) of these mice confirmed that SWCNT exposure accelerated atherosclerosis progression. Consistent with the larger size of the plaques, the number of macrophages and VCAM-1-positive cells were proportionally higher in the BCA plaques from SWCNT-exposed mice compared with plaques in PBS-treated mice. In the evaluated sections, there was no evidence that SWCNT exposure resulted in increased immunostaining in the vascular wall, which was not affected by plaque formation. The involvement of systemic inflammation and aortic mtDNA damage in SWCNT-related atherosclerosis progression was evaluated. The plasma MCP-1, IL-12, IL-6, TNF- α , and IFN- γ levels were not significantly different in ApoE^{-/-} mice treated with PBS or SWCNT. However, the aortas of SWCNT-exposed ApoE^{-/-} mice demonstrated significantly increased mtDNA damage.

Overall remarks, attachments

Attached full study report

Applicant's summary and conclusion

Conclusions

Respiratory exposure to high concentrations of mostly agglomerated SWCNTs provokes not only pulmonary toxicity but vascular effects related to mitochondrial oxidative modifications and accelerated atheroma formation.

Endpoint study record: CNI SWCNT Acute toxicity: Pharyngeal aspiration.003**Administrative Data****Study result type** experimental result**Reliability** 2 (reliable with restrictions)**Rationale for reliability incl. deficiencies** Meets generally accepted scientific standards, well documented and acceptable for assessment**Data source****Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Shvedova et al.	2008	Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis	Am J Physiol: Lung Cell Mol. Physiol. 295(4): L552-565					

Materials and methods**Type of method**

in vivo

Test materials**Test material identity**

Identifier	Identity
other:	CNI SWCNT

Test animals**Species**

mouse

Sex

female

Details on test animals and environmental conditions

Specific pathogen-free adult female C57BL/6 mice (8–10 wk) were supplied by The Jackson Laboratory (Bar Harbor, ME) and weighed 20.0 +/- 1.9 g when used. Animals were housed one mouse per cage receiving HEPA filtered air in Association for Assessment and Accreditation of Laboratory Animal Care-approved National Institute for Occupational Safety and Health (NIOSH) animal facilities. All animals were acclimated in the animal facility under controlled temperature and humidity for 1 wk before use. Animals were supplied with water and certified chow 5020 (Purina Mills, Richmond, IN) ad libitum in

accordance with guidelines and policy set forth by the Institute of Laboratory Animals Resources, National Research Council. All experimental procedures were conducted in accordance with a protocol approved by the NIOSH Institutional Animal Care and Use Committee.

Administration / exposure

Route of administration

other: Pharyngeal aspiration

Duration of treatment / exposure

Single pharyngeal aspiration: 0, 5, 10, and 20 µg per mouse

No. of animals per sex per dose

12 mice /Group

Examinations

Any other information on materials and methods incl. tables

A suspension of SWCNT (0, 5, 10, and 20 µg per mouse) was used for single pharyngeal aspiration to groups of C57BL/6 mice, whereas the corresponding control mice were administered sterile Ca²⁺+Mg²⁺-free PBS vehicle. Dose-dependent inflammatory response and pulmonary toxicity were evaluated, and the concentration of 10 µg per mouse of SWCNT was chosen for time course study. Mice were euthanized on days 1, 7, and 28 following the exposure. All experiments were repeated three times. Inflammation was evaluated by total cell counts, cell differentials, and accumulation of cytokines in the bronchoalveolar lavage (BAL) fluid. Pulmonary toxicity was assessed by elevation of lactate dehydrogenase (LDH) level in acellular BAL fluid. Fibrogenic responses to exposed materials were assessed by morphometric measurements and collagen deposition. Respiratory changes after SWCNT exposures were assessed by measurement of breathing patterns using whole body plethysmography.

Overall remarks, attachments

Remarks on results including tables and figures

Followings are observed: early inflammatory response and oxidative stress culminating in the development of multifocal granulomatous pneumonia and interstitial fibrosis.

Endpoint study record: CNI SWCNT Acute toxicity: Pharyngeal aspiration.004

Administrative Data

Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Mercer et al.	2008	Alteration of deposition pattern and pulmonary response as a result of improved dispersion of aspirated single-walled carbon nanotubes in a mouse model	Am J Physiol: Lung Cell Mol. Physiol. 294(1): L87-97					

Materials and methods

Type of method

in vivo

Test materials

Test material identity

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNT were purified from a highpressure carbon monoxide production process (HiPCO) with 2 wt% of contaminants (CNI, Houston, TX). DSWCNT is dispersed SWCNT and preparation method is as follows. Two hundred milligrams of purified SWCNT (low iron) were initially suspended in 60 ml of distilled water, passed through a 200-mesh stainless steel screen to grossly separate the material, and sonicated for 10 min. The solution was then expanded to 200 ml with acetone and placed in an ultrasonic bath maintained at room temperature for 24 h. When dispersed in acetone, there was an approximately 150-fold increase in the volume of the SWCNT sample compared with that in distilled water. This was due to the dissociation of nanotubes as the nonionic solvent, acetone, reduced the van der Waals forces binding the CNTs (27). The dispersed SWCNT were then filtered from the solution using two stages of a 20-m nylon mesh screen, which had a 4µm hole diameter (CMN-0029-C, Small Parts, Miami Lakes, FL). The acetone solution containing the DSWCNT was then passed through a 0.2µm polytetrafluoroethylene (PTFE) filter, which had been weighed before use. After collection on the filter, the DSWCNT were washed several times with distilled water to remove residual acetone. To assess the size distribution of the DSWCNT and SWCNT samples, a sample of each was taken, diluted to decrease loading density, and filtered onto a 25-mm diameter, 0.1-µm pore polycarbonate filter (VCTP02500 isopore membrane; Millipore, Billerica, MA). The polycarbonate filter was then washed with distilled water and dried. Micrographs of the CNT particles on the filter were then taken with a scanning electron microscope. The area equivalent diameter of the DSWCNT and SWCNT was then determined. The distribution of average area equivalent diameter of the DSWCNT was approximately normally distributed with a mean of 0.69 µm and a variance of 0.49 with a maximum area equivalent diameter of 3.7 µm. The area equivalent diameter of the SWCNT was approximately normally distributed with a mean of 15.2 µm and a variance of 3.2µm.

Test animals

Species

mouse

Strain

other: C57BL/6

Sex

male

Details on test animals and environmental conditions

Specific pathogen-free adult female C57BL/6 mice (24 wk) were supplied by The Jackson Laboratory (Bar Harbor, ME). Animals were individually housed in Association for Assessment and Accreditation of Laboratory Animal Care-accredited National Institute for Occupational Safety and Health animal facilities in microisolator cages for at least 1 wk before use. Autoclaved Beta Chip bedding (Northeastern Products, Warrensburg, NY) was changed weekly. Animals were supplied with water and Harlan Teklad 7913, NIH-31 modified mouse/rat diet, irradiated (Harlan Teklad, Madison, WI), and housed under controlled light, temperature, and humidity conditions. Animals were monitored on a daily basis for morbidity, illness, and adverse effects, with none noted. All methods involving animals were conducted under protocols approved by our Institutional Animal Care and Use Committee.

Administration / exposure

Route of administration

other: Pharyngeal aspiration

Vehicle

other: PBS

Details on exposure

After anesthetization, the animal was placed on a board in a near vertical position and the animal's tongue was extended with padded forceps. A suspension of the material to be aspirated (50 l/mouse) at the desired concentration or PBS vehicle was placed on the back of the tongue. A slight pull of the tongue results in a reflex gasp and aspiration of the droplet. The tongue was held, and the animal was monitored for a few breaths after aspiration. To eliminate the possibility of incidental food aspiration, food was removed 4 h before the procedure; water was removed 1 h before the procedure.

Duration of treatment / exposure

Single pharyngeal aspiration: 10 µg per mouse

Post exposure period

1h, 1d, 7d and 30 d

No. of animals per sex per dose

6 mice /Group

Further details on study design

Animals were studied at 1 h after pharyngeal aspiration to determine the initial deposition pattern and at 1 day, 7 day, and 1 mo postexposure to determine the acute inflammatory response, chronic fibrotic response, and fate of the aspirated carbon nanotube (CNT). A PBS-treated group was used as the control. Six mice, given DSWCNT or SWCNT, were studied at each time point for measurement of connective tissue and alveolar responses by morphometry. Bronchoalveolar lavage (BAL) analysis was done at each time point with six animals per group given DSWCNT. Additional groups were given gold-labeled DSWCNT (n 4) or quantum dot-labeled DSWCNT (n 4). These groups were studied at 1 h and at 1, 3, and 7 days after aspiration. To control for the possibility of unbound label, additional groups of mice were given aspirations of 0.1 g of colloidal gold or 0.25 g of quantum dots. The doses were chosen based on the amount of bound gold or quantum dots in the 10 g/mouse gold-labeled DSWCNT and 10 g/mouse quantum dot-labeled DSWCNT studies, respectively. These animals were studied 1 day after aspiration. A morphometric analysis of the fate of these unbound labels was conducted to identify how the lung processed unbound label.

Results and discussions**Details on results**

Micrographs of lungs exposed to DSWCNT or SWCNT demonstrate a similar pattern of inflammatory response, i.e., a rapid but transient PMN recruitment. No significant airway involvement in the inflammatory process was observed. The number of lavage macrophages was elevated between day 1 and 7. However, macrophages observed by transmission electron microscopy were generally normal in appearance and did not reveal morphological evidence of activation, such as flattening of the cell or increased cell ruffling and filopodia. The PMN response was similar in time course and magnitude to that previously reported for aspiration of less dispersed SWCNT (shvedova 2008). Although CNT are unreactive to light microscopic stains, large agglomerates, 2–20 μ m in diameter, can readily be identified in lung sections from animals given the less dispersed SWCNT because the mass of SWCNT in the section is sufficient to block light transmission. The lung response to these large agglomerates of less dispersed SWCNT results in infiltration and encasement by epithelioid macrophages to form a connective tissue-rich granulomatous inflammation that is typical of the lungs response to an insoluble particle. Because of its much smaller size, the DSWCNT deposition could not be easily observed. Only rarely was it possible to identify deposition of DSWCNT in ordinary light microscopic sections. Such deposits were typically 1–3 μ m in diameter. Fortunately, the gross distribution of all size classes of CNT was easily discernable in the silver-enhanced sections from animals given gold-labeled SWCNT or DSWCNT. The distributions of gold-labeled, less dispersed SWCNT were concentrated near the proximal alveolar region. This is the main area where granulomatous lesions form after administration of less dispersed SWCNT preparations. Submicrometer deposits of this SWCNT preparation can also be identified throughout the alveolar region. It is estimated that these submicrometer structures constitute 20% of the total of less dispersed SWCNT material in the lungs based on the morphometric point counting of these sections. Silver-enhanced sections from lungs of mice given the gold-labeled DSWCNT showed a much finer and diffuse pattern of deposition. DSWCNT were identified throughout the interstitial spaces and in the vicinity of the pleura. Occasional deposits were also identified within alveolar macrophages, but these were 5% of the total by point counting of silver-enhanced sections. Deposition of DSWCNT in conducting airways was minimal. The engulfment of DSWCNT by alveolar macrophages was relatively rare. By 7 days, only the large agglomerates remained in the air spaces. Deposition of DSWCNT, with the majority of the nanotubes incorporated within the alveolar interstitial spaces within the first day was found. In a normal lung, the edges of the alveolar septa that contain the

alveolar entrance rings are the most prominent site of collagen. The collagen distribution of mice 1 day after aspiration of dispersed SWCNT or DSWCNT was not different from the PBS-treated groups. Areas of significant collagen accumulation in the alveolar wall were found 7 and 30 days after aspiration of dispersed SWCNT and DSWCNT (arrows); however, the distribution of such collagen accumulation differed. Most of the abnormal collagen deposition in the SWCNT-treated lungs was associated with agglomerates of SWCNT in the lungs. In contrast, collagen accumulation in DSWCNT-treated lungs was widely distributed within the alveolar walls throughout the lungs. Morphological evaluation indicated that DSWCNT caused more extensive interstitial collagen accumulation than the less dispersed SWCNT.

Any other information on results incl. tables

Basic results for the PBS and DSWCNT groups prepared for morphometric analysis by vascular perfusion fixation are given in Table 1. In this experiment, age of the mice at exposure was staggered so that there were no significant differences between groups for body weight, fixed lung volume, alveolar surface area, or alveolar region gas volume at the time of death.

Table 1. Body weight and lung morphometry after DSWCNT exposure

Group	Body Weight, g	Lung Volume, ml	Alveolar Surface Area, cm ²	Alveolar Gas Vol., ml
PBS	33.0±1.5	0.89±0.3	567±60	0.51±0.03
DSWCNT	33.5±1.5	0.99±0.14	586±83	0.57±0.09
1 h	33.5±1.5	0.99±0.14	586±83	0.57±0.09
1 day	32.7±1.2	0.98±0.04	512±44	0.53±0.03
7 day	34.1±0.7	1.06±0.08	521±42	0.57±0.04
30 day	33.6±0.7	0.92±0.04	564±26	0.50±0.03

Values are means±SE. Mice were given 10 µg of dispersed single-wall carbon nanotubes (DSWCNT) at indicated times before death or were given PBS (control).

Overall remarks, attachments

Remarks on results including tables and figures

Figure 3 presents a quantitation of BAL cell counts for lungs after pharyngeal aspiration of 10 µg/lung DSWCNT, demonstrating a transient neutrophil influx that resolved within 7 days. This PMN response was similar in time course and magnitude to that previously reported for aspiration of less dispersed SWCNT (Shvedova 2008).

The average thicknesses of collagen in the alveolar wall for the PBS and DSWCNT groups are given in Fig. 9. As shown in Fig. 9, DSWCNT dramatically increased the collagen content of the alveolar wall in a time-dependent manner. At 1 h and 1 day after aspiration, collagen content was comparable to that of the PBS control. A significant increase was observed at day 7 and progressed, without further exposure, by day 30. This time course is consistent with prior study (Shvedova 2008) of lung collagen changes for less dispersed SWCNT, in which significant collagen production was noted 7 days postexposure with continued increases at longer time points. However, DSWCNT were more potent in inducing interstitial collagen accumulation than an equal mass aspiration of the less dispersed SWCNT preparation.

Illustration (picture/graph)

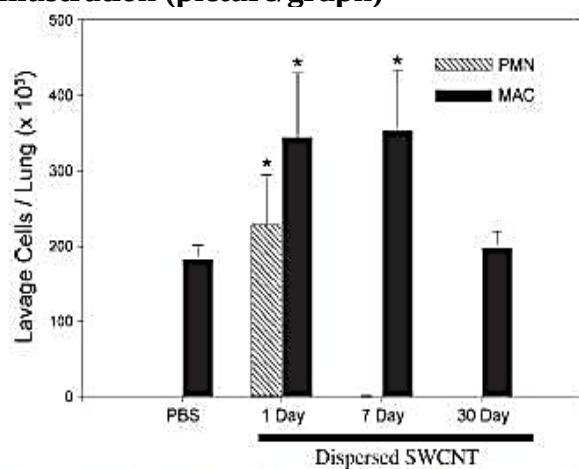


Fig. 3. Cell differential of bronchoalveolar lavage (BAL) samples from mice after pharyngeal aspiration of 10 µg/mouse of DSWCNT (1 day, 7 days, and 30 days) or PBS. PMNs in BAL demonstrated a transient response during the first few days after aspiration, which resolved by day 7. Lavage macrophages (MAC) were elevated above control levels (PBS) but returned to control values at 30 days. Values are means $\pm$ SE; $n = 6$ mice. * $P < 0.05$ vs. PBS-treated control mice.

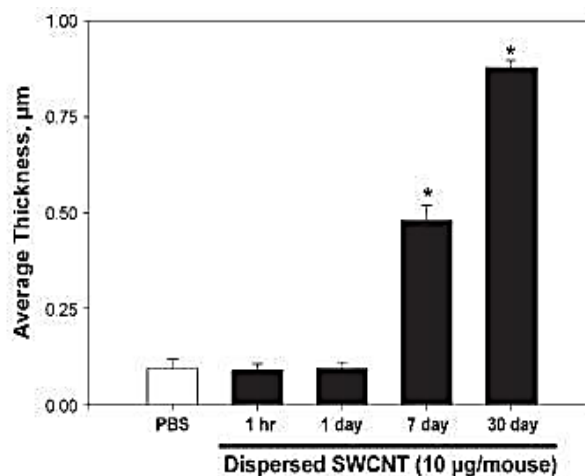


Fig. 9. Morphometric changes in alveolar wall collagen fiber content. Results from determination of the average thicknesses of alveolar wall connective tissue fibers at various times after aspiration of DSWCNT are given. At 7 days, there was significant accumulation of connective tissue fibers in the alveolar wall, which continued to increase through 30 days after aspiration. Values are means $\pm$ SE; $n = 6$ mice. * $P < 0.05$ vs. PBS-treated control mice.

Applicant's summary and conclusion

Executive summary

There are at least three principal pathways for dissemination of SWCNT in the lungs. In general, 80% of the less dispersed material deposits in the lungs as large agglomerates that result in rapidly forming granulomatous lesions in the alveolar region immediately proximal to the small airways. The remaining portion of less dispersed nanotubes appears to be aspirated as smaller structures and/or is dispersed by the lung milieu into a finer form, which is widely distributed throughout the air spaces of the lung parenchyma and is rapidly incorporated into the alveolar interstitial wall, inducing increased collagen production that progresses in the absence of persistent inflammation. A relatively small fraction of the purified SWCNT was found to be engulfed or otherwise processed by alveolar macrophages. Improved dispersal of SWCNT results in aspiration of smaller structures that are more prone to enter the alveolar walls. The pulmonary response to DSWCNT is a more potent interstitial fibrotic reaction in the absence of granuloma formation.

Endpoint study record: CNI SWCNT Acute toxicity: Pharyngeal aspiration.005

Administrative Data

Study result type

experimental result

Reliability

2 (reliable with restrictions)

Rationale for reliability incl. deficiencies

Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Shvedova et al.	2007	Vitamin E Deficiency Enhances Pulmonary Inflammatory Response and Oxidative Stress Induced by Single Walled Carbon Nanotubes in C57BL/6 Mice	Toxicol Appl Pharmacol. 2007 June 15; 221(3): 339–348.					

Materials and methods

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	CNI SWCNT

Details on test material

SWCNTs (CNI Inc., Houston, TX) were produced by the high pressure CO (HiPco) disproportionation technique, employing CO in a continuous-flow gas-phase as the carbon feedstock and Fe(CO)₅ as the iron-containing catalyst precursor (non-purified SWCNTs) (Bronikowski et al., 2001). Purified SWCNTs were prepared by acid treatment to remove metal contaminates (Gorelik et al., 2000). Specific surface area was measured at –196 °C by the nitrogen absorption–desorption technique (Brunauer Emmet Teller method, BET) using SA3100 Surface Area and Pore Size Analyzer, (Beckman Coulter Inc, Fullerton, CA), while diameter was measured by transmission electron microscopy (TEM). The mean diameters of purified SWCNTs were 1–4 nm. Surface area of purified SWCNTs was 1040 m²/g. Chemical analysis of total elemental carbon and metal (iron) in SWCNTs was performed at the Chemical Exposure and Monitoring Branch (DART/NIOSH, Cincinnati, OH). Elemental carbon in SWCNTs was assessed according to NIOSH Manual of Analytical Methods (NMAM) (Bronikowski et al., 2001), while metal content (iron) was analyzed using nitric acid dissolution and inductively coupled plasma-atomic emission spectrometry (ICP-AES) performed according to NMAM method 7300 for trace metals. Analysis performed by NMAM 5040 revealed that purified SWCNTs were comprised of 99.7 wt % elemental carbon. For purity assessment of HiPco SWCNTs, we used several standard analytical techniques including thermo gravimetric analysis with differential scanning calorimetry (TGA–DSC), thermoprogramming oxidation (TPO), and Raman and near-infrared (NIR) spectroscopy (Birch, 2003; Arepalli et al., 2004; Dresselhaus et al., 2004). Comparative analytical data obtained by TGA–DSC, TPO, NIR and Raman spectroscopy revealed that more than 99% of carbon content in the SWCNT HiPco product was accountable in a carbon nanotube morphology.

Test animals**Species**

mouse

Strain

C57BL

Sex

female

Details on test animals and environmental conditions

Specific pathogen-free adult female C57BL/6 mice (7–8 wk) were supplied by Jackson Lab (Bar Harbor, MN) and weighed 21.60 ± 0.26 g at time of use. Mice were given a vitamin E deficient diet (contained less than 10 IU/kg vitamin E) or basal diet (50 IU/kg of diet) for a total of 24 weeks. The basal diet (Basal Diet™ 5755, Test Diet, Purina Mill, Richmond, IN) is a purified, synthetic diet that provides all the essential nutrients to support maintenance, growth, gestation and lactation in laboratory rodents. Vitamin E deficient diet (Purina Mill, Richmond, IN) is based on the Basal Diet™ 5755 from which α -tocopherol was removed. Animals were daily supplied with fresh diets refrigerated at 4 °C. Mice were housed one mouse per cage in AAALAC-approved NIOSH animal facilities one week prior to use. Beta Chips (Northeastern Products Corp., Warrensburg, NY) were used for bedding and changed weekly. Animals were supplied with water and the respective diets ad libitum, in accordance with guidelines and policy set forth by the Institute of Laboratory Animals Resources, National Research Council.

Administration / exposure**Route of administration**

other: Pharyngeal aspiration

Vehicle

other: phosphate-buffered saline (PBS)

Details on exposure

Mouse pharyngeal aspiration was used for particulate administration (Rao et al., 2003). Briefly, after anesthesia with a mixture of ketamine and xylazine (62.5 and 2.5 mg/kg subcutaneous in the abdominal area), the mouse was placed on a board in a near vertical position, and the animal's tongue extended with lined forceps. A suspension (approximately 50 μ l) of particulates prepared in PBS (SWCNTs at a dose of 0.0 and 40 μ g/mouse) was placed posterior in the throat and the tongue held until the suspension was aspirated into the lungs. Control mice were administered sterile Ca^{2+} + Mg^{2+} -free phosphate-buffered saline (PBS) as a vehicle. All mice in SWCNT and PBS groups survived this exposure procedure. This technique provided good distribution of particles widely disseminated in a peri-bronchiolar pattern within the alveolar region as was detected by histological evaluation. The mice revived unassisted after approximately 30–40 min. Animals treated with the particulates and PBS recovered easily after anesthesia with no behavioral or negative health outcomes. Mice were sacrificed on days 1, 7 and 28 days following exposure.

Duration of treatment / exposure

Single pharyngeal aspiration

Post exposure period

Animals were killed 1, 7, 28 days following exposures.

Doses / concentrations

Basis nominal conc. (0, 40µg per mouse)

No. of animals per sex per dose

12 or 6 or 3 mice per sex per dose (different for endpoints)

Control animals

yes, concurrent vehicle

Further details on study design

Mice were weighed and sacrificed with intraperitoneal injection of sodium pentobarbital (>100mg/kg) and exsanguinated. The trachea was cannulated with a blunted 22 gauge needle, and BAL was performed using cold sterile PBS at a volume of 0.9 ml for first lavage (kept separate) and 1.0ml for subsequent lavages. Approximately 5 ml of BAL fluid per mouse was pooled and collected in sterile centrifuge tubes. Pooled BAL cells were washed in PBS by alternatecentrifugation (800 × g for 10 min at 4 °C) and resuspension. Cell-free first fraction BAL aliquots were frozen or kept until processed. The degree of inflammatory response induced by the pharyngeal aspirated particulates was estimated by quantitating total cells, macrophages, polymorphonuclear leukocytes (PMNs) and lymphocytes recovered by BAL. Cell counts were performed using an electronic cell counter equipped with a cell sizing attachment (Coulter model Multisizer II with a 256C channelizer, Coulter Electronics, Hialeah, FL). Alveolar macrophages (AM), PMNs, and lymphocytes (LM) were identified by their characteristic cell shape in cytopsin preparations stained with Diffquick (Fisher Scientific, Pittsburgh, PA), and differential counts of BAL cells was carried out. Three hundred cells per slide were counted. Lung Lavage Fluid Cytokine Analysis, Total Protein and Lactate Dehydrogenase (LDH) Activity in the BAL Fluids, Lung Fixation and Histopathology, Lung Collagen Measurements were conducted.

Results and discussions

Details on results

Body weight: Body weight was assessed throughout the experimental schedule to monitor the general health of the animals given vitamin E-deficient/sufficient diets and exposed to the particles. At 1 day post exposure, pharyngeal aspiration of SWCNTs (40µg/mouse) caused a significant weight loss compared to controls. No significant difference in the body weight was caused by vitamin E deficiency as compared to the control vitamin E-sufficient group 1 day after the exposure to PBS. Weight gains among groups were not significantly different at 7 days post exposure. The SWCNT depression of weight gain was significant again at 28 days postexposure as compared to animals exposed to PBS. At this time point, no significant difference in body weight was caused by vitamin E deficiency. Depletion of vitamin E and oxidative stress response in the lung: Lung is a fast “vitamin E-reactive” tissue where dietary manipulations cause significant changes in vitamin E content both in terms of its depletion by deficient diets as well as its elevation after supplementation (Shvedova et al., 2002). In line with this, our results indicate that

C57BL/6 mice kept for twenty weeks on a vitamin E-deficient diet responded by a remarkable depletion of vitamin E levels in the lung, i.e., a decrease of 90-fold compared to the control (kept on vitamin E sufficient diet) animals. The induced vitamin E deficiency resulted in depletion of other antioxidants (GSH and ascorbate). SWCNT-induced accumulation of lipid peroxidation products was also enhanced by vitamin E deficiency. Thus generalized oxidative stress rather than depletion of vitamin E alone was induced by dietary manipulations. This is in line with a well accepted concept that individual low molecular weight radical scavengers are participants of a coordinated network rather than independent antioxidant players (Kagan and Packer, 1994). Next we assessed oxidative stress responses to SWCNTs in vitamin E-sufficient and deficient animals. A more severe depletion of pulmonary antioxidants was detected in SWCNT treated vitamin E-deficient mice as compared to controls. Inflammatory response: In agreement with our previous results, SWCNTs caused a robust, acute (1 day post exposure) inflammatory response in the lung of exposed C57BL/6 mice as evidenced by increased: 1) total number of inflammatory cells, 2) number of PMNs, 3) released LDH, 4) BAL fluid total protein content, and 5) BAL fluid levels of pro-inflammatory cytokines (TNF- α and IL-6). Morphologic findings were consistent with the rapid development of granulomatous bronchointerstitial pneumonia. Inflammatory reaction at one day after aspiration of SWCNTs (40 μ g/mouse) was comprised of PMNs with a variable histiocytic response. These SWCNT-induced changes were significantly more pronounced in the lungs of vitamin E-deficient animals than in mice fed basal diet. Profibrotic response, assessed by the content of TGF- β 7 days after the exposure to SWCNTs, was also elevated to a greater extent in vitamin E-deficient animals as compared to control mice. The nature of the inflammatory response evolved with time and at 28 days postexposure, PMNs were no longer a major inflammatory component and the type of inflammation was leading to histiocytic granulomatous lesions (Fig. 5C & 5D). Evaluation of fibrosis at 28 days post exposure revealed a higher level of collagen deposition and a greater extent of thickening of the alveolar septa (measured morphometrically) in SWCNT-treated, vitamin E-deficient vs control mice.

Overall remarks, attachments

Illustration (picture/graph)

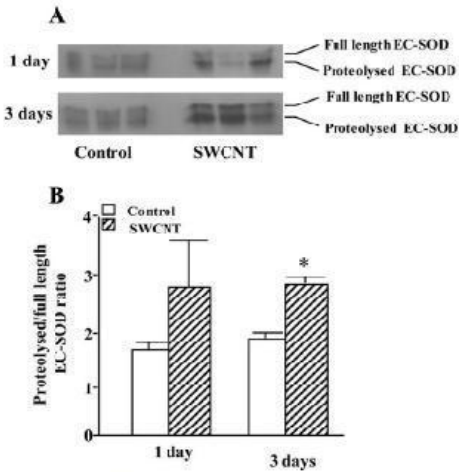
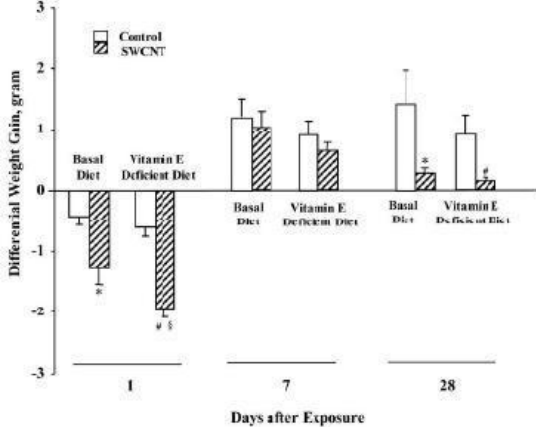


Fig. 3. Changes in EC-SOD following SWCNT treatment. Bronchoalveolar lavage fluid EC-SOD levels are increased in SWCNT-treated mice compared to control mice by western blot analysis (A). Denatometry also shows that the ratio of proteolysed to full length EC-SOD is greatly increased 3 days after SWCNT treatment (B). Mean ± SE (n = 3 mice/group). *p < 0.05 vs control group.

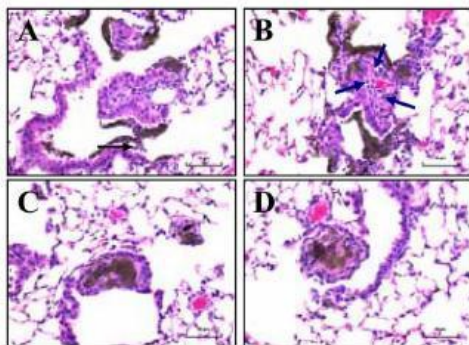


Fig. 5. Light micrographs of H&E-stained sections from lung of mice given basal or vitamin E-deficient diet in response to SWCNT (40 µg/mouse) 1 day post pharyngeal aspiration: A - basal diet, B - vitamin E-deficient diet, 28 days post pharyngeal aspiration; C - basal diet, D - vitamin E-deficient diet. Arrows indicated accumulation of PMNs. Magnification ×400.

Applicant's summary and conclusion

Conclusions

The vitamin E-deficient diet caused a 90-fold depletion of α -tocopherol in the lung tissue and resulted in a significant decline of other antioxidants (GSH, ascorbate) as well as accumulation of lipid peroxidation products. A greater decrease of pulmonary antioxidants was detected in SWCNT-treated vitamin E-deficient mice as compared to controls. Lowered levels of antioxidants in vitamin E-deficient mice were associated with a higher sensitivity to SWCNT-induced acute inflammation (total number of inflammatory cells, number of polymorphonuclear leukocytes, released LDH, total protein content, and levels of pro-inflammatory cytokines, TNF- α and IL-6) and enhanced profibrotic responses (elevation of TGF- β and collagen deposition). Exposure to SWCNTs markedly shifted the ratio of cleaved to full length extracellular superoxide dismutase (EC-SOD). Given that pulmonary levels of vitamin E can be manipulated through diet, its effects on SWCNT induced inflammation may be of practical importance in optimizing protective strategies.

Endpoint study record: CNI SWCNT Acute toxicity: Intratracheal instillation.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Miyawaki et al.	2008	Toxicity of Single-Walled Carbon Nanohorns	ACSNano, Vol.2, No.2, 213-226					

Materials and methods

Type of method

in vivo

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)
no

Test material identity

Identifier	Identity
other: CNI	HiPco

Details on test material

The nominal size of the SWCNTs was 1.0 ± 0.2 nm in diameter and several hundred nanometers to several micrometers long. However, the SWCNTs were rarely individual units; instead, they were mostly aggregated into bundles. The purity of the SWCNTs was >90%; metal catalyst (Fe) residue accounted for about 5-10 wt%.

Test animals

Species

rat

Strain

Wistar

Sex

male

Details on test animals and environmental conditions

SPF male rats (4 weeks old upon arrival) of the Wistar Hannover GALAS variety (BrlHan: WIST@Jcl(GALAS)) were obtained from CLEA Japan, Inc. The animals were housed in stainless steel suspended cages (two or three animals/cage) during 8 day acclimation under controlled laboratory conditions (20-26°C, 40-70% relative humidity, 12 h/12 h light/dark cycle). They had free access to standard pellets (FR 2, Funabashi Farms Co., Ltd.) and water. At the time of installation, the animals were 5 weeks old and weighed 124-140g. They were treated in accordance with the Institutional Animal Care and Use Guidelines.

Administration / exposure

Route of administration

intratracheal

Vehicle

other: saline, 0.025% w/v Tween 80

Details on exposure

A suspension of test materials was prepared by mixing 0.15 g of the test materials with a small amount of 0.025% w/v Tween 80 (Wako Pure Chemical Industries, Ltd.)/saline (Otsuka Pharmaceutical Factory, Inc.) solution using a mortar and pestle and then adding the 0.025% w/v Tween 80/saline solution to bring the total volume up to 20 mL (final concentration: 7.5 mg/mL). Surfactant Tween 80 was used to obtain a good dispersion of the test materials in the solution, enabling homogeneous instillation into all pulmonary lobes. A 0.3 mL/animal (~2.3 mL/kg of body weight) portion of the suspension was intratracheally instilled into unanesthetized but immobilized rats using a 1 mL disposable syringe barrel with a common metal oral sonde. The dosage was calculated to be 2.25 mg/animal (=17.3 mg/kg of body weight), which is comparable to a dose of 0.5 mg/animal (~17 mg/kg for mice of about 30 g body weight) used in a study of pulmonary toxicity after a single intratracheal instillation of SWCNT suspension.

Analytical verification of doses or concentrations

no

Duration of treatment / exposure

Single administration

Post exposure period

Seven or 90 days after instillation of the test material

Doses / concentrations

2.25mg/mouse

No. of animals per sex per dose

4 mice for 7d and 5 mice for 50 d.

Further details on study design

Rats into which only Tween 80/saline was instilled served as a vehicle control. Seven or 90 days after instillation, each animal was euthanized by exsanguination under pentobarbital anesthesia. Their organs and tissues were autopsied, and the heart, lungs, kidneys, liver, and spleen were weighed. These organs, the brain (only for the 90 day group), and other organs/tissues showing macroscopic abnormalities were fixed in 10% buffered formalin, embedded in paraffin, thin sectioned, stained with hematoxylin eosin, and observed using an optical microscope. Body weight was measured on the day of instillation (day 0), at 1, 3, 5, 7 days after instillation, and thereafter once a week.

Results and discussions**Details on results**

There was no statistically significant difference in body weight gain between the vehicle control group and the SWCNT group. For SWCNT groups, foreign body granuloma together with inflammatory cell infiltration and foamy macrophage in the intra-alveolar spaces were observed for one of the four animals in the 7 day group, and one of the five animals in the 90 day group showed formation of foreign body granuloma in which many black particles were completely surrounded by the granuloma. Although there was not much difference in the degree of the lesions of the foreign body granuloma observed for both periods, it is difficult to predict the long-term outcome of the lesions at the moment.

Overall remarks, attachments

Illustration (picture/graph)

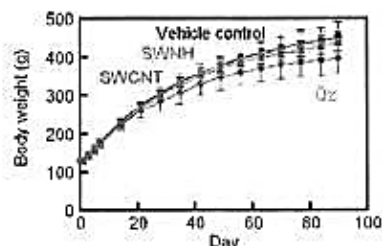


Figure 5. Time profiles of body weight change of rats after single-dose intratracheal instillation: (black) vehicle control group; (red) SWNH group; (blue) SWCNT group; (green) Qz group ($n = 5$ for each group). Weights are mean $\pm$ standard deviation.

TABLE 5. Histopathological Findings for Intratracheal Instillation Test

organ	finding	degree	Tween 80/saline		SWNH		SWCNT		Qz	
			day 7 autopsy, 4 animals	day 90 autopsy, 5 animals	day 7 autopsy, 4 animals	day 90 autopsy, 5 animals	day 7 autopsy, 4 animals	day 90 autopsy, 5 animals	day 7 autopsy, 4 animals	day 90 autopsy, 5 animals
lungs	foreign body granuloma	+	0	0	0	0	0	1	0	0
		++	0	0	0	0	1	0	0	0
	inflammatory cell infiltration	+	0	0	0	0	1	0	0	0
	foamy macrophage in intra-alveolar spaces	$\pm$	0	0	1	1	0	0	0	0
		+	0	0	0	0	1	0	0	1
		++	0	0	0	0	0	0	2	1
	granuloma sarcoido-like	++	0	0	0	0	0	0	2	0
	granulomatous inflammation	++	0	0	0	0	0	0	0	2
		+++	0	0	0	0	0	0	0	3
	anthracosis	+	0	0	0	3	0	0	0	0
	fibrin deposition in intra-alveolar spaces	+	0	0	0	0	0	0	0	2
		++	0	0	0	0	0	0	0	2
		+++	0	0	0	0	0	0	0	1
	fibrosis	+	0	0	0	0	0	0	0	3
heart	focal infiltration of mononuclear cells	$\pm$	0	3	0	0	0	2	0	0
kidneys	basophilic tubules	$\pm$	0	1	0	0	0	0	0	0
liver	microgranuloma	+	0	1	0	0	0	0	0	0
spleen	abnormality		0	0	0	0	0	0	0	0
brain	abnormality		NA	0	NA	0	NA	0	NA	0
thoracic lymph node	sarcoido-like granulomatous inflammation	+	0	0	0	0	0	0	0	2
		++	0	0	0	0	0	0	0	2
		+++	0	0	0	0	0	0	0	1

*Vehicle control, NA: not applicable.

Applicant's summary and conclusion

Conclusions

Male Wistar rats were exposed to SWCNT (CNI, HiPco) by intratracheal instillation at 2.25 mg/rat (17.3 mg/kg). Formation of foreign body granuloma in the lungs was observed. Severity of pathological findings was similar at 7 days and 90 days post-instillation (not recovered).

Endpoint study record: CarboLex SWCNT Acute toxicity: Intratracheal instillation.001**Administrative Data**

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Chou et al.	2008	Single-Walled Carbon Nanotubes Can Induce Pulmonary Injury in Mouse Model	NANO LETTERS 2008 Vol. 8, No.2 437-445					

Materials and methods**Type of method**

in vivo

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

no

Test material identity

Identifier	Identity
other: CarboLex	

Details on test material

SWCNT (99.5% purity; CarboLex, KY) particles possess fibrous structure with diameters about 12 nm and lengths ranging from tens of nanometers to several micrometers.

Test animals**Species**

mouse

Strain

ICR

Sex

male

Details on test animals and environmental conditions

ICR mice (8 weeks old) in the study were treated according to the Animal Care and Use Program guidelines accredited by National Health Research Institutes(NHRI) in Taiwan.

Administration / exposure

Route of administration

intratracheal

Vehicle

other:

Details on exposure

The mice (about 25 g) were administered with 0.5 mg of SWCNT (95% purity;CarboLex, KY) dispersed in a cell culture surfactant, Pluronic F-68, at a final concentration of 0.1% (w/v) by intratracheal instillation as described in the literature (Lam et al. 2004,Leong et al. 1998). Pluronic F-68 (cell culture grade) was used as the experimental controls.

Analytical verification of doses or concentrations

no

Duration of treatment / exposure

Single administration

Post exposure period

3 or 14 days after instillation of the test material

Doses / concentrations

0.5mg/mouse

Results and discussions

Details on results

A significant inflammatory response and pathological changes were observed in the SWCNT-treated mice on histopathological examination of the lungs. At the early stage (day 3), the foamy-like macrophages loaded with SWCNT were found to have accumulated in alveolae (Figure 1 B,F). The effects persisted and progressed to give rise to multifocal macrophage-containing granulomas around the sites of SWCNT aggregation at the 14-day postexposure (Figure 1 C,D,G, H). The granulomas are mainly composed of aggregates of macrophages laden with SWCNT particles.

Overall remarks, attachments

Illustration (picture/graph)

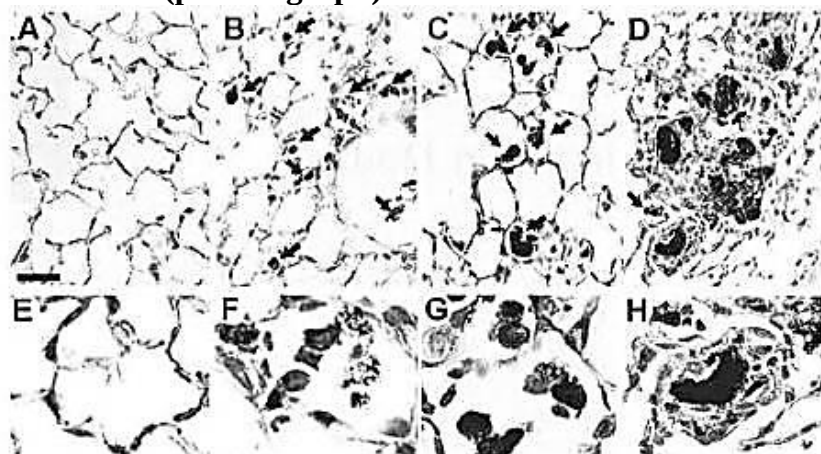


Figure 1. Hematoxylin and eosin staining of mouse lung tissue (A–H) and THP-1 derived macrophages (I,J). (A,E) Fluoronic F-68-treated group acts as the solvent control. (B,F) Early response (3 days) of the mouse lung tissue to a single dose of 0.5 mg of SWCNT. (C,D,G,H) Two weeks response of the mouse lung tissue to a single dose of 0.5 mg of SWCNT. (F,G) SWCNT-loaded foamy-like macrophages in the alveolae; (H) multifocal macrophage-containing granuloma around the sites of SWCNT aggregates. (A–D) Original magnification $\times 100$, bar = 100 μm ; (E–H) $\times 400$. The black arrows shown in panels B and C indicate the SWCNT-loaded foamy-like macrophages.

Applicant's summary and conclusion

Conclusions

ICR mice were exposed to SWCNT (Carbolex) by intratracheal instillation at 0.5 mg/kg. Foamy macrophages phagocytosing CNT was observed at 3 days post-instillation and multifocal granuloma and foamy macrophages at 14 days.

Endpoint study record: HiPCO SWCNT Intratracheal instillation.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Lam et al.	2004	Pulmonary Toxicity of Single-Wall Carbon Nanotubes in Mice 7 and 90 Days After Intratracheal Instillation	TOXICOLOGICAL SCIENCES 77, 126-134 (2004)					

Materials and methods**Type of method**

in vivo

Test materials**Identity of test material same as for substance defined in section 1 (if not read-across)**

no

Test material identity

Identifier	Identity
other: RNT(raw SWCNT, HiPco product of Rice)	
other: PNT(purified HiPco product of Rice)	
other: CNT(CarboLex's electric-arc product)	

Details on test material

RNT:raw SWCNT Fe 26.9%, Ni 0.78%, Cu0.26%, Mo0.96%, Zn0.01%PNT:purified SWCNT Fe 2.14%, Ni 0.00%, CNT:Fe 0.53%, Ni 25.99%, Y 5.01%, Al0.15%, Zn0.15%, Co0.02%

Test animals**Species**

mouse

Strain

B6C3F1

Sex

male

Details on test animals and environmental conditions

Male mice (B6C3F1, 2 months old), free of known rodent pathogens, were obtained from Charles River (Indianapolis, IN). The types of pathogens screened can be found in Charles River's Rodent Health Monitoring Summary (Charles River, 1998). The animals were housed in groups of 4 or 5 in

polycarbonate cages (with HEPA air filters) in the AAA LAC-accredited vivarium at the Johnson Space Center (JSC). Animals were allowed to acclimate at this facility (with a 12-h light-dark cycle) for at least one week before being used in the study. The mice had free access to tap water and Purina Formulab Chow No. 50008 (Ralston Purina Co., St. Louis, MO). They were cared for and used humanely according to NASA Animal Care and Use Program guidelines. The animals weighed about 30 g when the dust treatments were administered.

Administration / exposure

Route of administration

intratracheal

Vehicle

other: heat inactivated mouse serum

Details on exposure

The NT suspensions (2 mg/ml (0.1 mg/50 µl) or 10 mg/ml (0.5 mg/50 µl)) were prepared by briefly shearing (2 min in a small glass homogenizing tube) and subsequently sonicating (0.5 min) NT samples in heat-inactivated mouse serum (Sigma, St Louis, MO). Brief sonication does not shorten or change the fundamental nature of NTs (Hauge, personal communication). Serum inactivation was performed in a water bath heated to 56°C for 0.5 h. Serum, used by Leong et al. (1998) and found in our pilot studies to be the best dispersing vehicle for NT, was also used to suspend other test dusts. Samples that were not freshly generated on the day of dosing were resonicated before the instillation. Each sample was vortexed just before an aliquot was drawn for instillation. After being anesthetized with 3 to 5% isoflurane in a small chamber, individual mice were secured on an inclined plastic platform and anesthesia continued via a small nose cone. The trachea was exposed by a 1-cm incision on the ventral neck skin for instillation of the dust suspension (Lam et al., 2002a). The instillation procedures for mice reported by these authors and the intratracheal fast instillation/nebulization procedure for rats used by Leong et al. (1998) were modified to ensure that instilled material was delivered into the lungs of mice with a good distribution.

Analytical verification of doses or concentrations

no

Duration of treatment / exposure

Single administration

Post exposure period

Seven or 90 days after instillation of the test material

Doses / concentrations

0.1 mg or 0.5 mg/mouse

No. of animals per sex per dose

4 mice for 7d and 5 mice for 50 d.

Examinations

Positive control

Quartz(Min-U-Sil-5)

Results and discussions

Details on results

All animals treated with 0.1 mg per mouse (low dose, LD) of CNT (containing Ni and Y) showed no overt clinical signs. However, 5 of the 9 mice treated with 0.5 mg (high dose, HD) of this product died (2/4 in the 7-d group and 3/5 in the 90-d group). All deaths occurred 4 to 7 days after instillation of the CNT. The deaths were generally preceded by lethargy, inactivity, and body-weight losses. These symptoms were also seen in the HD mice that survived. Mice in the HD 90-d CNT group (including those that died within the first week) lost about 27% of their body weight (pretreatment: 30.9 ± 1.1 g; posttreatment: 22.5 ± 0.9 g) by the first week. Symptoms in the two surviving mice disappeared after one week, and the animals started to gain weight. The iron-containing NTs (RNT and PNT) did not cause deaths in the mice. Mild signs of inactivity, hypothermia (felt cold to the touch), piloerection, and occasionally shivering (when provoked by picking up and laying down) were most noticeable 8 to 12 h after treatment with the HD RNT; these symptoms disappeared soon after this time. These clinical signs were not observed in the mice treated with PNT. Body weight losses, seen in the first week with the HD CNT, were not observed with RNT or PNT. In the distribution of black particles in lungs 90 d after they were instilled with 0.5 mg of CB or NTs, some of the lungs had a relatively uniform particle distribution, while others did not. At the microscopic level, the lungs of dead animals of the HD CNT groups showed congestion and postmortem histopathological changes. The lungs of the 4 (2 from the 7-d and 2 from the 90-d group) surviving mice treated with HD CNT had large aggregates of particles in macrophages in the alveolar space; some of these aggregates were also found in the interstitium, forming granulomas. Some interstitial inflammation was apparent. Granulomas were not detected in the LD groups (7 d and 90 d) given CNT. The lungs of mice in the HD 7-d study that were treated with either RNT or PNT showed prominent granulomas. Most of these microscopic nodules were located beneath the bronchial epithelium and were present throughout most of the lung fields. Some appeared to extend into the bronchias polyps. The granulomas consisted of macrophages laden with black particles, and had very few lymphocytes, neutrophils, eosinophils, or other inflammatory cells. The macrophages had abundant granular cytoplasm with indistinct borders, a characteristic of activated macrophages or epithelioid cells. The black particles were almost entirely contained within these granulomas. Some of the lungs from HD 90-d NT-treated groups appeared grossly abnormal. The micrographs of lungs from these groups showed the persistence of granulomas that contained particle-laden macrophages and NT particles. The lung lesions were generally more pronounced than those of the HD 7-d groups; some also had necrosis, interstitial inflammation that had extended into the alveolar septa, and peribronchial inflammation. Granulomas and other pulmonary lesions were also seen in some of the LD HiPco-NT-treated mice (Table 2), but to a mild degree. As expected, heat-inactivated mouse serum did not produce any clinical signs, or gross and microscopic lesions. The mice of the negative (CB) or positive (quartz) control groups also did not show any clinical signs that could be attributed to treatment. Aside from the presence of black particles that appeared predominantly in alveoli, the lungs of the mice in CB groups were microscopically normal. The lungs of the LD quartz groups were also normal. Quartz at HD induced an increase in the number of alveolar macrophages in the lungs, and some of these cells contained particles. Quartz also produced mild to moderate alveolar and interstitial inflammation. The results for the 7-d and 90-d groups were generally similar. One of the mice in the 7-d group had a low-grade granulomatous reaction. The HD 90-d group had increased clusters of peribronchiolar lymphocytes.

Any other information on results incl. tables**Table 2. Incidence of Pulmonary Lesions in Mice 90 Days After Intratracheal Instillation with Nanotubes***

Dust dose (mg)	Type of lung lesion	Carbon black	Quartz	RN T	PNT	CN T
0.1	Inflammation	0	I	3	2	0
0.1	Granulomas	0	0	5	2	0
0.5	Inflammation	0	4	3	5	0
0.5	Granulomas	0	0	5	5	5**

*Mice (5/group) were each instilled with 0.1 or 0.5 mg of test material and euthanized 90 days after the single treatment. Lungs were microscopically examined by a pathologist who had no knowledge of the treatment of each animal.

**including 3 mice that died in the first week.

Applicant's summary and conclusion**Conclusions**

For the test conditions described here and on an equal-weight basis, that if NTs reach the lungs, they are much more toxic than carbon black and can be more toxic than quartz, which is considered a serious occupational health hazard in chronic inhalation exposures.

Endpoint study record: Dupont SWCNT Intratracheal instillation.001**Administrative Data**

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Warheit et al.	2004	Comparative Pulmonary Toxicity Assessment of Single-wall Carbon Nanotubes in Rats	TOXICOLOGICAL SCIENCES 77, 117-125 (2004)					

Materials and methods

Type of method

in vivo

Test materials

Identity of test material same as for substance defined in section 1 (if not read-across)

no

Test material identity

Identifier	Identity
other:	

Details on test material

SWCNT soot generated via a laser ablation process (Rinzler et al., 1998) was obtained from GAM Reynolds and D. I. Roach of DuPont Central Research. The nominal size of single-wall nanotubes is 1.4 nm diameter $\times$ $> 1 \mu\text{m}$ length. However, the nanotubes merely exist as individual units and exist primarily as agglomerated "ropes" of nanotubes of ~ 30 nm diameter. The soot is composed of about 30-40% amorphous carbon (by weight) and 5% each of nickel and cobalt, with the balance being the carbon nanotube agglomerates.

Test animals

Species

rat

Strain

other: CrI:CD(SD)IGS BR

Sex

male

Details on test animals and environmental conditions

Groups of male CrI:CD (SD)IGS BR rats (Charles River Laboratories, Inc., Raleigh, NC) were used in this study. The rats were approximately 8 weeks old at study start (mean weights in the range of 240-255 grams). All procedures using animals were reviewed and approved by the Institutional Animal Care and Use Committee, and the animal program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

Administration / exposure

Route of administration

intratracheal

Vehicle

other: All particles were prepared in a volume of 1.0% Tween 80 and phosphate-buffered saline (PBS)

and subjected to polytron dispersement.

Details on exposure

Groups of rats were intratracheally instilled with 1 or 5 mg/kg of SWCNT, quartz-crystalline silica particles (Q), or carbonyl iron (CI) particles. All particles were prepared in a volume of 1.0% Tween 80 and phosphate-buffered saline (PBS) and subjected to polytron dispersement. Groups of PBS and PBS-Tween instilled rats served as controls. The lungs of PBS, PBS-Tween, and particle-exposed rats were evaluated by bronchoalveolar lavage at 24 h, 1 week, 1 month, and 3 months post-exposure. For the morphological studies, additional groups of animals were instilled with the particle types listed above as well as PBS, PBS-Tween, and graphite particles. These studies were dedicated for lung tissue analyses and consisted of cell proliferation assessments and histopathological evaluations of the lower respiratory tract. Similar to the BAL fluid studies, the intratracheal instillation exposure period was followed by 24-hour, 1-week, 1-month, and 3-month recovery periods.

Frequency of treatment

Single administration

Post exposure period

The intratracheal instillation exposure period was followed by 24-hour, 1-week, 1-month, and 3-month recovery periods.

Doses / concentrations

Basis nominal conc. (1 or 5 mg/kg of SWCNT)

No. of animals per sex per dose

No data

Further details on study design

Groups of male rats were exposed via intratracheal instillation to (1) vehicle control-PBS, (2) additional vehicle control-PBS + 1% Tween 80, (3) SWCNT in 1% Tween + PBS at 1 and 5 mg/kg, (4) Min-U-Sil crystalline quartz particles in 1% Tween + PBS at 1 and 5 mg/kg, and (5) carbonyl iron particles in 1% Tween + PBS at 1 and 5 mg/kg. All particle types were suspended in phosphate-buffered saline and 1% Tween 80.

Examinations

Any other information on materials and methods incl. tables

The lung of sham and particulate-exposed rats were lavaged with a warmed PBS solution as described previously (Warheit *et al.*, 1991, 1997).

Briefly, the lungs were removed from the thoracic cavity and lavaged with a PBS solution that had been heated to ~37°C. A 10ml syringe was used to fill the lungs with 8ml of PBS per wash. The lungs were gently manipulated after insertion of the PBS and during the withdrawal of lavage fluid. The first recovered 12 ml of lavaged fluids was used for BAL fluid analyses, and an additional 30 ml was collected for cell counts and differentials. Methodologies for cell counts, differentials, and pulmonary biomarkers in lavaged fluids were conducted as previously described (Warheit *et al.*, 1991, 1997). All biochemical assays were performed on BAL fluids using a Roche Diagnostics (BMC)/Hitachi 717 clinical chemistry

analyser using Roche Diagnostics (BMC)/Hitachi reagents. Lactate dehydrogenase (LDH), alkaline phosphatase (ALP), and lavage fluid protein were measured using Roche Diagnostics (BMC)/Hitachi reagents. Lactate dehydrogenase is a cytoplasmic enzyme and is used as an indicator of cell injury. Alkaline phosphatase activity is a measure of Type II alveolar epithelial cell secretory activity, and increased ALP activity in BAL fluids is considered to be an indicator of Type II cell toxicity. Increases in BAL fluid protein concentrations generally are consistent with enhanced permeability of vascular proteins into alveolar regions.

Results and discussions

Details on results

SWCNT

- Related Mortality; Exposure to high-dose (5 mg/kg) carbon nano tubes produced mortality in ~15% of the SWCNT-instilled rats within 24 h postinstillation. Upon further extensive investigation, this mortality was determined to result from mechanical blockage of the upper air ways by the instillate and was not due to inherent pulmonary toxicity of the instilled SWCNT particulate. Thus, it is believed that the mortality caused by exposure to SWCNT 5 mg/kg was an artifact of the exposure regimen, due, in large part, to the nature of the carbon nanotubes, which are highly electrostatic and do not disperse into individual nanotubes (1 nm diameter X 1 µm length), but instead form "nanoropes" that consist of agglomerates of 10-100 nanotubes. In addition, careful observations of the animals indicated that the rats that survived the 5 mg/kg SWCNT dose for a 24-hour period appeared normal throughout the duration of the study, as evidenced by subsequent normal eating behavior and weight gain throughout the pe period. Instillation of SWCNT at 1 mg/kg or exposures to any other particle types did not produce mortality in exposed rats.

Lung Weights; Lung weights of rats increased with increasing age (i.e., increased pe time periods following instillation). Lung weights in high-dose SWCNT exposed rats were significantly increased versus controls at 24 h, 1 week, and 1 month but not at 3 months pe (data not shown). Pulmonary Injury and Inflammation; The numbers of cells recovered by bronchoalveolar lavage from the lungs of high-dose quartz-exposed (5 mg/kg) groups were significantly higher than any of the other groups for all pe time periods (Fig. 2A). Intratracheal instillation exposures of several particle types produced a short-term, pulmonary inflammatory response, as evidenced by an increase in the percentages/numbers of SAL-recovered neutrophils, measured at 24 h postexposure. However, only the exposures to quartz particles (1 and 5 mg/kg) produced sustained pulmonary inflammatory responses, as measured through 3 months pe. Transient increases in BAL fluid lactate dehydrogenase values were measured in the lungs of high-dose (5 mg/kg) SWCNT-exposed rats at 24 h post exposure, but were not sustained through the other pe time periods. In contrast, exposures to 5 mg/kg quartz particles produced a sustained increase in BAL fluid LDH values through the 3-month pe period. Transient increases in BAL fluid microprotein values were measured in the lungs of high-dose (5 mg/kg) SWCNT exposed rats at 24 h pe, but were not different from controls at 1 week pe. In contrast, exposures to 5 mg/kg quartz particles produced a sustained increase in BAL fluid microprotein values at 24 h, 1 month, and 3 months pe. Transient increases in BAL fluid alkaline phosphatase values were measured only in the lungs of quartz-exposed rats at 24 h pe (1 mg/kg) and at the 1-week (5 mg/kg) pe time periods (data not shown).

Overall remarks, attachments

Remarks on results including tables and figures

It has been demonstrated that under the conditions of this test, pulmonary exposures to carbon nanotubes in rats produced a dose-dependent series of multifocal granulomas, evidence of a foreign tissue body reaction. These multifocal granulomas consisted of macrophage-like multinucleated giant cells and appeared to be associated with and surrounded/sequestered a black carbon nanotube bolus. The

distribution of these lesions was non uniform, with some lobes containing many granulomas and others containing few if any granulomas. There appeared to be no dose-response relationship, concomitant with a possible regression of lesions occurring from the 1-month to 3-month post-exposure periods. The development of these lesions is not consistent with a normal dust-related paradigm, where lesions are not ed initially at the sites of particle deposition, i.e., the junctions of the terminal bronchiole and adjacent alveolar ducts. Moreover, the pulmonary bioassay/bronchoalveolar lavage biomarker results were not predictive of this lesion.

Two recent exposure assessment studies have been conducted at the workplace where carbon nanotubes are either manufactured or utilized. Both assessments have reported very low aerosol exposure levels of respirable-sized carbon nanotubes, ranging from not detectable to <0.1 mg/m³.

These findings give credence to the hypothesis that, due to their electrostatic nature and tendency to agglomerate into nanorope structures, exposures at the workplace to respirable-sized carbon nanotubes are extremely low. As a consequence, the pulmonary toxicity study findings

Of multifocal granulomas that have been reported here in may not have physiological relevance, and maybe related to the instillation of a bolus of agglomerated nanotubes (i.e., nanoropes). Therefore, to reconcile the unusual adverse findings of this study with the potential risk associated with inhaling carbon nanotubes, its relevance at the pulmonary effects of carbon nanotubes must be evaluated by generating aerosols of SWCNT and, thus, conducting an inhalation toxicity study of carbon nanotubes in rats.

Endpoint study record: Helix SWCNT Acute toxicity: Pharyngeal aspiration.001

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Mangum et al.	2006	Single-Walled Carbon Nanotube (SWCNT)-induced interstitial fibrosis in the lungs of rats is associated with increased levels of PDGF mRNA and the formation of unique intercellular carbon structures that bridge alveolar macrophages In Situ	Particle and Fibre Toxicology 2006, 3:15					

Materials and methods**Type of method**

in vivo

GLP compliance

no data

Test materials**Test material identity**

Identifier	Identity
other:	Helix SWCNT

Details on test material

High-purity SWCNT were purchased from Helix Material Solutions, Richardson, TX [19]. These SWCNT were produced by a chemical vapor deposition process yielding particle external diameters of less than 2 nm with lengths ranging from 0.5 to 40 microns and a purity > 90% as determined by transmission electron microscopy (TEM), scanning electron microscopy (SEM), Raman spectroscopy and thermogravimetric analysis (TGA). SWCNT contained less than 5% amorphous carbon. The specific surface area of SWCNT was 300–600 m²/g. The elemental composition of SWCNT as determined by energy dispersive X-ray diffraction was 89.6% carbon, 6.1% oxygen, 2.6% cobalt (Co), and 1.7% molybdenum (Mo). Co and Mo were metal catalysts used in the chemical vapor deposition process.

Test animals**Species**

rat

Strain

other: pathogen-free CDF (F344)/CrIBR rats

Sex

female

Details on test animals and environmental conditions

Rats were purchased from Charles River Breeding Laboratories, Kingston, NY, and housed in an International Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited humidity and temperature controlled facility. Rats were housed in microisolator cages on Alpha-dri cellulose bedding and supplied water and cereal-based diet NIH07 (Zeiger Brothers., Gardners, PA) ad libitum. The animal studies were approved by the CIIT Centers for Health Research Institutional Animal Care and Use Committee.

Administration / exposure**Route of administration**

other: Pharyngeal aspiration

Vehicle

other:

Details on exposure

Dry SWCNT were milled in a Retsch Mixer Mill (Retsch Inc., Newtown, PA) for 5 minutes at 30 cycles per second. The milled nanoparticles were then suspended in a biocompatible nonionic surfactant, 1% Pluronic F-68 (BASF Corp., Florham Park, NJ) in PBS and wet milled for an additional 5 min. The CB and SWCNT suspensions were further diluted with PBS to achieve the desired final dosing concentration of 0.1% Pluronic F-68. Animals were anesthetized with isofluorane and ~100 µl volume of either CB, SWCNT or V2O5 (dose concentration equivalent of 2 mg of particles per kg of bodyweight) was placed at the back of the throat while holding the rat's tongue until the suspension was aspirated into the lungs. Control rats were administered an equivalent volume of PBS with 0.1% Pluronic F-68 surfactant.

Duration of treatment / exposure

Single pharyngeal aspiration: 0, 2 mg per kg of bodyweight

Frequency of treatment

Single pharyngeal aspiration

Post exposure period

1 and 21 days following particle exposure

No. of animals per sex per dose

5 animals per sex per dose

Further details on study design

The objective of this study was to determine whether SWCNT are fibrogenic in the lungs of rats after exposure by oropharyngeal aspiration. Moreover, it was sought to determine whether SWCNT increase mRNA levels of growth factors that mediate a fibrogenic response. It was hypothesized that SWCNT would increase the expression of pro-fibrotic growth factors (PDGF, CTGF, TGF-β1) and thereby induce interstitial fibrosis. Oropharyngeal aspiration is a standard method for delivering particle suspensions to the lung and results in better particle distribution throughout the lung as compared to intratracheal instillation. Quantitative real time RT-PCR is the optimal method for measuring changes in growth factor mRNA levels in the lungs of exposed animals and changes in mRNA can be normalized for several standard housekeeping genes (described below under Taqman quantitative real time RT-PCR). Animals were acclimated for two weeks and then randomly assigned into treatment groups according to body weight prior to particle exposure. Rats were exposed to 2 mg/kg of SWCNT by oropharyngeal aspiration. Negative control animals were treated with Ca²⁺ and Mg²⁺ -free phosphate-buffered saline (PBS) with 0.1% pluronic. Carbon black (CB) particles were used as a negative control particle that does not cause fibrosis. Vanadium pentoxide (V2O5) was used as a positive control for causing a fibrotic response in the lungs of rats. Animals were killed by pentobarbitol overdose at 1 and 21 days following particle exposure.

The lungs were lavaged with PBS. The left lungs were used to assess lung cell proliferation and histopathology. The right lung lobes were snapfrozen in liquid nitrogen stored at -80°C until used for RNA isolation and assessment of gene expression by real time quantitative RT-PCR.

Examinations

Positive control

Vanadium pentoxide (V2O5) was used as a positive control for causing a fibrotic response in the lungs of rats.

Results and discussions

Details on results

SWCNT stimulate the formation of focal interstitial fibrotic lesions in the lungs of rats: Clusters of carbon-laden macrophages were observed in the lungs of either CB or SWCNT-exposed rats after 21 days. No fibrotic lesions were observed in the lungs of CB-exposed rats. Relatively small trichrome-positive fibrotic lesions (~100 µm diameter) were observed within the alveolar regions of SWCNT-exposed rats and were adjacent to clusters of alveolar macrophages that contained carbonaceous materials. These fibrotic foci were sparsely distributed within the alveolar region of the lung and affected less than 5% of the total lung tissue. LDH and total protein levels measured in BALF collected from SWCNT-exposed rats were not statistically changed from controls or CB groups. V2O5 caused the formation of fibrotic lesions in the alveolar region and around airways, consistent with previous observation of V2O5-induced lung injury in rats [Bonner 2000]. Intercellular carbon structures that bridge alveolar macrophages are readily identifiable in cytopins of BAL from SWCNT exposed rats: Differential analysis of BAL cell cytopins from CB or SWCNT-exposed rats showed no overt inflammatory response, with greater than 95% macrophages and the remaining 5% or less comprised of neutrophils or lymphocytes. A small percentage of macrophages in either the CB or SWCNT groups contained carbon inclusions. An even smaller number of BAL macrophages from the SWCNT-exposed rats were linked by readily identifiable carbon bridges at 21 days post-exposure. These carbon bridges composed of SWCNT were ~5 µm in length and possessed a unique appearance characterized by fanned ends extending into the cytoplasm of each macrophage, giving the overall appearance of an hourglass. These structures were not apparent in BAL cytopins from rats treated for 1 day with SWCNT. Although some macrophages were joined by SWCNT aggregates at day 1, these were not the well-formed hourglass structures that were readily identifiable at 21 days. Carbon bridges were not observed in BAL cells isolated from CB-exposed rats. The results of the cytology and carbon bridge assessment are shown in Fig. 3. There were no differences in the total numbers of macrophages per field between CB and SWCNT-exposed groups (Fig. 3A). In both CB and SWCNT groups, approximately 5% of the total cells per field contained carbon inclusions of a similar size range (0.1 to 5 µm in diameter). In the SWCNT groups only, approximately one fourth of the carbon inclusions within cells were identified as carbon bridges. No significant differences were observed in the numbers of mitotic figures, i.e., macrophages undergoing cell division, between CB and SWCNT groups. SWCNT induce cell proliferation in small granulomatous lesions and cause carbon bridge formation between macrophages in situ: The light hematoxylin counterstain used for BrdU immunohistochemistry revealed a wide distribution of CB and SWCNT aggregates possessing a similar size range of 0.5 to 5 µm in diameter within the alveolar region. A background labeling index of ~1% BrdU-positive cells were present in the alveolar region or bronchus-associated lymphatic tissue (BALT) of saline vehicle-exposed control rats (Fig. 5). No significant increase in BrdU incorporation was observed in the alveolar region of CB-exposed rats, although carbon-containing macrophages were sparsely distributed within the alveolar region and were observed clustered in alveolar spaces. SWCNT-exposed rats contained BrdU-positive interstitial cells and epithelial cells in the alveolar region and these were limited to sparse fibroproliferative lesions. A small number of alveolar

macrophages joined by carbon bridges were observed in situ within the alveolar region of SWCNT-exposed rats. These in situ carbon bridges between macrophages were not observed in the lungs of CB-exposed rats. SWCNT caused a significant increase in the BrdU labeling index at both day 1 and day 21 (Fig. 5). SWCNT and CB caused a slight but not statistically significant increase in lymphocytes within BALT at day 1 or day 21 (Fig. 5). V2O5 caused a significant increase in the BrdU labeling index in the alveolar region and particularly within BALT at day 1 and day 21 (Fig. 5). Profibrotic growth factors and procollagen gene expression in lungs from CB- and SWCNT-exposed rats: Quantitative real-time RT-PCR was performed on whole lung mRNA from CB, SWCNT, and V2O5-exposed rats to measure changes in the gene expression of a variety of profibrotic growth factors or procollagen (COL1A2). Platelet-derived growth factor (PDGF-A, -B, and -C) mRNA levels were significantly increased in SWCNT-exposed rats at day 1, although less than 2-fold over saline controls (Fig. 6A). SWCNT also increased OPN mRNA levels day 1 (~5-fold) and day 21 (~2-fold) although neither was statistically significant. V2O5 caused a significant increase in PDGF-A, -B and -C, PDGF receptor-alpha (PDGF-R α), transforming growth factor- β 1 (TGF- β 1), OPN, and connective tissue growth factor (CTGF) at 21 days post-exposure. Procollagen mRNAs were not induced by either CB or SWCNT, but were decreased 5-fold by V2O5 (Fig. 6B).

Overall remarks, attachments

Illustration (picture/graph)

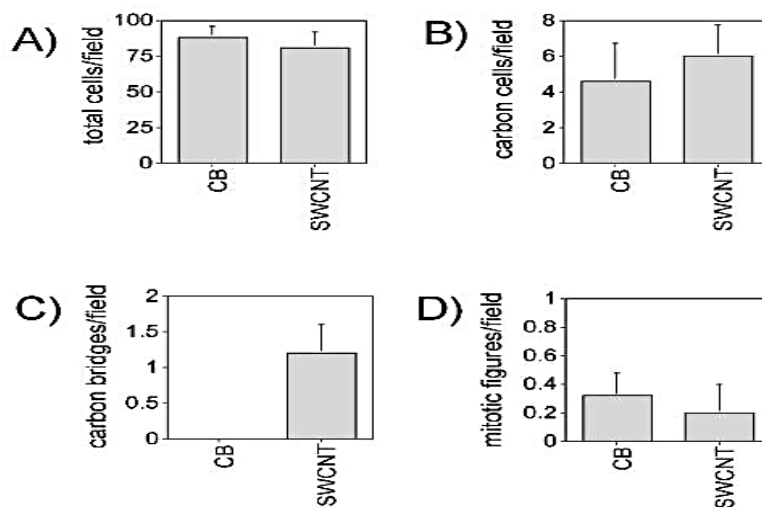


Figure 3
Quantitation of BAL cells in cytopins for assessment of carbon bridge formation between macrophages from 21 day SWCNT-exposed or carbon black (CB)-exposed rats. Each bar represents the mean $\pm$ SD of five animals. Ten fields were counted per animal and at least 500 cells per animal were counted. (A) Total cells/field, (B) Numbers of cells per field containing carbon inclusions, (C) Carbon bridges per field (only found in SWCNT-exposed rats), and (D) Mitotic figures per field.

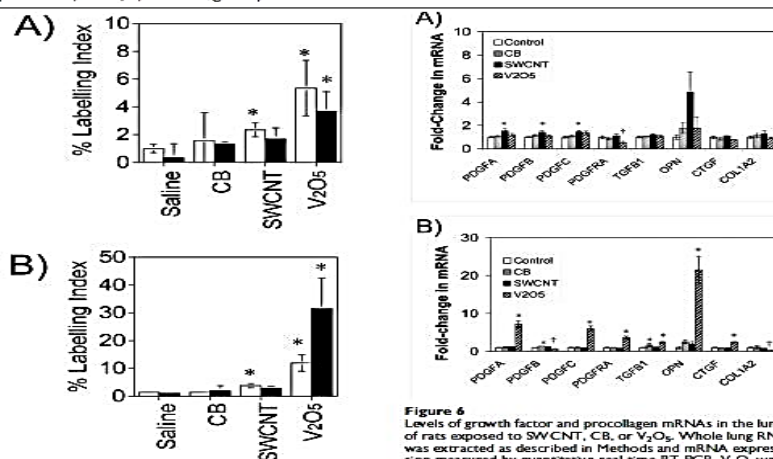


Figure 5
Cell proliferation at (A) day 1 and (B) day 21 post-exposure in the lungs of mice exposed by oropharyngeal aspiration to saline vehicle containing 0.1% Pluronic F-68, carbon black (CB), single-walled carbon nanotubes (SWCNT) or vanadium pentoxide (V_2O_5). Open bars represent the labeling index in the alveolar region of the lung (open bars) and solid bars represent the labeling index in the bronchus-associated lymphatic tissue. Labelling indices were measured as described in Methods and are reported as the percentage of BrdU-labeled cells of the cells counted (minimum of 400 cells counted). *P < 0.05 compared to saline control.

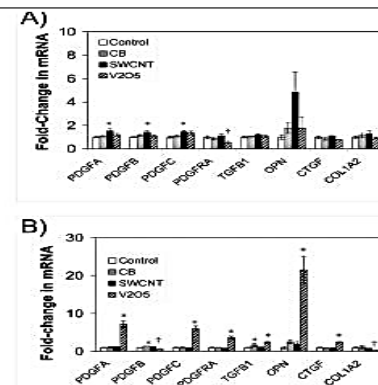


Figure 6
Levels of growth factor and procollagen mRNAs in the lungs of rats exposed to SWCNT, CB, or V_2O_5 . Whole lung RNA was extracted as described in Methods and mRNA expression measured by quantitative real-time RT-PCR. V_2O_5 was used as a positive control for inducing mRNA levels of pro-fibrogenic mediators. (A) 1 day post-exposure. (B) 21 days post-exposure. Control animals were treated with vehicle alone (PBS with 0.1% Pluronic F-68). Data are presented as mean values $\pm$ SEM from three to five animals per group and expressed as the fold-change in mRNA relative to an 18S rRNA housekeeping gene. *P < 0.05 significantly increased or †P < 0.05 significantly decreased compared to control.

Applicant's summary and conclusion

Conclusions

Despite similar size and specific surface area of carbon black nanoparticles and SWCNT used in this study, only SWCNT caused

1. interstitial fibrotic lesions in the lungs of rats,
2. significant increases in lung PDGF mRNA levels, and
3. formation of unique carbon bridge structures between alveolar macrophages in situ. These differences between carbon black nanoparticles and SWCNT suggest that the fibrogenic activity of SWCNT is due to factors that make them unique from carbon black nanoparticles, including their unique shape or the presence of contaminating metal catalysts used in the manufacturing process. Finally, carbon bridge structures that form between macrophages are likely due to unique

structural characteristics of SWCNT. Intercellular carbon bridge structures that link macrophages serve as a useful biomarker of SWCNT exposure in macrophages retrieved by bronchoalveolar lavage.

7.10 Exposure related observations in humans

7.11 Toxic effects on livestock and pets

7.12 Additional toxicological information

Endpoint study record: Additional toxicological information.001; Aneuploidy in vitro

Administrative Data

other:OECD/WPMN

Purpose flag supporting study
Study result type experimental result
Reliability 2 (reliable with restrictions)

Rationale for reliability incl. deficiencies Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Sargent et al.	2009	Induction of aneuploidy by single-walled carbon nanotube	Environ Mol Mutagen, 50(8):208-17	US NIOSH				

Materials and methods

Test materials

Test material identity

Identifier	Identity
other:	CNI SWCNTs

Method

Any other information on materials and methods incl. tables

Cultured primary and immortalized human airway epithelial cells were exposed to SWCNT or a positive control, vanadium pentoxide. After 24 hr of exposure to either SWCNT or vanadium pentoxide, fragmented centrosomes, multiple mitotic spindle poles, anaphase bridges, and aneuploidy chromosome number were observed.

Overall remarks, attachments**Remarks on results including tables and figures**

Nanotubes within the nucleus that were in association with cellular and mitotic tubulin as well as the chromatin. Disruption of the mitotic spindle by SWCNT was observed.

Endpoint study record: Additional toxicological information.002; In Vitro Pulmonary Toxicity

Administrative Data

other:OECD/WPMN

Purpose flag supporting study

Study result type experimental result

Data source**Reference**

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Dreher K. et al.	2013			US EPA				

Materials and methods**Test materials**

Identity of test material same as for substance defined in section 1 (if not read-across)

yes

Test material identity

Identifier	Identity
other:	Carbolex SWCNT
other:	Helix SWCNT
other:	CNI SWCNT
other:	NASA SWCNT

Details on test material

Helix Materials Solutions Inc. SWCNTs; Purity: 90% w/w, Impurities < 2% w/w, Diameter approximately 1.3nm, Length approximately 0.5-40µm Unidym (Carbon Nanotechnologies Inc); Purity: 90% w/w, Impurities < 2% w/w, Diameter approximately 0.8-1.2nm, Length approximately 100-1,000µm Nanotubes are bundled in ropes.

Method

Any other information on materials and methods incl. tables

In Vitro Pulmonary Toxicity: Use of BEAS2B cells (Human airway epithelial cell line). Cellular cytotoxicity assessed for the five different methods; comparative gene expression; and in vitro wound repair assay.

Applicant's summary and conclusion

Executive summary

- 1) Cellular responses were dependent on SWCNT method of synthesis (CVD > ARC > Laser > HiPco).
- 2) The most reliable methods to assess cellular cytotoxicity were trypan blue staining and live/dead cell counting or fluorescent staining and flow cytometry for live/dead cell counting. WST-1, LDH release, or MTT assays were found inappropriate to assess SWCNT cytotoxicity effects on BEAS2B cells.
- 3) Results demonstrated that BEAS2B cells can detect different carbon allotropes.
- 4) SWCNTs were more toxic to BEAS2B cells when compared to either Printex90, Nanographite fibers or Diesel exhaust particles.
- 5) SWCNTs produced by different methods were found to induce different gene expression profiles in BEAS2B cells. In addition, SWCNTs were found to induce different gene expression profiles in BEAS2B when compared to either Printex90 or Diesel exhaust particles.
- 6) SWCNTs were found to inhibit in vitro airway wound repair in the following order of potency based on production method (Laser = CVD > ARC > HiPco).

Endpoint study record: Additional toxicological information.003; CNI SWCNT toxicity in SMC

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Raja et al.	2007	Impact of carbon nanotube exposure, dosage and aggregation on smooth muscle cells	Toxicology Letters 169 (2007) 51-63					

Materials and methods

Test materials

Test material identity

Identifier	Identity
other:	CNI SWCNT

Details on test material

As-produced SWNT (CAS No. 7782-42-5), generated by the HiPco method using an iron catalyst (Carbon Nanotechnologies Inc., Houston, TX), were subjected to mineral acid-treatment (Liu et al., 1998) to minimize the iron catalyst content and render the SWNT dispersible in water by carboxylation at the nanotube defect sites. The SWNT were sonicated in a 3:1 (v/v) mixture of H₂SO₄ and HNO₃ (Mallinckrodt Baker Inc., Paris, KY) using a Branson 5510 ultrasonic cleaner (Branson Ultrasonic Corporation, Danbury, CT) and refluxed. The SWNT-acid mixture was cooled to room temperature, diluted with reverse osmosis-purified (RO) water to remove any residual acid and passed through a 0.45 µm filter (Millipore Corporation, Bedford, MA). The SWNT residue was washed with 28-30% (w/v) ammonium hydroxide (Mallinckrodt Baker Inc.) and RO water to neutralize excess acid. The SWNT were re-suspended from the filter membrane into fresh RO water by sonication and the suspension pH was measured to be 7. The SWNT suspension was centrifuged at 2465 x g for 20 min and the supernatant decanted, to remove large SWNT aggregates, and obtain a suspension containing only finely dispersed SWNT. A fine dispersion allows control over SWNT dosage of corresponding test media. Several batches of acid-treated SWNT were combined to obtain a stock SWNT aqueous suspension and stored at room temperature. The suspension was used a week later to prepare the test media and was stable for at least 4 months. Stability was visually assessed and for the duration the suspension was observed, there was no visible residue or pellet. The suspension concentration was determined by lyophilizing 10 ml of the SWNT stock suspension in duplicate and the lyophilized residue was weighed. The concentration was found to range between 0.18 and 0.22 mg/ml.

Method

Any other information on materials and methods incl. tables

Cell culture

Rat aortic smooth muscle cells (SMC) were the generous gift of Dr. Jan P. Stegemann at Rensselaer. The SMC were grown in a sterile 1:1 (v/v) mixture of Dulbecco's Minimal Essential Medium and Ham's F-12 medium (1:1 DMEM/F-12, MediaTech Inc. Herndon, VA) supplemented with 8.92% fetal bovine serum (FBS, Hyclone Inc., Logan, UT), 1.79 mM L-glutamine (MediaTech) and 89.29 units/ml penicillin/89.29 µg/ml streptomycin (Hyclone) at 37°C in 5% CO₂. The medium was changed every alternate day and the cells were passaged upon confluency. Cells at low passage were seeded at a concentration of 2.7x10⁴ cells/cm² in 96-well plates and allowed to grow for 54 h until reaching 70-75% confluency. Freshly prepared test media was added and incubated for periods of 1, 2.5 and 3.5 days. At the end of each incubation time, the cells were fixed in 4% formaldehyde (Fisher Scientific, Fair Lawn, NJ).

Nanostructured media.

SWNT medium (0.1 mg/ml) was centrifuged at 600xg for 10 min to pellet any SWNT aggregates. The pellet was washed five times with RO water and after each wash the pellet was centrifuged and RO water carefully aspirated. The pellet was air-dried onto a clean silicon wafer for SEM and compared to acid-treated SWNT dispersed in RO water. Likewise, the activated carbon-based medium (0.1 mg/ml) was briefly agitated to re-suspend the activated carbon particles and medium was air-dried onto a clean silicon wafer for SEM. Media (without SWNT or activated carbon) were also deposited and evaporated onto a

silicon wafer as controls.

Filtered media.

Filtered media was concentrated to increase the probability of isolating suspended nanostructured material. Five millilitre samples of SWNT (0.1 and 0.01mg/ml) and activated carbon (0.1mg/ml) filtered media were concentrated approximately 10-15-fold using a Savant ISS110 Speed-Vac system set at a medium drying rate for 100 min. The concentrated filtered media was cooled to 4 °C to precipitate suspended particles. The resultant particles were centrifuged at 600xg for 10 min and washed five times in RO water to remove salts prior to deposition on clean silicon wafers for SEM. SMC growth in response to nanostructured material media SMC growth was monitored for 3.5 days following exposure to the SWNT and activated carbon media. At various time points, the SMC were fixed, stained and imaged to determine cell number per unit growth area.

Overall remarks, attachments

Remarks on results including tables and figures

Both large aggregates/particles as well as finely suspended nanoparticles resulted in a significant decline in SMC growth. A comparison between the SWNT-based nanomaterial and filtered media shows that the finely dispersed nanoparticles were actually a significant cause of cell growth inhibition, despite their low (<1%, v/v) concentration in the media. These particles were observed to be composed of SWNT as well as non-SWNT matter such as amorphous and graphitic carbon. Thus, all the observed effects of the acid-treated SWNT cannot be solely attributed to the SWNT. The presence of the amorphous and graphitic carbon particles may vary with the method and catalyst chosen for the nanotube production, the subsequent chemical treatment protocols (e.g. acid-treatment), and nanotube type (SWNT or MWNT). These particles may be undesirable in SWNT or MWNT-based applications related to nanomedicine, such as drug delivery and gene therapy and optimization of the nanotube related processes and processing parameters may avoid or minimize the presence of impurities.

Applicant's summary and conclusion

Conclusions

The results of this study implied that the size of the particles or aggregates had a noticeable effect on the SMC growth kinetics. While each type of material might have affected the cells by a different mechanism, the overall results indicate that SWNT and related nanomaterials adversely impact the cell growth to a greater extent than other common carbon nanostructured, microparticulate material such as activated carbon.

Endpoint study record: Additional toxicological information.004; SES research SWCNT in vivo vascular study

Administrative Data

Purpose flag	supporting study
Study result type	experimental result
Reliability	2 (reliable with restrictions)
Rationale for reliability incl. deficiencies	Meets generally accepted scientific standards, well documented and acceptable for assessment

Data source

Reference

Reference type	Author	Year	Title	Bibliographic source	Testing laboratory	Report no.	Owner company	Company study no.	Report date
publication	Radomski et al.	2005	Nanoparticle-induced platelet aggregation and vascular thrombosis	British Journal of Pharmacology (2005) 146, 882–893					

Materials and methods

Test materials

Details on test material

SWNT were purchased from SES Research, Houston, TX, U.S.A.

Method

Any other information on materials and methods incl. tables

Human platelets, aggregation and ATP release;

Blood was obtained from healthy volunteers who had not taken any drugs known to affect platelet function for 2 weeks prior to the study. Washed platelets were isolated and resuspended in Tyrode's solution (2.5108 platelets ml⁻¹), as previously described (Radomski & Moncada, 1983). Platelets were preincubated for 2 min at 37°C in a whole blood ionized calcium lumi-aggregometer (Chronolog) prior to the addition of particles (0.2–300 mg ml⁻¹). Prostacyclin (PGI₂), S-nitrosoglutathione (GSNO), aspirin, 2-methylthio-AMP, phenanthroline, ethylenediamine tetraacetic acid (EDTA) and Go⁶⁴⁶⁶ were preincubated with platelets for 1 min before the addition of particles. Platelet aggregation was studied for 8 min and analysed using Aggro-Link data reduction system (Chronolog) (Radomski et al., 2001; 2002; Jurasz et al., 2003). The release of ATP was measured by luciferin-luciferase using lumiaggregometer as previously described (Sawicki et al., 1997; Chung et al., 2002).

Rat vascular thrombosis;

Rodent ferric chloride model of carotid artery thrombosis (Kurz et al., 1990; Wang & Xu, 2005) with the following modifications was used. Wistar–Kyoto rats were anesthetized with ketamine/xylazine solution (0.09 ml 100 g⁻¹ body weight) IM (thigh). The rats were placed in dorsal recumbency on a heated (37°C) surgical board. The trachea was palpated and a midline incision from the caudal end of the larynx to the suprasternal notch was made. The left carotid artery was then isolated and a Parafilm M sheet (720 mm) was placed under the carotid artery in order to prevent chloride-induced tissue damage. To initiate thrombosis, a patch (68 mm) of micro-filter paper saturated with FeCl₃ solution (70% wv⁻¹) was placed

on the carotid artery upstream from the flow probe. Simultaneously, a 0.5 ml suspension (50 mg/ml) of PM in 0.9% NaCl solution was injected intravenously into the femoral vein. The blood flow in carotid artery was monitored continuously with an ultrasonic flow probe (Transit Time Flowmeter TTFM Type 700, Hugo Sachs Elektronik) and an in-house data acquisition system.

Overall remarks, attachments

Remarks on results including tables and figures

Incubation of platelets with SWNT resulted in a concentration-dependent increase in platelet aggregation. The efficacy was larger than MWNT (SES Research) and SRM1648 (Standard particulate matter, average size 1.4 µm).

Infusions of SWNT significantly accelerated time and the rate of development of carotid artery thrombosis in rats and it was more effective than SRM1648.

Applicant's summary and conclusion

Conclusions

SWNT has the ability to activate platelets and enhance vascular thrombosis.

8. ANALYTICAL METHODS

9. RESIDUES IN FOOD AND FEEDINGSTUFFS

10. EFFECTIVENESS AGAINST TARGET ORGANISMS

11. GUIDANCE ON SAFE USE

12. LITERATURE SEARCH

13. ASSESSMENT REPORTS

14. INFORMATION REQUIREMENTS

ANNEXE 1: Acute and Repeated Oral Toxicity of Nikkiso SWCNT and MWCNT (TG423-407).pdf

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.37, No.3, ***-***, 2012

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Original Article

No toxicological effects on acute and repeated oral gavage doses of single-wall or multi-wall carbon nanotube in rats

Mariko Matsumoto¹, Hideki Serizawa², Masao Sunaga³, Hina Kato¹, Mika Takahashi¹,
Mutsuko Hirata-Koizumi¹, Atsushi Ono¹, Eiichi Kamata¹, Akihiko Hirose¹

¹Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences,
1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

²Bozo Research Center Inc., 1284, Kamado, Gotenba-shi, Shizuoka 412-0039, Japan

³Safety Research Institute for Chemical Compounds Co., Ltd., 363-24 Shinei, Kiyota-ku, Sapporo-shi,
Hokkaido 004-0839, Japan

(Received November 29, 2011; Accepted January 25, 2012)

ABSTRACT — Three female Crl:CD(SD) rats/group were dosed with single wall carbon nanotube (SWCNT) or multi wall carbon nanotube (MWCNT) four times by gavage at a total of 50 mg/kg bw or 200 mg/kg bw (four equally divided doses at one-hour intervals). Acute oral doses of SWCNT and MWCNT caused neither death nor toxicological effects, and thus the oral LD₅₀ values for SWCNT and MWCNT were considered to be greater than 50 mg/kg bw and 200 mg/kg bw, in rats respectively. Five or ten Crl:CD(SD) rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 12.5 mg/kg bw/day groups). Six or twelve Crl:CD(SD) rats/sex were dosed with MWCNT once daily by gavage at a dose of 0 (control), 0.5, 5.0 or 50 mg/kg bw/day for 28 days with a 14-day recovery period (0 and 50 mg/kg bw/day groups). Based on no toxicological effects, the no observable adverse effect levels (NOAELs) of repeated dose toxicity of SWCNT and MWCNT were considered to be 12.5 mg/kg bw/day and 50 mg/kg bw/day (the highest dose tested), respectively. It was suggested that SWCNT and MWCNT dosed by gavage reached the gastro-intestinal tract as agglomerates and were mostly excreted via feces.

Key words: Single wall carbon nanotube, Multi wall carbon nanotube, Acute oral toxicity, Repeated oral dose toxicity, Rat

INTRODUCTION

Nanomaterials possess different physico-chemical properties from bulk materials. Therefore, it is necessary to develop specialized approaches to testing their effects on human health and on the environment. Our study group has worked on the establishment of a human health risk assessment methodology of nanomaterials since 2005. As part of efforts, our co-researchers reported carcinogenic potential of intraperitoneal administration of multi wall carbon nanotube (MWCNT) in p53 heterozygous mice (Takagi *et al.*, 2008) and intrascrotal injection of MWCNT in intact Fischer 344 rats (Sakamoto *et al.*, 2009) and also suggested that nano-sized particles can be transferred to other organs. Subsequently, Sakamoto *et al.* (2010) showed that expression of renal carcinoma/mesothelin can be used as a biomarker of mesothelial proliferative lesions

induced by intrascrotal administration of MWCNT.

In parallel of the establishment of our study group, the OECD Working Party on Manufactured Nanomaterials (WPMN) was established to assess the safety of the use of nanomaterials for human health and the environment in 2006, and the Sponsorship Programme on the Testing on Manufactured Nanomaterials was launched in 2007 (OECD, 2011). The aim of the Sponsorship Programme is to fill data gaps between existing data and a desired data set for 13 nanomaterials including single wall carbon nanotubes (SWCNTs) and MWCNTs. Japan has volunteered to act as the Joint Lead Sponsors with the US for the evaluation of mammalian toxicology for fullerenes (C60), SWCNTs and MWCNTs in the Sponsorship Programme. We recently reported no toxicological effects of gavage doses of fullerene C60 up to 1,000 mg/kg bw/day in rats (Takahashi *et al.*, 2012). After the 29-day

Correspondence: Akihiko Hirose (E-mail: hirose@nihs.go.jp)

administration period, blackish feces and black content of the stomach and large intestine were observed. Pulmonary C60 was not detected in the liver, spleen, or kidney at the end of administration period. This study indicated that Pulmonary C60 dosed by gavage was excreted via feces and not distributed in major organs.

We also conducted acute and repeated dose toxicity studies of SWCNT and MWCNT by gavage, target endpoints of the Sponsorship Programme. In some past studies, the physical-chemical properties of novel materials are not clear due to the diversity of carbon nanotubes (CNTs) with respect to purity, production methods, purification methods or surface treatments/coatings (Kobayashi *et al.*, 2008; OECD, 2011). The clear characterization and good control of the size and shape of nano-sized particulate materials are essential for ensuring the reproducibility and reliability of tests. Therefore, well-defined Japanese SWCNT and MWCNT were set as principal samples in this Sponsorship Programme to evaluate nanomaterial toxicity.

Major current uses of MWCNTs are electronics applications such as super-capacitors and batteries and structural composite applications such as sporting equipment and conductive sheets (OECD, 2010a) and the future applications of MWCNTs include medical uses and fibers (Kobayashi *et al.*, 2008; MHLW, 2010). Major emerging uses of SWCNTs in future are super-capacitors, high speed transistors, fuel cells, super high strength wires (OECD, 2010a). A total volume of production and import of MWCNTs was 500 tons in 2008 in Japan, and it is expected to increase in future (MHLW 2010). Oral exposure to CNTs may occur through the ingestion from food contact products or agricultural foods that utilize CNTs from environment (Matsunaga *et al.*, 2011).

Many toxicity studies have been available for SWCNTs or MWCNTs dosed by pleural/peritoneal aspiration (Enslin *et al.*, 2009; Shvedova *et al.*, 2008), intravenous injection (Yang *et al.*, 2008), intratracheal aspiration (Iwano *et al.*, 2008; Elgish *et al.*, 2008; Iwano *et al.*, 2008) and inhalation (Shvedova *et al.*, 2004) in rats and mice, but this will be the first report to show the results of acute and repeated dose toxicity of SWCNT and MWCNT by gavage in rats according to the OECD guidelines (TG 423 and TG 407). Although a gavage dose is not likely to be representative of the anticipated exposure scenario, the findings of our studies will be useful to characterize the future of CNTs toxicity and for risk assessment in humans.

MATERIALS AND METHODS

Acute and repeated dose toxicity studies for SWCNT or MWCNT were performed in the Genetech Laboratory, Bunko Research Center Inc. or the Safety Research Institute for Chemical Compounds Co., Ltd., respectively. These studies were conducted in compliance with the OECD Guidelines 423, Acute Oral Toxicity; the OECD Guideline 407, Repeated Dose 28-Day Oral Toxicity Study in Rodents and the Guideline for 28-Day Repeated Dose Toxicity Test in Mammalian Species (Chemical Substances Control Law of Japan) under GLP. The SWCNT studies were conducted in compliance with the Act on Welfare and Management of Animals (Act No. 100 of October 1, 1972), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No. 83 of the Ministry of Environment, dated April 26, 2004) and the Guidelines for Proper Conduct of Animal Experiments (June 1, 2006). The MWCNT studies were conducted in compliance with the Guidelines for Animal Experimentation (May 22, 1987), along with the above described Acts and Standards.

Animals

CRJCD(10) rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). Female rats (SWCNT: 8 weeks old, MWCNT: 9 weeks old) were used for acute toxicity studies, and male and female rats (SWCNT: 8 weeks old, MWCNT: 5 weeks old) were used for repeated dose toxicity studies. Rats were individually housed in metal cages with wire mesh bottoms and received a basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum*. Rats were maintained at room temperature, a humidity of 50 ± 20%, 12–15 air changes per hour and a 12 hr dark/12 hr light cycle.

Chemicals and dosing

Principal single-wall carbon nanotubes (SWCNT, purity > 99%, Lot No.: SW113P-SW1486/SW1487) and principal multi-wall carbon nanotubes (MWCNT, purity > 99%, Lot No.: 04-12-1041-40) supplied by HiMark Co., Ltd. (Shizuoka, Japan) were used. These chemicals are principal samples in the OECD Sponsorship Programme on the Testing on Manufactured Nanomaterials. The structure of SWCNTs is a honeycomb carbon lattice rolled into a cylinder, and the helix morphology is in sheet form consisting of straight SWCNTs (with a diameter of around 2 nm) bundles with diameters of several decade nanometers. The structure of MWCNTs is honeycomb carbon lattices rolled into a multi-layer tubular shape,

Gavage dose toxicity of SWCNTs and MWCNTs in rats

and the basic morphology is particles consisting of entangled MWCNTs with a diameter of around 30 nm. Both test materials were not coated or modified. The test materials were stored in a polycarbonate bottle with an airtight stopper to prevent dissemination at room temperature, and were accurately weighed and added to gum acacia (vehicle). This vehicle was chosen based on the results of the preliminary investigation with commonly used vehicles, in which CNTs showed the best dispersion state in 5% gum acacia of aqueous solution. The mixture of test materials was homogenized using an ultrasonic homogenizer (SWCNT: UR-200P, TOMY Seiko Co., Ltd., Tokyo, Japan; MWCNT: VC-130, Sonics & Materials Inc., Newtown, CT, USA) and a compact ultrasonic cleaning bath (SWCNT: US-1, As One Co., Ltd., Tokyo, Japan; MWCNT: USC-6, Iwaki Glass Co., Ltd., Chiba, Japan). The homogeneity of test suspensions was confirmed microscopically (Figs. 1 and 2).

Acute toxicity studies for SWCNT and MWCNT were conducted in a stepwise procedure. As a first step, three female rats group were dosed with SWCNT or MWCNT four times by gavage at a total of 50 mg/kg bw or 200 mg/kg bw (four equally divided doses at one-hour inter-

vals), respectively. Because oral toxicity of the test materials was expected to be low, dosage levels were determined based on the maximum doses which could be prepared and administered. The concentrations of 0.625 mg/ml SWCNT and 2.5 mg/ml MWCNT were confirmed to be the limit to prepare. In addition, ethically, a dosing volume of 20 ml/kg was limit for gavage dosing and four times seemed to be the limit for the number of dosing times. In the first step, no deaths or adverse effects were found for either SWCNT or MWCNT dosing. Therefore, a second step was carried out with the same regimen to confirm the acute toxicity of the test materials.

Five or ten rats/sex were dosed with SWCNT once daily by gavage at a dose of 0 (control), 0.125, 1.25 or 12.5 mg/kg bw/day for 28 days. Five animals/sex at 0 and 12.5 mg/kg bw/day were used as the recovery groups and were observed for 14 days after the administration period. Six or twelve rats/sex were dosed with MWCNT once daily by gavage at a dose of 0 (control), 0.5, 5.0 or 50 mg/kg bw/day for 28 days. Six animals/sex at 0 and 50 mg/kg bw/day were used as the recovery groups and were observed for 14 days after the administration period. A high dose was set with the maximum doses which could be prepared, as described above, and the middle and low



Fig. 1. Microscopic views of SWCNT suspensions ($\times 400$). (A) 0.125 mg/kg bw/day (0.00625 mg/ml); (B) 1.25 mg/kg bw/day (0.0625 mg/ml); (C) 12.5 mg/kg bw/day (0.625 mg/ml).



Fig. 2. Microscopic views of MWCNT suspensions ($\times 400$). (A) 0.5 mg/kg bw/day (0.025 mg/ml); (B) 5.0 mg/kg bw/day (0.25 mg/ml); (C) 50 mg/kg bw/day (2.5 mg/ml).

doses were set with a common ratio of 10.

Observations

As for the acute toxicity studies, rats were observed for 14 days. Clinical observation was performed consecutively for several hours after administration and once or twice a day from the next day of administration. Animals were weighed just prior to administration, and 1, 3, 5, 7, 10 and 14 days (MWCNT) or 1, 3, 7 and 14 days (SWCNT) after administration. Necropsy was performed 14 days after administration.

As for the repeated dose studies, all males and females in the MWCNT study were observed twice per day, every day during administration and recovery periods, and in the SWCNT were observed three times per day, every day during the administration and once a day during the recovery period. A detailed clinical observation was carried out one day before administration, on days 7, 14, 21 and 28 days of the administration period and days 7 and 14 of the recovery period in the MWCNT study, and once a week in the SWCNT study. A functional examination was carried out in the fourth week of the administration period and in the second week of the recovery period. Body weight was measured on days 1, 4, 7, 14, 21 and 28 of the administration period and on days 7 and 14 of the recovery period in the MWCNT study, and on days 1, 4, 7, 10, 14, 17, 21, 24 and 28 of the administration period and on days 1, 3, 7, 10 and 14 of the recovery period. Food consumption was measured on days 1, 7, 14, 21 and 28 of the administration period, and on days 7 and 14 of the recovery period. Hematological examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Clinical chemistry examinations were performed on blood samples obtained from fasted rats just prior to necropsy. Necropsy was performed under anesthesia on the day following the end of the administration or recovery period. The external surfaces of rats were examined and a gross internal examination was performed. Organ weights were measured and histopathological evaluations were performed on the organs. Urinary samples were collected for 3 or 4, and 20 hr in the fourth week of the administration period, and in the second week of the recovery period. Urine volume was calculated and a urinary examination was conducted.

Data analysis

For the SWCNT study, continuous data from the administration period were analyzed by the Bartlett test for homogeneity of distribution. When homogeneity was recognized, data were analyzed by the Dunnett test, whereas heterogeneous data were analyzed by the

Dunnett-type mean rank test between the control group and individual treatment groups. For the recovery group data, homogeneity of variance was tested by the F-test. When homogeneity was recognized, the difference in mean values between the control group and treatment groups was analyzed by a Student's t-test, whereas heterogeneous data were analyzed by an Aspin-Welch's t-test.

For the MWCNT study, continuous data were analyzed by the Bartlett test for homogeneity of distribution. When homogeneity was recognized, the Dunnett test was conducted for comparison between the control group and individual treatment groups after a one-way layout analysis of variance. If not homogenous, the data were analyzed using the Kruskal-Wallis test followed by a Mann-Whitney's U-test. Qualitative data were analyzed by the Kruskal-Wallis test followed by Mann-Whitney's U-test.

RESULTS AND DISCUSSION

Acute oral doses of SWCNT and MWCNT caused neither death nor toxicological effects on the clinical observation and body weight. Thus, the oral LD₅₀ values for SWCNT and MWCNT were considered to be greater than 50 mg/kg bw and 200 mg/kg bw in rats, respectively (data not shown).

A 28-day dose of SWCNT caused no death in both sexes. There were no differences in the clinical observation, detailed clinical observation, body weight and food consumption (Table 1) or histopathological examination (Table 2). In the functional examination, significantly low values of landing foot splay were observed in all the treatment groups in males at the end of the administration period (90 ± 11 , 61 ± 15 , 64 ± 18 and 75 ± 10 mm at 0, 0.125, 1.25 and 12.5 mg/kg bw/day, respectively). However, it was due to the high value in the control group and was not considered to be toxicological effects. In urinalysis, a significantly low urine volume was observed during the administration period in females at 1.25 mg/kg bw/day and above (Table 3). However, these values were within the historical background data of the test facility (Mean $\pm$ S.D.: 8.3 ± 4.0 ml/24 hr), and these were not dose dependent. In the hematological examination, significantly high erythrocyte counts in females and lymphocyte counts and basophil counts in males were observed at 12.5 mg/kg bw/day at the end of the administration period (Table 4). However, these changes were considered to be incidental because there were no changes in related parameters. In the serum biochemistry examination, significantly high alanine aminotransferase and triglyceride levels were observed in females in the 0.125 mg/kg bw/day group but not in the high dose groups at

Table 1. Body weight and food consumption in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Body weight (g)								
Day 1	216 ± 7	212 ± 6	214 ± 8	214 ± 8	162 ± 5	165 ± 6	160 ± 7	161 ± 7
Day 4	243 ± 8	238 ± 8	241 ± 11	241 ± 10	174 ± 8	175 ± 5	172 ± 8	172 ± 7
Day 7	274 ± 12	267 ± 9	273 ± 16	270 ± 14	187 ± 9	186 ± 6	181 ± 9	184 ± 8
Day 14	335 ± 18	322 ± 16	337 ± 29	331 ± 20	211 ± 14	210 ± 10	198 ± 12	209 ± 10
Day 21	390 ± 26	368 ± 27	388 ± 34	378 ± 24	231 ± 18	234 ± 11	213 ± 17	227 ± 15
Day 28	426 ± 31	399 ± 34	426 ± 39	413 ± 30	250 ± 22	246 ± 15	227 ± 18	245 ± 17
Food consumption (g/rat/day)								
Day 0 - 1	25 ± 2	24 ± 1	25 ± 2	25 ± 1	18 ± 2	20 ± 1	18 ± 2	19 ± 2
Day 1 - 7	27 ± 2	26 ± 1	26 ± 2	26 ± 1	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 7 - 14	29 ± 3	28 ± 2	29 ± 3	29 ± 2	19 ± 2	19 ± 2	17 ± 1	19 ± 1
Day 14 - 21	29 ± 3	28 ± 3	29 ± 3	29 ± 2	20 ± 2	20 ± 2	18 ± 1	19 ± 2
Day 21 - 28	29 ± 4	27 ± 3	29 ± 2	28 ± 2	20 ± 3	20 ± 2	18 ± 1	20 ± 2

Table 2. Histopathological findings of rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
	Grade							
Number of animals examined [⊗]	5	0	0	5	5	5	5	5
Kidney								
Tubular regeneration	+	2	-	1	0	1	0	0
Liver								
Vacuolation of midzonal hepatocyte	+	0	-	1	0	-	-	0
Vacuolation of periportal hepatocyte	+	0	-	0	2	-	-	3
	++	0	-	0	1	-	-	0
Focal necrosis	+	1	-	0	0	-	-	1
Microgranuloma	+	5	-	3	5	-	-	5
Lung (bronchus)								
Focal hemorrhage	+	1	-	1	0	-	-	0
Cell infiltration	+	0	-	1	0	-	-	0
Accumulation of foamy cell	+	0	-	1	0	-	-	0
Spleen								
Lymphoid hyperplasia	++	1	-	0	0	-	-	0

Grade; +: slight change; ++: mild change, -: Not applicable

[⊗] :The histopathological examination was carried out in 5 animals at 0 and 12.5 mg/kg bw/day and the kidney was examined in 5 females at 0.125 and 12.5 mg/kg bw/day.**Table 3.** Water intake and urinalysis in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
Dose (mg/kg bw/day)	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Water intake (ml/24 hr)	35 ± 6	30 ± 5	36 ± 4	35 ± 6	30 ± 8	31 ± 13	25 ± 5	26 ± 4
Urine volume (ml/24 hr)	17.7 ± 4.7	14.2 ± 2.7	16.7 ± 5	15.3 ± 3.6	12.8 ± 4.5	8.1 ± 1.5	5.5 ± 2.3**	8.1 ± 3.1*
Osmolality (mOsm/kg)	1696 ± 407	1710 ± 455	1459 ± 208	1758 ± 352	1874 ± 507	2046 ± 239	2296 ± 463	2104 ± 394

*: Significantly different from the control group ($p < 0.05$).**: Significantly different from the control group ($p < 0.01$).

Table 4. Hematology in rats dosed with SWCNT by gavage for 28 days

Sex	Male				Female			
	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Dose (mg/kg bw/day)								
RBC ($\times 10^6/\mu\text{l}$)	841 $\pm$ 32	845 $\pm$ 30	819 $\pm$ 26	816 $\pm$ 83	810 $\pm$ 43	841 $\pm$ 13	858 $\pm$ 18	859 $\pm$ 34*
Hemoglobin (g/dl)	15.5 $\pm$ 0.6	15.6 $\pm$ 0.4	15.7 $\pm$ 0.6	15.2 $\pm$ 1.6	15.4 $\pm$ 0.7	15.9 $\pm$ 0.4	15.9 $\pm$ 0.4	16.2 $\pm$ 0.7
Hematocrit (%)	45.7 $\pm$ 1.4	45.5 $\pm$ 1.3	46 $\pm$ 1.8	44.8 $\pm$ 4.7	43.6 $\pm$ 2.3	45.5 $\pm$ 1	45.4 $\pm$ 1.1	46.3 $\pm$ 1.9
MCV (fl)	54.3 $\pm$ 1.3	53.8 $\pm$ 0.9	56.1 $\pm$ 1	54.9 $\pm$ 2	53.9 $\pm$ 2.5	54.1 $\pm$ 1.2	52.9 $\pm$ 0.9	53.9 $\pm$ 1.3
MCH (pg)	18.5 $\pm$ 0.5	18.5 $\pm$ 0.4	19.2 $\pm$ 0.3	18.6 $\pm$ 0.8	19 $\pm$ 0.8	19 $\pm$ 0.5	18.5 $\pm$ 0.4	18.8 $\pm$ 0.4
MCHC (g/dl)	34 $\pm$ 0.3	34.4 $\pm$ 0.1	34.2 $\pm$ 0.2	34 $\pm$ 0.2	35.3 $\pm$ 0.3	35 $\pm$ 0.3	35 $\pm$ 0.4	34.9 $\pm$ 0.1
Reticulocyte (%)	2.4 $\pm$ 0.4	1.9 $\pm$ 0.5	2.6 $\pm$ 0.3	2.8 $\pm$ 1.5	1.8 $\pm$ 0.3	1.5 $\pm$ 0.2	1.5 $\pm$ 0.1	1.7 $\pm$ 0.4
WBC ($\times 10^6/\mu\text{l}$)	89.1 $\pm$ 14.0	110.5 $\pm$ 24.2	105.5 $\pm$ 23.7	123.8 $\pm$ 34.8	70 $\pm$ 22.4	67.8 $\pm$ 11.4	81.0 $\pm$ 30.8	64.1 $\pm$ 10.9
Differential leukocyte counts ($\times 10^6/\mu\text{l}$)								
Lymphocyte	70.6 $\pm$ 9.7	86 $\pm$ 14.8	76.7 $\pm$ 13.6	99.9 $\pm$ 25.2*	52.4 $\pm$ 18	53.3 $\pm$ 13.7	64.1 $\pm$ 31.2	48.9 $\pm$ 11.3
Neutrophil	14.8 $\pm$ 3.2	20.1 $\pm$ 8.9	24.6 $\pm$ 16.3	19.2 $\pm$ 8.2	14.7 $\pm$ 3.4	11.8 $\pm$ 2.8	14.1 $\pm$ 5.4	12.7 $\pm$ 4.7
Eosinophil	1.0 $\pm$ 0.8	1.1 $\pm$ 0.7	0.9 $\pm$ 0.1	0.9 $\pm$ 0.4	1.0 $\pm$ 0.7	0.9 $\pm$ 0.2	0.9 $\pm$ 0.4	1.0 $\pm$ 0.3
Basophil	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1	0.3 $\pm$ 0.1	0.5 $\pm$ 0.3*	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	0.3 $\pm$ 0.3	0.1 $\pm$ 0.1
Monocyte	1.9 $\pm$ 0.8	2.3 $\pm$ 1.5	2.4 $\pm$ 1	2.6 $\pm$ 1.8	1.3 $\pm$ 0.7	1.3 $\pm$ 0.4	1.2 $\pm$ 0.5	1.2 $\pm$ 0.3

RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.

*: Significantly different from the control group ($p < 0.05$).

Table 5. Serum biochemistry in rats dosed with SWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.125	1.25	12.5	0	0.125	1.25	12.5
AST (IU/l)	68 $\pm$ 4	68 $\pm$ 5	62 $\pm$ 4	64 $\pm$ 6	60 $\pm$ 7	86 $\pm$ 23	79 $\pm$ 29	72 $\pm$ 11
ALT (IU/l)	28 $\pm$ 5	30 $\pm$ 4	25 $\pm$ 3	28 $\pm$ 7	19 $\pm$ 2	34 $\pm$ 10**	27 $\pm$ 10	22 $\pm$ 1
LDH (IU/l)	54 $\pm$ 8	53 $\pm$ 7	52 $\pm$ 5	54 $\pm$ 6	52 $\pm$ 3	63 $\pm$ 16	57 $\pm$ 12	56 $\pm$ 9
γ -GTP (IU/l)	1 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 0	1 $\pm$ 1	1 $\pm$ 0	1 $\pm$ 0
ALP (IU/l)	647 $\pm$ 198	686 $\pm$ 82	655 $\pm$ 73	663 $\pm$ 84	415 $\pm$ 127	359 $\pm$ 102	378 $\pm$ 34	470 $\pm$ 102
T. bile acid ($\mu\text{mol/l}$)	15.1 $\pm$ 12.7	12.5 $\pm$ 4.9	10.5 $\pm$ 7.5	7.6 $\pm$ 2.9	12.2 $\pm$ 2.3	15.4 $\pm$ 4.9	11.3 $\pm$ 3.1	11.6 $\pm$ 6.3
T. cholesterol (mg/dl)	50 $\pm$ 5	48 $\pm$ 14	52 $\pm$ 7	50 $\pm$ 6	55 $\pm$ 7	57 $\pm$ 14	66 $\pm$ 16	50 $\pm$ 17
Triglyceride (mg/dl)	56 $\pm$ 19	55 $\pm$ 22	50 $\pm$ 18	59 $\pm$ 24	14 $\pm$ 3	29 $\pm$ 12*	21 $\pm$ 11	14 $\pm$ 6
Phospholipid (mg/dl)	93 $\pm$ 8	92 $\pm$ 14	94 $\pm$ 10	93 $\pm$ 10	100 $\pm$ 11	108 $\pm$ 18	123 $\pm$ 20	95 $\pm$ 26
T. bilirubin (mg/dl)	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0
Glucose (mg/dl)	126 $\pm$ 13	121 $\pm$ 5	135 $\pm$ 18	141 $\pm$ 12	113 $\pm$ 11	106 $\pm$ 11	122 $\pm$ 8	123 $\pm$ 23
BUN (mg/dl)	13 $\pm$ 2	14 $\pm$ 3	12 $\pm$ 1	14 $\pm$ 2	16 $\pm$ 2	16 $\pm$ 2	15 $\pm$ 2	16 $\pm$ 2
Creatinine (mg/dl)	0.24 $\pm$ 0.02	0.24 $\pm$ 0.02	0.25 $\pm$ 0.02	0.25 $\pm$ 0.03	0.27 $\pm$ 0.02	0.26 $\pm$ 0.04	0.27 $\pm$ 0.02	0.28 $\pm$ 0.01
Sodium (mmol/l)	144 $\pm$ 1	144 $\pm$ 1	144 $\pm$ 1	143 $\pm$ 1	144 $\pm$ 1	143 $\pm$ 1	143 $\pm$ 1	143 $\pm$ 2
Potassium (mmol/l)	4.6 $\pm$ 0.1	4.6 $\pm$ 0.1	4.6 $\pm$ 0.1	4.7 $\pm$ 0.2	4.3 $\pm$ 0.2	4.4 $\pm$ 0.3	4.2 $\pm$ 0.2	4.2 $\pm$ 0.2
Chloride (mmol/l)	105 $\pm$ 1	106 $\pm$ 1	105 $\pm$ 1	106 $\pm$ 1	107 $\pm$ 1	107 $\pm$ 1	107 $\pm$ 2	107 $\pm$ 1
Calcium (mg/dl)	10 $\pm$ 0.2	9.8 $\pm$ 0.2	10.2 $\pm$ 0.3	9.8 $\pm$ 0.2	10 $\pm$ 0.3	10 $\pm$ 0.1	9.9 $\pm$ 0.1	10 $\pm$ 0.1
I. phosphorus (mg/dl)	7.8 $\pm$ 0.3	7.5 $\pm$ 0.4	7.9 $\pm$ 0.5	7.5 $\pm$ 0.6	7 $\pm$ 0.3	7 $\pm$ 0.6	6.9 $\pm$ 0.9	6.9 $\pm$ 0.8
T. protein (g/dl)	5.8 $\pm$ 0.1	6 $\pm$ 0.1	6 $\pm$ 0.1	5.9 $\pm$ 0.2	6.4 $\pm$ 0.3	6.2 $\pm$ 0	6.2 $\pm$ 0.2	6.1 $\pm$ 0.1
Albumin (g/dl)	3.2 $\pm$ 0	3.2 $\pm$ 0.1	3.2 $\pm$ 0.1	3.2 $\pm$ 0.1	3.6 $\pm$ 0.2	3.5 $\pm$ 0.1	3.6 $\pm$ 0.1	3.5 $\pm$ 0.1
Albumin/Globulin	1.21 $\pm$ 0.09	1.14 $\pm$ 0.07	1.15 $\pm$ 0.08	1.23 $\pm$ 0.06	1.31 $\pm$ 0.12	1.31 $\pm$ 0.09	1.43 $\pm$ 0.08	1.35 $\pm$ 0.07

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; LDH: lactate dehydrogenase; ALP: Alkaline phosphatase; BUN: Blood urea nitrogen

Table 6. Absolute and relative organ weights in rats dosed with SWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.125	1.25	12.5	0	0.125	1.25	12.5
Body weight ^a	406 ± 23	371 ± 13	390 ± 34	379 ± 21	232 ± 10	227 ± 13	212 ± 18	229 ± 16
Primary (mg) ^a	12.3 ± 1.3	11.3 ± 2	11.7 ± 1.2	10.6 ± 0.8	14.9 ± 1.1	12.9 ± 2	12.6 ± 1.8	13.2 ± 2.4
(mg%) ^a	3 ± 0.3	3.1 ± 0.5	3 ± 0.4	2.8 ± 0.2	6.4 ± 0.4	5.7 ± 0.7	6 ± 0.8	5.8 ± 0.7
Thymus (mg)	21.9 ± 4.2	18 ± 2.4	20.2 ± 2.8	17.8 ± 2.1	14.2 ± 0.8	13.2 ± 1.5	13.2 ± 2.3	14.3 ± 1.7
(mg%) ^a	5.4 ± 0.8	4.8 ± 0.6	5.2 ± 0.7	4.7 ± 0.4	6.1 ± 0.5	5.7 ± 1	6.2 ± 1.3	6.4 ± 0.8
Thyroid (mg)	619 ± 121	538 ± 123	430 ± 96	541 ± 79	484 ± 40	480 ± 62	439 ± 112	410 ± 85
(mg%) ^a	152 ± 29	144 ± 34	111 ± 12	143 ± 18	208 ± 13	211 ± 32	206 ± 58	199 ± 28
Heart (g)	1.34 ± 0.08	1.22 ± 0.11	1.32 ± 0.16	1.28 ± 0.11	0.82 ± 0.07	0.86 ± 0.06	0.78 ± 0.08	0.83 ± 0.08
(g%) ^a	0.33 ± 0.04	0.33 ± 0.03	0.34 ± 0.03	0.34 ± 0.03	0.35 ± 0.02	0.38 ± 0.02*	0.37 ± 0.02	0.37 ± 0.02
Liver (g)	11.94 ± 1.17	11 ± 1.22	12.02 ± 1.54	11.52 ± 1.16	6.57 ± 0.48	6.59 ± 0.54	6.06 ± 0.66	6.42 ± 0.89
(g%) ^a	2.97 ± 0.16	2.96 ± 0.16	3.08 ± 0.34	3.03 ± 0.15	2.83 ± 0.12	2.9 ± 0.13	2.86 ± 0.18	2.94 ± 0.18
Spleen (g)	0.81 ± 0.2	0.73 ± 0.12	0.71 ± 0.1	0.64 ± 0.01*	0.5 ± 0.09	0.48 ± 0.07	0.42 ± 0.07	0.44 ± 0.06
(g%) ^a	0.20 ± 0.04	0.19 ± 0.02	0.18 ± 0.02	0.17 ± 0.01	0.22 ± 0.02	0.21 ± 0.03	0.2 ± 0.02	0.2 ± 0.02
Kidney (g)	2.9 ± 0.15	2.69 ± 0.18	2.94 ± 0.2	2.71 ± 0.1	1.81 ± 0.09	1.75 ± 0.16	1.67 ± 0.12	1.71 ± 0.14
(g%) ^a	0.72 ± 0.03	0.73 ± 0.03	0.75 ± 0.03	0.72 ± 0.04	0.78 ± 0.03	0.77 ± 0.03	0.79 ± 0.03	0.76 ± 0.02
Adrenal (mg)	72 ± 12	59 ± 14	67 ± 9	62 ± 8	60 ± 11	61 ± 8	62 ± 7	71 ± 12
(mg%) ^a	18 ± 3	16 ± 3	17 ± 3	16 ± 2	26 ± 4	27 ± 2	29 ± 2	32 ± 7
Testis (g)	3.35 ± 0.33	3.37 ± 0.33	3.39 ± 0.24	3.05 ± 0.42				
(g%) ^a	0.82 ± 0.04	0.91 ± 0.08	0.85 ± 0.07	0.81 ± 0.11				
Epididymis (mg)	907 ± 66	911 ± 50	865 ± 97	869 ± 123				
(mg%) ^a	224 ± 8	247 ± 22	223 ± 27	234 ± 32				
Ovary (mg)					87.4 ± 8.8	84.9 ± 4.5	81.3 ± 12.3	81.9 ± 11.6
(mg%) ^a					37.7 ± 4.1	37.4 ± 1.8	38.5 ± 5.5	41.1 ± 8.7
Uterus (mg)					470 ± 143	334 ± 52	480 ± 197	407 ± 94
(mg%) ^a					202 ± 56	148 ± 25	218 ± 45	180 ± 38

* Significantly different from the control group (p < 0.05).

^a Values are given as the mean ± S.D.

^a Absolute organ weight.

^a Relative organ weight.

the end of the administration period (Table 5). In organ weight measurements, a significantly low absolute weight of the spleen was observed in males in the 12.5 mg/kg group at the end of the administration period (Table 6). However, there were no changes in the relative weight and histopathological examination of the spleen. A significantly high relative weight of the heart was observed in females in the 0.125 mg/kg group but not in the high-dose groups at the end of the administration period. At necropsy, a dark red focus in the lung was observed sporadically in the control group and 12.5 mg/kg bw/day group at the end of the administration and recovery periods. At the histopathological examination, focal hemorrhage in the lung was observed in the animals showed a dark red focus. Blackish contents were observed in the caecum of animals in the treatment groups at the end of administration period. However, the histopathological examination revealed no effects in these organs. The contents in the intestines were not observed after the recovery period. Significant

differences noted at the end of administration period all disappeared by the end of the recovery period.

A 28-day dose of SWCNT caused no death in both sexes. Black foci were observed in both sexes in all the treatment groups. No effects were found on detailed clinical observation, functional examination, body weight and food consumption (Table 7), organ weights, urinalysis and histopathology (Table 8). In the hematology examination, eosinophil counts were significantly low in females in the 5.0 and 50 mg/kg bw/day groups at the end of the administration period (Table 9) and in the 50 mg/kg bw/day group at the end of recovery period ($1.13 \pm 0.38 \times 10^6/\mu\text{L}$ vs. $0.73 \pm 0.23 \times 10^6/\mu\text{L}$), but the changes were within the historical background data of the test facility ($0.9 \pm 0.26 \times 10^6/\mu\text{L}$). A few female animals incidentally showed high values for eosinophils in the control group, and a significant decrease in eosinophil counts was considered to be due to the high value of eosinophils in the control group. Therefore, it was not considered to be clinically

Table 7. Body weight and food consumption in rats dosed with MWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.5	5	50	0	0.5	5	50
Body weight (g)								
Day 1	183.7 ± 11.1	185.7 ± 9.7	186.7 ± 11.4	186.8 ± 8.4	149.7 ± 8.6	150.2 ± 10.8	150.5 ± 10.3	149.0 ± 10.3
Day 4	212.9 ± 13.3	213.2 ± 10.9	215.5 ± 14.0	215.0 ± 10.9	161.7 ± 9.9	161.8 ± 9.4	160.8 ± 10.7	159.1 ± 10.8
Day 7	242.0 ± 15.0	242.0 ± 14.4	244.5 ± 13.8	241.5 ± 13.0	172.0 ± 11.9	172.7 ± 15.4	172.0 ± 15.9	168.7 ± 13.3
Day 14	305.7 ± 18.5	304.5 ± 16.8	308.3 ± 17.5	305.3 ± 17.3	197.4 ± 12.3	193.0 ± 19.8	198.0 ± 19.9	194.0 ± 17.7
Day 21	362.4 ± 20.0	359.5 ± 17.2	367.2 ± 23.3	359.7 ± 24.1	219.7 ± 16.1	215.2 ± 22.1	218.8 ± 22.0	213.2 ± 19.9
Day 28	399.7 ± 24.1	392.2 ± 21.8	401.8 ± 30.0	394.5 ± 27.2	237.8 ± 18.7	227.7 ± 17.3	236.8 ± 23.2	230.4 ± 22.2
Food consumption (g/rat/day)								
Day 0 - 1	22.42 ± 2.02	22.00 ± 2.37	22.00 ± 1.67	22.58 ± 1.56	16.42 ± 2.19	16.83 ± 2.64	17.00 ± 1.26	17.50 ± 2.39
Day 1 - 4	23.33 ± 1.49	23.33 ± 1.45	23.90 ± 1.62	23.33 ± 1.12	16.76 ± 1.17	16.38 ± 0.98	16.17 ± 1.60	16.48 ± 1.68
Day 4 - 7	25.36 ± 1.75	24.95 ± 2.02	24.98 ± 1.55	24.65 ± 1.17	16.96 ± 1.39	16.77 ± 1.85	17.00 ± 2.31	17.10 ± 1.86
Day 7 - 14	26.94 ± 1.92	26.20 ± 1.62	26.53 ± 1.99	26.14 ± 1.18	18.27 ± 1.47	17.22 ± 1.02	17.92 ± 2.22	17.74 ± 1.82
Day 14 - 21	28.98 ± 1.42	28.25 ± 1.47	28.33 ± 3.34	27.69 ± 1.44	19.31 ± 1.96	18.50 ± 1.52	18.83 ± 2.33	18.62 ± 2.30
Day 21 - 28	29.09 ± 1.89	27.62 ± 2.14	28.18 ± 2.94	28.03 ± 2.26	19.66 ± 1.94	18.62 ± 1.86	19.20 ± 2.66	19.32 ± 2.51

Table 8. Histopathological findings of rats dosed with MWCNT by gavage for 28 days

Sex Dose (mg/kg bw/day)	Grade	Male				Female			
		0	0.5	5	50	0	0.5	5	50
Number of animals examined ^(a)		6	0	1	6	6	0	0	6
Lung	+								
Aggregation of alveolar macrophage	+	1	-	-	0	0	-	-	0
Mineralization of artery	+	0	-	-	0	1	-	-	0
Pancreas									
Atrophy of focal acinar cell	+	1	-	-	0	0	-	-	0
Ileum									
Diverticulum	+	0	-	1	0	1	-	-	0
Liver									
Microgranuloma	+	3	-	-	2	4	-	-	2
Kidney									
Tubular epithelium regeneration	+	2	-	-	1	0	-	-	0
Hyaline droplet in proximal tubular epithelium	+	0	-	-	1	0	-	-	0
Eosinophilic body in proximal tubular epithelium	+	0	-	-	1	0	-	-	0
Focal inflammatory cell infiltration in cortex	+	0	-	-	0	0	-	-	1

Grade; +: slight change. -: Not applicable.

^(a) The histopathological examination was carried out in 6 animals at 0 and 50 mg/kg bw/day and in animals which showed gross findings at 0.5 and 5 mg/kg bw/day.

or toxicologically important. Table 10 shows the results of serum biochemistry in rats dosed MWCNT. A significantly low γ -globulin fraction in females in the 50 mg/kg group was not accompanied by significant differences in other fractions, and no changes were noted in related parameters such as white blood cell counts. This was also considered to be due to a high value in the control group. Gamma-GTP in males in the 0.5 mg/kg group was significantly lower than that in the control group, but it was

not dose-dependent. These changes in serum biochemistry were not observed at the end of the recovery period. Necropsy at the end of the administration period revealed swelling of the submandibular lymph node in one male in the 0.5 mg/kg group. This change was not dose-dependent and was not considered to be toxicologically important. Grayish green/dark green contents in the cecum, colon and/or rectum were observed in both sexes at 5.0 mg/kg bw/day and higher. These contents in the intes-

Table 9. Hematology in rats dosed with MWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.5	5	50	0	0.5	5	50
RBC ($\times 10^6/\mu\text{l}$)	878.8 $\pm$ 39.3	865.2 $\pm$ 27.7	862.8 $\pm$ 18.9	878.7 $\pm$ 35.5	879.3 $\pm$ 30.3	866.8 $\pm$ 48.2	855.8 $\pm$ 15.6	864.5 $\pm$ 24.0
Hemoglobin (g/dl)	17.20 $\pm$ 0.45	16.97 $\pm$ 0.56	17.12 $\pm$ 0.33	17.08 $\pm$ 0.28	16.77 $\pm$ 0.45	16.90 $\pm$ 0.82	16.50 $\pm$ 0.24	16.58 $\pm$ 0.43
Hematocrit (%)	48.57 $\pm$ 0.88	48.00 $\pm$ 1.56	48.72 $\pm$ 0.81	47.95 $\pm$ 1.09	46.13 $\pm$ 0.94	46.77 $\pm$ 1.94	45.98 $\pm$ 0.79	45.80 $\pm$ 1.45
MCV (fl)	55.33 $\pm$ 1.73	55.52 $\pm$ 1.89	56.47 $\pm$ 0.87	54.62 $\pm$ 1.86	52.48 $\pm$ 1.39	54.05 $\pm$ 2.16	53.73 $\pm$ 0.92	52.98 $\pm$ 1.12
MCH (pg)	19.58 $\pm$ 0.47	19.60 $\pm$ 0.41	19.85 $\pm$ 0.18	19.45 $\pm$ 0.68	19.07 $\pm$ 0.54	19.50 $\pm$ 0.68	19.30 $\pm$ 0.39	19.20 $\pm$ 0.37
MCHC (g/dl)	35.42 $\pm$ 0.40	35.35 $\pm$ 0.38	35.13 $\pm$ 0.30	35.63 $\pm$ 0.24	36.35 $\pm$ 0.38	36.12 $\pm$ 0.33	35.88 $\pm$ 0.40	36.22 $\pm$ 0.37
Reticulocyte (%)	3.683 $\pm$ 0.405	3.603 $\pm$ 0.448	3.545 $\pm$ 0.534	3.445 $\pm$ 0.434	2.907 $\pm$ 0.730	3.265 $\pm$ 0.431	3.013 $\pm$ 0.557	2.875 $\pm$ 0.644
WBC ($10^6/\mu\text{l}$)	128.57 $\pm$ 38.56	116.95 $\pm$ 15.35	116.48 $\pm$ 29.80	129.37 $\pm$ 27.96	91.07 $\pm$ 12.67	109.07 $\pm$ 27.58	88.83 $\pm$ 26.32	79.32 $\pm$ 18.52
Differential leukocyte counts ($\times 10^4/\mu\text{l}$)								
Lymphocyte	111.07 $\pm$ 35.91	100.13 $\pm$ 12.75	102.98 $\pm$ 27.80	111.30 $\pm$ 23.02	78.73 $\pm$ 11.18	95.97 $\pm$ 27.81	78.45 $\pm$ 26.20	68.18 $\pm$ 18.27
Neutrophil	12.63 $\pm$ 3.89	11.82 $\pm$ 2.65	9.95 $\pm$ 2.83	13.88 $\pm$ 5.17	9.32 $\pm$ 3.07	10.58 $\pm$ 4.12	8.48 $\pm$ 2.78	8.88 $\pm$ 4.68
Eosinophil	1.43 $\pm$ 1.02	1.67 $\pm$ 0.31	1.18 $\pm$ 0.64	1.07 $\pm$ 0.39	1.68 $\pm$ 0.89	1.08 $\pm$ 0.41	0.87 $\pm$ 0.33*	0.88 $\pm$ 0.34*
Basophil	0.07 $\pm$ 0.05	0.00 $\pm$ 0.00	0.03 $\pm$ 0.05	0.02 $\pm$ 0.04	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.02 $\pm$ 0.04	0.00 $\pm$ 0.00
Monocyte	3.37 $\pm$ 0.81	3.33 $\pm$ 0.76	2.33 $\pm$ 1.00	3.10 $\pm$ 0.88	1.33 $\pm$ 0.62	1.43 $\pm$ 0.48	1.02 $\pm$ 0.52	1.37 $\pm$ 1.18

RBC: Red blood cell; MCV: Mean corpuscular volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; WBC: White blood cell.
 *: Significantly different from the control group ($p < 0.05$).

Table 10. Serum biochemistry in rats dosed with MWCNT by gavage for 28 days

Dose (mg/kg bw/day)	Male				Female			
	0	0.5	5	50	0	0.5	5	50
T. protein (g/dl)	5.67 $\pm$ 0.18	5.65 $\pm$ 0.12	5.57 $\pm$ 0.23	5.70 $\pm$ 0.28	5.80 $\pm$ 0.32	5.65 $\pm$ 0.23	5.87 $\pm$ 0.21	5.75 $\pm$ 0.16
Glucose (mg/dl)	164.7 $\pm$ 8.2	162.2 $\pm$ 18.9	168.3 $\pm$ 16.8	173.5 $\pm$ 13.9	137.3 $\pm$ 17.3	134.5 $\pm$ 20.1	136.5 $\pm$ 13.4	138.8 $\pm$ 18.6
Triglyceride (mg/dl)	50.7 $\pm$ 11.5	48.7 $\pm$ 18.3	39.7 $\pm$ 13.5	44.7 $\pm$ 9.5	10.0 $\pm$ 2.5	11.2 $\pm$ 3.7	10.7 $\pm$ 4.1	11.2 $\pm$ 5.0
T. cholesterol (mg/dl)	59.5 $\pm$ 12.1	51.7 $\pm$ 9.3	56.8 $\pm$ 11.4	56.0 $\pm$ 10.1	56.0 $\pm$ 5.8	56.7 $\pm$ 9.0	60.8 $\pm$ 7.7	62.8 $\pm$ 13.7
BUN (mg/dl)	14.42 $\pm$ 2.12	13.53 $\pm$ 2.22	14.32 $\pm$ 1.57	15.22 $\pm$ 2.61	16.02 $\pm$ 1.36	17.22 $\pm$ 2.80	16.08 $\pm$ 4.45	15.77 $\pm$ 4.60
Creatinine (mg/dl)	0.497 $\pm$ 0.027	0.483 $\pm$ 0.023	0.502 $\pm$ 0.035	0.502 $\pm$ 0.022	0.505 $\pm$ 0.037	0.513 $\pm$ 0.043	0.507 $\pm$ 0.058	0.508 $\pm$ 0.045
T. bilirubin (mg/dl)	0.047 $\pm$ 0.008	0.038 $\pm$ 0.008	0.043 $\pm$ 0.008	0.040 $\pm$ 0.009	0.055 $\pm$ 0.012	0.063 $\pm$ 0.012	0.060 $\pm$ 0.021	0.057 $\pm$ 0.008
AST (IU/l)	67.3 $\pm$ 7.4	64.2 $\pm$ 7.7	66.3 $\pm$ 4.2	67.7 $\pm$ 10.0	69.3 $\pm$ 5.2	65.0 $\pm$ 4.7	61.5 $\pm$ 5.8	69.7 $\pm$ 11.6
ALT (IU/l)	26.2 $\pm$ 3.2	26.2 $\pm$ 2.6	25.7 $\pm$ 2.6	25.2 $\pm$ 2.6	20.3 $\pm$ 2.9	22.7 $\pm$ 2.4	21.2 $\pm$ 3.4	22.2 $\pm$ 4.4
ALP (IU/l)	688.8 $\pm$ 46.3	693.3 $\pm$ 122.5	614.2 $\pm$ 112.8	700.7 $\pm$ 171.2	423.8 $\pm$ 44.8	412.8 $\pm$ 91.3	333.2 $\pm$ 65.3	398.7 $\pm$ 138.4
γ -GTP (IU/l)	0.85 $\pm$ 0.21	0.62 $\pm$ 0.12*	0.68 $\pm$ 0.12	0.70 $\pm$ 0.14	1.22 $\pm$ 0.19	1.18 $\pm$ 0.12	0.95 $\pm$ 0.20	1.20 $\pm$ 0.27
Calcium (mg/dl)	10.18 $\pm$ 0.30	9.90 $\pm$ 0.14	9.93 $\pm$ 0.21	10.07 $\pm$ 0.27	9.88 $\pm$ 0.08	9.87 $\pm$ 0.46	10.00 $\pm$ 0.39	9.78 $\pm$ 0.43
I. phosphorus (mg/dl)	8.78 $\pm$ 0.49	8.67 $\pm$ 0.22	8.53 $\pm$ 0.48	8.77 $\pm$ 0.36	8.03 $\pm$ 0.42	8.02 $\pm$ 0.67	7.93 $\pm$ 0.38	7.70 $\pm$ 0.91
Sodium (mEq/l)	143.2 $\pm$ 1.2	143.2 $\pm$ 0.8	143.3 $\pm$ 0.8	143.8 $\pm$ 1.0	144.0 $\pm$ 1.4	143.5 $\pm$ 1.9	143.8 $\pm$ 1.5	144.3 $\pm$ 0.8
Potassium (mEq/l)	4.873 $\pm$ 0.268	4.923 $\pm$ 0.265	4.858 $\pm$ 0.117	4.762 $\pm$ 0.331	4.593 $\pm$ 0.372	5.108 $\pm$ 0.420	4.862 $\pm$ 0.351	4.755 $\pm$ 0.900
Chloride (mEq/l)	104.3 $\pm$ 1.9	105.0 $\pm$ 0.6	105.2 $\pm$ 1.2	105.0 $\pm$ 0.9	107.3 $\pm$ 2.1	107.2 $\pm$ 1.9	106.8 $\pm$ 1.9	106.7 $\pm$ 1.6
Albumin (%)	51.03 $\pm$ 2.11	51.93 $\pm$ 1.67	52.93 $\pm$ 1.25	53.30 $\pm$ 2.68	54.83 $\pm$ 2.31	55.53 $\pm$ 1.87	55.65 $\pm$ 1.94	55.88 $\pm$ 1.40
Alpha-1 globulin (%)	22.93 $\pm$ 3.10	23.35 $\pm$ 1.51	21.20 $\pm$ 2.81	21.47 $\pm$ 3.08	17.60 $\pm$ 2.54	17.93 $\pm$ 2.17	19.28 $\pm$ 2.38	19.32 $\pm$ 0.65
Alpha-2 globulin (%)	7.03 $\pm$ 0.34	6.65 $\pm$ 0.45	7.10 $\pm$ 0.51	6.98 $\pm$ 0.55	6.95 $\pm$ 0.77	7.45 $\pm$ 0.29	6.40 $\pm$ 0.75	6.57 $\pm$ 0.45
Beta globulin (%)	15.48 $\pm$ 0.98	14.72 $\pm$ 1.09	15.15 $\pm$ 1.1	14.48 $\pm$ 0.44	15.27 $\pm$ 0.85	14.57 $\pm$ 0.99	14.40 $\pm$ 1.11	14.35 $\pm$ 0.9
Gamma globulin (%)	3.52 $\pm$ 0.60	3.35 $\pm$ 0.63	3.62 $\pm$ 0.95	3.57 $\pm$ 0.43	5.35 $\pm$ 1.29	4.52 $\pm$ 0.76	4.27 $\pm$ 1.04	3.88 $\pm$ 0.67*
Albumin/Globulin	1.045 $\pm$ 0.089	1.083 $\pm$ 0.074	1.123 $\pm$ 0.057	1.153 $\pm$ 0.128	1.218 $\pm$ 0.115	1.252 $\pm$ 0.091	1.257 $\pm$ 0.101	1.268 $\pm$ 0.070

BUN: Blood urea nitrogen; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; ALP: Alkaline phosphatase.

*: Significantly different from the control group ($p < 0.05$).

tines were not observed after the recovery period. Based on the absence of toxicological effects, the no observable adverse effect levels (NOAELs) of repeated dose toxicity of SWCNT and MWCNT were considered to be 12.5 mg/kg bw/day and 50 mg/kg bw/day in rats (the highest dose tested), respectively.

Our findings showed no toxicological effects of orally administered CNTs, but available data show CNTs, under some conditions, may induce harmful effects. Both SWCNTs and MWCNTs showed the capacity to induce toxicity such as inflammation and fibrosis in the lung when administered by pharyngeal aspiration, intratracheal instillation or inhalation (Shvedova *et al.*, 2008; Erdelyi *et al.*, 2009; Inoue *et al.*, 2008; Pauluhn, 2010; Warheit *et al.*, 2004; Lam *et al.*, 2004; Han *et al.*, 2008). The carcinogenic potential of intraperitoneally administered MWCNT was observed in p53 heterozygous mice (Takagi *et al.*, 2008) and intrascrotal injection of MWCNT in intact Fischer 344 rats (Sakamoto *et al.*, 2009). The cytotoxic potential of CNTs was also observed in skin and lung cells *in vitro* (Jia *et al.*, 2005; Monteiro-Riviere *et al.*, 2005) although cytotoxicity can be influenced by various factors such as material impurities, length and size distribution, surface area, and so on (Hussain *et al.*, 2009). Intravenously injected CNTs increased oxidative stress markers in the lung and liver in mice (Yang *et al.*, 2008) or induced slight hepatotoxicity by inflammation and oxidative damage in mice (Ji *et al.*, 2009).

Deng *et al.* (2007) showed that intratracheally dosed taurine-functionalized MWCNT accumulated mainly in the lung and remained there until 28 days following administration whereas an oral gavage dose led to distribution in the small/large intestines and stomach, and about 74% was excreted in the feces after 12 hr. Intravenously injected taurine-functionalized MWCNT accumulated and remained until 28 days following administration in the liver, lung and spleen. The authors of this study suggested that taurine-functionalized-MWCNT cannot enter the blood circulation or be absorbed by the intestine tracts, and that routes of exposure influence the distribution of CNTs although the distribution pattern was for functionalized MWCNT. The findings of a study by Deng *et al.* (2007) together with our study suggested that SWCNTs and MWCNTs dosed by gavage reached the gastrointestinal tract as agglomerates and were rapidly excreted via feces in rats.

However, there is a study in which rats were dosed with SWCNT by gavage once at 0.64 mg/kg bw, and the levels of oxidatively damaged DNA increased in the liver and lung tissue (Folkman *et al.*, 2009). Furthermore, sig-

nificant increases in the number of resorptions, and fetal morphological and skeletal abnormalities, were observed in fetuses of CD-1 mouse dams that were administered functionalized CNTs by gavage at 10 mg/kg bw/day on day 9 of gestation (Philbrook *et al.*, 2011). Surface functionalization increases solubility of CNTs, and therefore absorbability of CNTs could have been increased in this study. However, these two studies suggested that CNTs could be absorbed from the gastro-intestinal tract into the blood circulation.

The findings of a study by Philbrook *et al.* (2011) are in discord from a study by Lim *et al.* (2011), in which pregnant SD rats were given MWCNTs by gavage at 0-1,000 mg/kg bw/day on days 6-19 of gestation, and no adverse effects were found in fetuses. Although controversial findings were observed in oral dose studies, an intravenous study obviously showed the teratogenic potential of CNTs (Pietroiusti *et al.*, 2011). Pregnant CD-1 mice were intravenously injected with SWCNT, oxidized-SWCNT and ultra oxidized-SWCNT at 0-30 µg/animal on day 5.5 of gestation. Increases in incidence of early miscarriages and fetal malformations were observed in all treatment groups at 0.1 µg/animal and higher. This study suggested that low-dose SWCNTs may induce fetal malformations. Our study group will undertake further teratogenic studies to confirm whether CNTs cause developmental toxicity.

In conclusion, rats were dosed with SWCNT or MWCNT once daily by gavage for 28 days with a 14-day recovery period, and no toxicological effects were found up to the highest dose tested. However, the possibility that CNTs dosed by oral administration are absorbed from the gastro-intestinal tract and persistent in the body for the long term cannot be ruled out, and a further study for chronic oral toxicity will be helpful to confirm the safety of CNTs. Because of the small size of CNTs, particles may spread over the entire body, and causing adverse effects like observed in intravenous studies, once absorbed.

ACKNOWLEDGMENTS

This study was undertaken under the safety programmes for existing chemicals funded by the Ministry of Health, Labour and Welfare (MHLW), Japan, and was supported by a Health and Labour Sciences Research Grant (Research on Risk of Chemical Substances: H21-Chemistry-Ippan-008) from the MHLW, Japan. The test materials were kindly provided from the National Institute of Advanced Industrial Science and Technology (AIST), Japan.