In conclusion, treatment of nZnO by IPS did not promote lung and mammary carcinogenesis in our carcinogenesis model. Although nZnO induced EHTB and FAIP, the lesions regressed rapidly along with clearance of surplus Zn^{2+} from the lung and serum. Thus, from a toxicological viewpoint, under the present experimental conditions, exposure of the lung to nZnO does not cause progressive neoplastic development or chronic fibrosis in the lung. These findings will be helpful in evaluating of the safety of nZnO used in biomedical applications, in which its use is of rather short duration, although long-term studies including inhalation studies are required to assess their occupational and environmental health hazards.

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Conflict of interest The authors declare that they have no conflict of interest.

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Original Article

An improved dispersion method of multi-wall carbon nanotube for inhalation toxicity studies of experimental animals

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ABSTRACT — A multi-wall carbon nanotube (MWCNT) product Mitsui MWNT-7 is a mixture of dispersed single fibers and their agglomerates/aggregates. In rodents, installation of such mixture induces inflammatory lesions triggered predominantly by the aggregates/agglomerates at the level of terminal bronchiole of the lungs. In human, however, pulmonary toxicity induced by dispersed single fibers that reached the lung alveoli is most important to assess. Therefore, a method to generate aerosol predominantly consisting of dispersed single fibers without changing their length and width is needed for inhalation studies. Here, we report a method (designated as Taquann method) to effectively remove the aggregate/agglomerates and enrich the well-dispersed singler fibers in dry state without dispersant and without changing the length and width distribution of the single fibers. This method is base on two major concept; liquid-phase fine filtration and critical point drying to avoid re-aggregation by surface tension. MWNT-7 was suspended in Tert-butyl alcohol, freeze-and-thawed, filtered by a vibrating 25 μm mesh Metallic Sieve, snap-frozen by liquid nitrogen, and vacuum-sublimated (an alternative method to carbon dioxide critical point drying). A newly designed direct injection system generated well-dispersed aerosol in an inhalation chamber. The lung of mice exposed to the aerosol contained single fibers with a length distribution similar to the original and the Taquann-treated sample. Taquann method utilizes inexpensive materials and equipments mostly found in common biological laboratories, and prepares dry powder ready to make well-dispersed aerosol. This method and the chamber with direct injection system would facilitate the inhalation toxicity studies more relevant to human exposure.

Key words: Multi-wall carbon nanotube, Dispersion, Metallic sieve, Tert-butyl alcohol, Sublimation, Critical point drying

INTRODUCTION

We previously reported that a certain make of multi-wall carbon nanotube (MWCNT) contained particles similar to asbestos fibers in size and shape, and was positive for mesotheliomagenesis in intraperitoneal injection studies using p53-heterozygous mice (Takagi *et al.*, 2008, 2012). The intraperitoneal injection study is a specialized method for the detection of mesotheliomagenic potential of inhaled fibrous materials (Pott *et al.*, 1994; Roller *et al.*, 1997; Poland *et al.*, 2008). For the assessment of general respiratory toxicity including non-cancerous endpoints, the inhalation studies are considered essential. As a surrogate for inhalation studies, pharyngeal aspiration and intratracheal spray methods are often used. However, in both methods, the suspension medium may modify the distribution and/or the toxicity of the test particles (Morimoto *et al.*, 2011; Oyabu *et al.*, 2011; Gasser *et al.*, 2012; Wang *et al.*, 2012). Dispersion methods without suspension media are reported. However, those are usually using, at least in part of the processes, rigorous sonication or mechanical milling resulting in certain degree of physiological changes in sample characteristics, such as shortening in length distribution of MWNCT (Muller *et al.*, 2005; Mitchell *et al.*, 2007; Ahn *et al.*, 2011). Changes in particle size and/or shape will also affect the nature

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and strength of toxicity of the test substances. Therefore, development of a dispersion method to generate the aerosol of concern without addition of chemicals and changes in particle dimensions is considered to be essential for the assessment of inhalation toxicity in humans.

Fibrous nanomaterial such as Mitsui MWNT-7 is a mixture of dispersed single fibers of various length and width, and their agglomerates and aggregates. When given as a mixture, the lung lesions were mainly seen as inflammatory and/or granulomatous lesions with various degree of fibrosis at the level of terminal bronchiole accompanying the aggregates and agglomerates. These lesions were considered to block and/or mask the changes induced by the single fibers that should have reached the alveolar ducts and alveoli (Warheit *et al.*, 2004; Muller *et al.*, 2005; Shvedova *et al.*, 2008; Porter *et al.*, 2009; Mercer *et al.*, 2011; Wang *et al.*, 2011). Therefore, assessment of the toxicity of single fibers needs well-dispersed sample without aggregate/agglomerate. In practical inhalation testing, the animal chamber air is rigorously agitated in order to ensure the homogeneity of aerosol in the chamber. Therefore, if the MWNT-7 as a mixture is used, the likelihood of aggregates/agglomerates reaching the nose of the animals is high. In contrast, human ambient air is less agitated; the aggregates/agglomerates may sediment away fast and dispersed single fibers may stay longer in the air to be inhaled by humans (Han *et al.*, 2008). In addition, humans have longer respiratory tract compared to rodents and may effectively filtered out aggregates/agglomerates before the air reaches the alveolar region.

Taking all into account, we concluded that it is essential to prepare a dispersed single fiber aerosol without aggregate/agglomerates, without additional chemical components, and without changes in size and shape of the single fiber component for the rodent inhalation studies in order to predict human inhalation toxicity. To date, one dispersion method is reported, i.e. the filtration system. Filtration by a sieve with its pore size smaller than the size of aggregates/agglomerate will not affect the size distribution of the single fibers (Kasai *et al.*, 2013). However, filtration in gaseous phase turns out to be ineffective in terms of yield of the filtrate. Filtration in liquid phase is much efficient (Mercer *et al.*, 2008; Tsuda, personal communication). However, in our experience, the difficulty is found in avoiding re-aggregation during the process of drying; the surface tension. To solve this problem, here we report a new improved dispersion method consisting of a combination of aqueous filtering and the concept of a drying method used for scanning electron microscopic (SEM) samples; the critical point drying.

MATERIALS AND METHODS

MWCNT, reagent and equipments

MWCNT (Mitsui MWNT-7) was kindly donated by Mitsui & Co., Ltd., Tokyo, Japan for use in toxicity studies (Takagi *et al.*, 2008). Tert-butanol (TB) of guaranteed reagent grade was used (CAS: 75-65-0, Kanto Chemical Co., Inc., Tokyo, Japan). Metallic Sieve (pore size 25 μm mesh, Seishin Enterprise Co., LTD., Tokyo, Japan) was used for filtration. Miniature coin type vibration motors used in cellular phones (Model FM34F, T.C.P. Co, Tokyo Japan; 13,000 rpm 1.8m²/sec) are attached to the extended filler rim (5cm in depth, custom-made, Seishin Enterprise Co., LTD.) of the metallic sieve (cf. Fig. 1c) to gain high yield of filtrate. Chemistry diaphragm pumps and pumping systems (Model; MD4C NT+AK+EK, Vacuubrand, Wertheim, Germany) was used for sublimation of the frozen TB suspension and recovery of TB. Glass wares such as funnel, filtering bottle, trap bottle and silicon stoppers (Sansyo Co., Ltd., Tokyo, Japan), laboratory bottles (Pyrex®, Asahi Glass Co. Ltd., Tokyo, Japan), were used.

Dispersion method ("Taquann" method)

An outline flowchart is shown in Fig. 1. TB (melting point 25.69°C) was heated up to 60°C by a mantle heater (Sibata Scientific Technology Ltd., Saitama, Japan). It is advised not to use water bath; TB is highly hygroscopic and becomes difficult to freeze and sublimate. A volume of 200 ml of TB and 0.2 g of MWCNT were transferred to a 500 ml laboratory bottle and agitated to make crude suspension. The bottle was put into an ice bath, occasionally shaken by hand, until the suspension starts to freeze and becomes sherbet-like half frozen state and kneaded by a stainless steel spatula until it becomes evenly gray without clear crystals of TB (Fig. 1a), and then kept overnight at -25°C. To the frozen suspension, 500 ml of TB pre-heated to 60°C, was added, capped and shaken hard until the liquefied suspension becomes evenly dark brown to gray in color (Fig. 1b). The bottle was further heated up to 60°C by a mantle heater and the suspension was immediately applied to vibrating metal sieve for filtration (Fig. 1c). The filtrate was collected through a funnel into a 1,000 ml laboratory bottle. Immediately after the filtration, approximately 1,500 ml of liquid nitrogen was poured onto the filtrate in the bottle to snap freeze the suspension (Fig. 1d). Then, the bottle was connected to the pumping system and vacuumed until TB was totally sublimated; leaving dispersed MWCNT (T-CNT for Taquann-treated MWCNT) in the bottle. The MWCNT was collected by a cyclone-suction bottle using conduc-

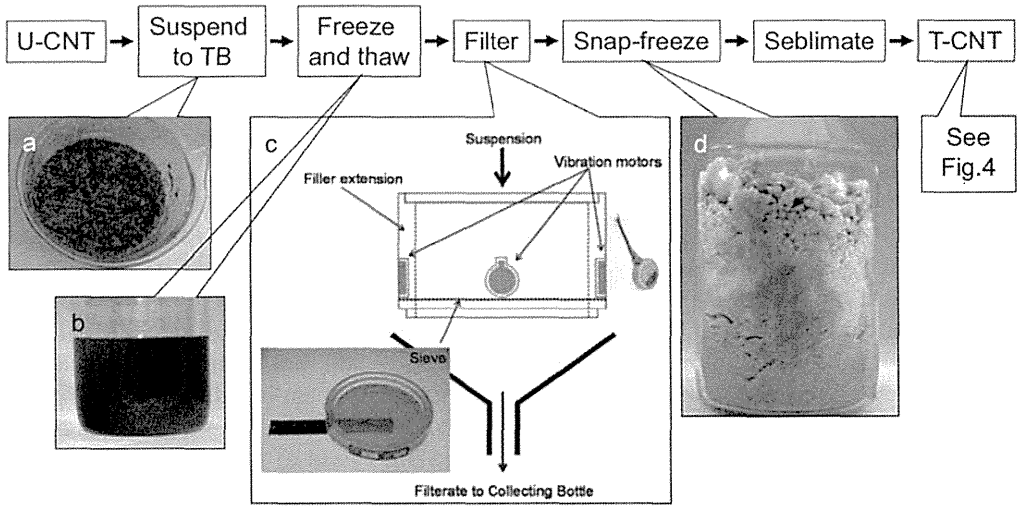

Fig. 1. Outline flowchart of the Taquann method. a) Half-frozen sherbet-like suspension of MWNT-7 kneaded (beaker was used for demonstration). b) Well-shaken liquefied suspension after adding 60°C TB (beaker was used for demonstration). c), Photograph of the sieve on a backlight box with a scale underneath (left inset), vibration motor (right inset), and a diagram of the filter unit with a filler extension and vibration motors. d) Snap-frozen filtrate.

tive silicon and aluminum tubing. In order to make a precise aliquot, a measured amount the collected T-CNT was resuspended to TB, and the suspension was aliquoted into proper containers, in this study into the newly designed cylindrical cartridge case (cf. Fig. 3), snap-frozen, and sublimated.

Aerosol generation system

An originally designed 105 L main exposure chamber (capacity of 16 mice per chamber), with a disposable electrostatic-free plastic bag inside, was prepared (Fig. 2, patent pending, manufactured by Sibata Scientific Technology Ltd.). Onto the plastic disposable top plate, a 20 L subchamber was connected with a 5 cm-diameter 10 cm long connecting pipe. To the subchamber, an injection port was connected, to which a newly designed cylindrical cartridge (manufactured by Sibata Scientific Technology Ltd.) containing dispersed T-CNT is loaded. The cartridge has a slide-valve air inlet at its base and four ejection holes at its top opening towards the subchamber lumen. The compressed air (0.8 M pascal) was injected five times with 0.2 sec duration and 10 sec interval to empty the T-CNT into the subchamber (Fig. 3). The carrier air flow from the subcamber to the main chamber was 15 L/min. Twenty-one cartridges were prepared for a two-hr exposure experiment, loading first two in 1 min for an initial boost and then one in every 6 min, resulting in generation of saw-tooth concentration wave with an average of 1.3 mg/m^3 (250 μg/cartridge) and 2.8 mg/m^3 (500 μg/cartridge).

Twelve C57BL/6NCrSlc male mice (SLC, Inc., Shizuoka, Japan), 10~11 weeks old, body weight of 23.8~30.8 g were placed in the cage suspended from the top plate of the inhalation chamber and exposed to 1 mg/m^3 of T-CNT for 2 hr a day for 5 days, lungs (excluding primary bronchi) were sampled and subjected to characterization of deposited fibers (see below).

The animal study was conducted in accordance with the Guidance for Animal Studies of the National Institute of Health Sciences under Institutional approval.

Real time particle counting and weight measurement

An optical particle counter (OPC) with a nominal detection limit of 300 nm (OPC-110GT, Sibata Scientific Technology Ltd.) and a condensation particle counter (CPC) with a nominal detection limit of 2.5 nm (ultrafine condensation particle counter 3776, Trust Science Innovation, MN, USA) were connected to the main chamber with a sample flow of 2.83 L (0.1cf) /min and 0.3 L/min respectively. The mass concentration of the chamber aerosol was calculated from the weight increase of polytetrafluoroethylene-glass fiber filter (Model T60A20, φ55mm, Tokyo Dylec Corp, Tokyo, Japan) after filtering the chamber aerosol by an Asbestos sampling pump (AIP-105, Sibata Scientific Technology Ltd.) at a rate of 1.5 L/min for 120 min (total of 180 L). Filter weight was measured by a microbalance (XP26V,

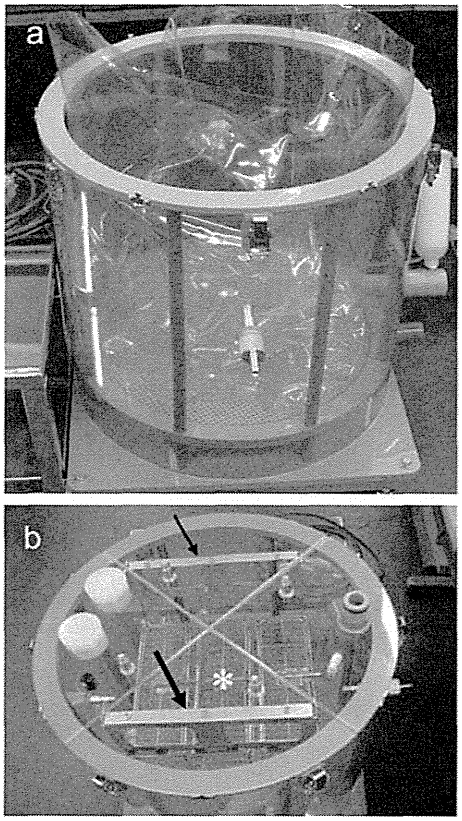

Fig. 2. Newly designed original inhalation chamber. a) Outer chamber and inner bag before top plate is in place. During operation, the space between the outer chamber and inner bag is negatively pressured to inflate the inner bag. B) Disposable top plate with tubing holes are placed on the chamber. The animal cages for 16 mice (asterisk) are suspended from the top place by a pair of hanger arms (arrows) (photo was taken without inner bag for better demonstration).

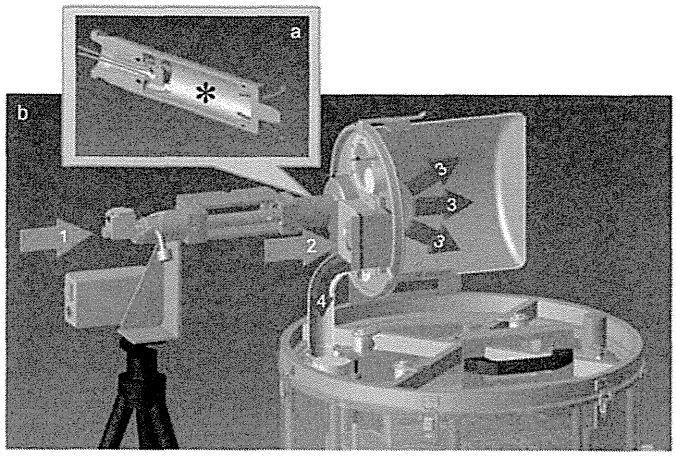

Fig. 3. A scheme of direct injection aerosol generation system. a) Upper inset shows the cut section of the injection cartridge (capacity; 23.5 ml). A slide valve opens when the cartridge is loaded to the subchamber. A measured amount of dispersed MWCNT is preloaded inside the cartridge shown in asterisk. b) Compressed air (Blue arrow 1) blows out the MWCNT through four small outlets of the cartridge into the subchamber (red arrows 3), where main flow air from the HEPA filtered inlet (blue arrow 2) mixes in. The air with the aerosol goes down the connection pipe to the main chamber (red arrow 4).

Mettler Toledo).

Characterization of the dispersed MWCNT

The T-CNT in TB suspension was mounted on a slide glass and observed under a light microscope using a pair of polarizing filters. Untreated MWCNT (U-CNT) from the bulk, 200 mg, was dispersed in to 500 ml of TB and sonicated for 30 min at 40W, 3.4 kHz (SU-3TH, Sibata Scientific Technology Ltd.) and observed.

A weight-measured aliquot of T-CNT was re-suspended, blotted on a Anopore™ Inorganic Aluminum Oxide Membrane Filters (Whatman GmbH, Dassel GE Healthcare, Hahnestrasse, Germany, pore size; 0.02 μm, φ13 mm, Anodisc 13) or a cellulose acetate/nitrocellulose membrane filter (MFTM- Millipore Membrane filters, 0.025 μm, φ13 mm, Merck Millipore, Billerica, MA, USA) and observed with a scanning electron microscope (SEM).

From the main chamber, the aerosol was collected at a rate of 5 L/min for 3 min on a Anopore™ Inorganic Aluminum Oxide Membrane Filters (Whatman GmbH, pore size; 0.1 um, Anodisc 25) joined to asbestos sampling pump (AIP-105, Sibata, Scientific Technology Ltd.). A scanning electron microscope (SEM) (VE-9800, Keyense Co., LTD., Osaka, Japan) was used for monitoring the details of the samples on the slide glasses and on the Anodiscs after osmium coating (HPC-1SW, Vacuum Device Inc., Ibaraki, Japan).

From the exposed mouse, lung lobes are collected and treated with lysis solution composed of 5 w/v% potassium hydroxide (Super Special Grade, Wako Pure Chemical Industries, Ltd., Osaka, Japan), 0.1w/v% Sodium dodecyl sulfate (SDS, for Biochemistry, Wako Pure Chemical Industries, Ltd.), 0.1 w/v% Ethylenediamine-N,N,N',N'-tetraacetic acid disodium salt dehydrate (EDTA 2Na, Dojindo laboratories, Kumamoto, Japan) and 2w/v% ascorbic acid (Super Special Grade, Wako Pure Chemical Industries, Ltd.) in ultra-pure water, dissolved at 80°C (Fig. 10b). Lung samples (approx. 200 mg) and 1.8 ml of

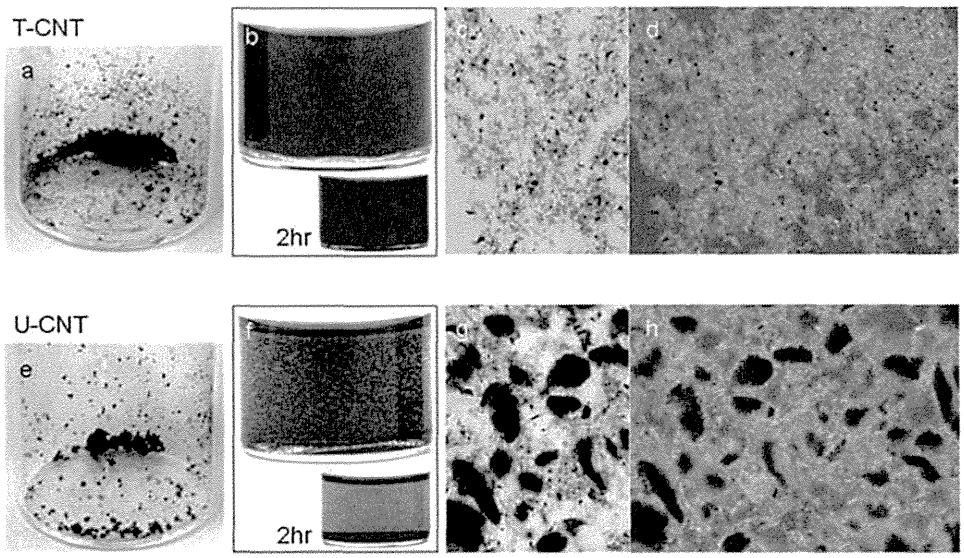

Fig. 4. Taquann-treated carbon nanotube (T-CNT) and untreated bulk carbon nanotube (U-CNT). a) final fine and dry powder of Taquann-treated MWCNT. b) Resuspended T-CNT to TB and placed for 5 min and 2 hr; T-CNT suspension is stable, compared to U-CNT, c) light microscopic view of the resuspended T-CNT on a slide glass, and d) under polarized light. e) course powder of U-CNT, f) Resuspended U-CNT to TB and placed for 5 min and 2 hr. g) light microscopic view of the resuspended U-CNT on a slide glass, and d) under polarized light. (diameter of the vials in a), b), e) and f) is 2.3 cm)

lysis solution in a centrifuge tube (DNA LoBindid tube 2.0 ml, Eppendorf, Hamburg, Germany) was incubated at 80°C for approx. 24 hr in an oven (HV-100, Funakoshi Co., Ltd., Tokyo, Japan), centrifuged at 20,000 g for 1 hr at 25°C (MX-207, Tomy Seiko Co., Ltd., Tokyo, Japan), and the pellet containing MWCNTs and SDS crystals was recovered. 1.8 ml of 70% ethanol was added to the tube and incubated at 80°C for 30 min to dissolve SDS crystals and centrifuged at 20,000 g for 1 hr at 25°C. 100 µl of 1w/w% Triton®X-100 (MP Biomedicals, Inc., Solon, OH, USA) was added to the pellet and dispersed by pipetting. One microliter of the suspension was placed on an inorganic aluminum oxide membrane filter (Anodisc 13, 0.02 µm φ13mm, Whatman GmbH) or the cellulose acetate/nitrocellulose membrane filter and filtrated on a funnel shape glass filter (SANSYO Co., LTD., Tokyo, Japan). The filter was dried at room temperature and osmium coated for SEM. For a reference of extraction efficiency, lung sample from untreated mouse was spiked with 1 µg T-CNT and measured alongside.

Lung tissue from eight mice were fixed with buffered 10% formalin (four with and four without inflation), paraffin embedded and processed routinely for H&E stained histology slides, and observed under a light microscope with or without polarizing filters (Olympus BX50 microscope with DP-70 image system, Olympus Corporation, Tokyo, Japan).

RESULTS

Characteristics of "Taquann"-dispersed MWCNT

Macroscopic and light microscopic views of the final product, the dried MWCNT after sublimation, i.e. "Taquann"-dispersed MWCNT (T-CNT) and, for comparison, untreated MWCNT from the bulk (U-CNT) are shown in Fig. 4. The powder of T-CNT is finer compared to U-CNT (Fig. 4a). The T-CNT resuspended very well to TB (Fig. 4b) and other solvents including 0.1 w/v% Sodium dodecyl sulfite and 0.1 w/v% Sodium dodecylbenzene sulfonate (not shown). Light microscopically, the resuspended T-CNT consists mostly of dispersed single fibers with smaller numbers of small aggregates corresponding to the mesh size of the metal sieve (Figs. 4c, 4d), whereas U-CNT was a mixture of large aggregates/agglomerates and single fibers among them (Figs. 4g, 4h). The T-CNT fibers slowly precipitated in the medium (cf. Figs. 4b and f), and are easily resuspended by gentle agitation. The yield of the T-CNT was approximately 5% of the U-CNT in weight. Re-filtration of the residue on the sieve resulted in negligible yield. The low power SEM views of the TB-resuspended T-CNT and U-CNT are shown in Fig. 5.

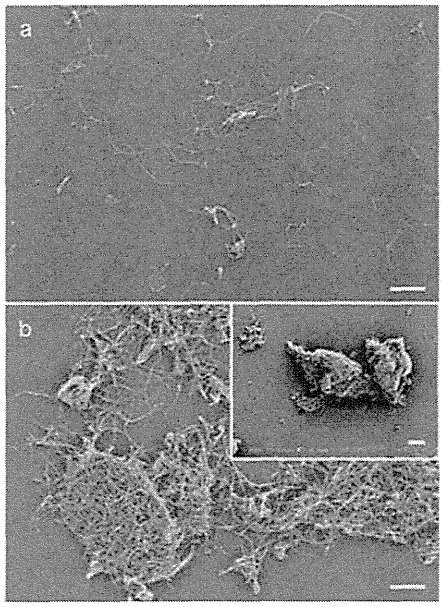

Fig. 5. Scanning electron microscopy of T-CNT and U-CNT resuspended in TB. a) T-CNT consists mainly of dispersed single fibers with few small aggregates/agglomerates smaller than the mesh size of the sieve, SEM x 1,000. b) U-CNT showing mixture of single fibers and large aggregates/agglomerates, SEM x 1,000. The length and width distribution of the single fibers of T-CNT were virtually identical to those of U-CNT. Inset; Lower power view to demonstrate larger aggregates/agglomerates measuring up to 300 μm in major axis SEM x 400. (scale bars are 10 μm)

Again, the majority of the particles of the T-CNT were the dispersed single fibers. The length and width distribution of single fibers counted on these SEM images are shown in Fig. 6. The length and width distribution was similar between single fibers of T-CNT and U-CNT, indicating that the mechanical shortening of the fibers is negligible for Taquann method.

The number of fibers per 10, 1 and 0.1 μg weight of T-CNT with length distribution was counted on SEM images (measured number of fibers are 959, 246, and 45 per designated area for calculation, respectively). The number of fibers calculated was 2.1 x 10^7/10 μg 4.1 x 10^6/1 μg and 3.3 x 10^5/0.1 μg. The distribution of the fiber length was similar to that shown in Fig. 6a, and the average length was 7.5 ± 4.7 μm (max 34 μm), 8.7 ± 6.4 μm (max 42 μm), and 7.0 ± 5.4 μm (max 26 μm) respectively. As a whole, T-CNT has roughly 3 x 10^6 fibers per 1 μg, mean length of approximately 7 μm with a length range up to 50 μm with a median of approximately 6.5 μm.

"Taquann"-dispersed MWCNT in the inhalation chamber

The T-CNT aerosol generated at an average concentration of 1 mg/m³ was sampled on the Anodisc and observed by a SEM (Fig. 7). The aerosol was composed mainly of well-dispersed single fibers and some small tangles of fibers admixed with a relatively small amount of non-fibrous particles.

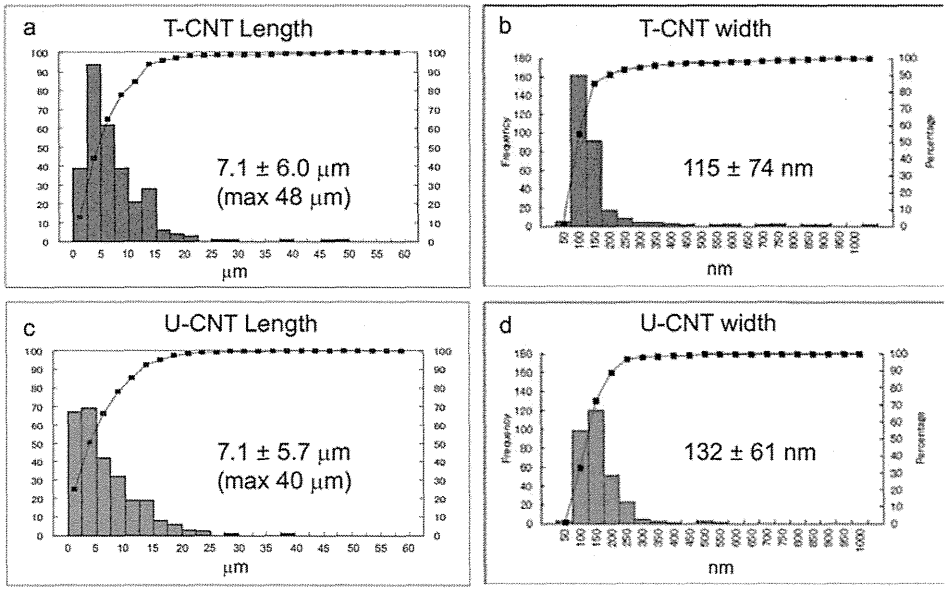

Fig. 6. Length and width of single fibers in T-CNT and U-CNT (measured by SEM on TB-resuspended samples). a) Length distribution and b) width distribution of Taquann-treated MWNT-7. c) Length distribution and d) width distribution of single fibers in the mildly sonicated suspension of the bulk MWNT-7 (mean ± s.d., n = 304 each).

Table 1. Aerosol particle count by optical particle counter (OPC) and condensation particle counter (CPC).

	Date of measurement	2013/4/29	2013/5/1	2013/5/3
Equipment	Mass concentration (mg/m³)	1.25	1.25	1.38
OPC	Average cpm* (/L) ± s.d.	627,096 ± 145,399	781,973 ± 138,610	821,272 ± 114,278
	K-value (mg/m³/cpm)	1.99 x 10^{-9}	1.60 x 10^{-9}	1.68 x 10^{-9}
CPC	Average cpm (/L) ± s.d.	859,692 ± 171,858	1,228,545 ± 223,371	1,317,873 ± 217,990
	K-value(mg/m³/cpm)	1.45 x 10^{-9}	1.02 x 10^{-9}	1.05 x 10^{-9}

*count per minute

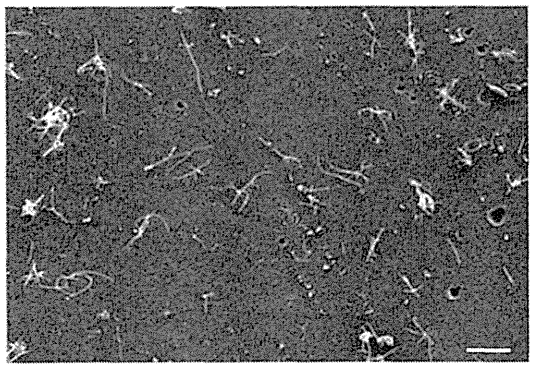

Fig. 7. T-CNT aerosol at a concentration of 1 mg/m³ in the main chamber was collected on the Anodisc filter (5 L/min for 3 min). SEM x 1,000. (scale bar is 10 μm)

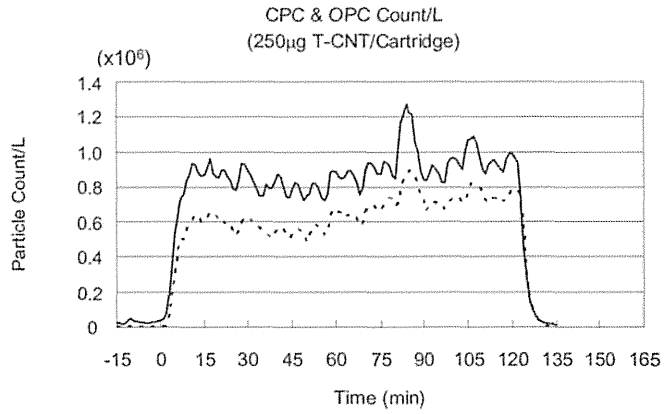

Fig. 8. A real time data from condensation particle counter (CPC, solid line) and optical particle counter (OPC, dotted line) from an inhalation chamber injected with T-CNT (250 μg/cartridge) from 0 min to 120 min with an average injection interval of 6 min (for detail see text).

From the amount of weight increase of polytetrafluoroethylene-glass fiber filter after sampling the chamber aerosol, the weight of aerosol per m³ of the chamber air (weight concentration) was calculated as approximately 1.3 mg/m³ (average of three measurements shown in Table 1). At the same time, the particle counts per m³ given by OPC and CPC were recorded (Fig. 8), and the K-value (mg/particle count in m³) was calculated (Table 1).

K-value (mg/m³/cpm), i.e. the weight concentration (mg/m³) divided by OPC or CPC count per minute (cpm) is often used as an indicator of the status of dispersion. Three measurements conducted with a few days' interval showed that not only the K-values itself but also the values used to calculate it were fairly stable over a period of days.

The length distribution of the T-CNT recovered from the lungs of two mice exposed in the whole body inhalation chamber 2 hr a day for 5 days at an average concentration of 1.8 mg/m³ of T-CNT are shown in Fig. 9 along with the data from the spiked lung tissue sample. The average length were 8.4 ± 5.0 μm and 8.3 ± 4.9 μm (Figs. 9a, 9b), comparable to that of the T-CNT in spiked lung tissue sample; 9.5 ± 5.2 μm (Fig. 9c) (width was qualitatively not different, data not shown). The total numbers of the fibers recovered were 5.1 x 10^6 and 3.2 x 10^6 from the inhaled lungs and 1.6 x 10^6 from the spiked lung; the weight of T-CNT deposited in the lung after 2 hr x 5 days of inhalation was roughly calculated as 3 μg/lung.

The fibers recovered from one of the mice were observed with SEM (Fig. 10a). Dispersed single fibers were found and some of which are longer that 20 μm (cf. Fig. 9). It was noted that EDTA and ascorbic acid in the lysis solution were effective in removing the debris from the SEM sample (Fig. 10b).

Histologically, the CNTs were found to distribute from bronchial lumen to peripheral alveolar spaces. In the bronchial lumen, the fibers were trapped in the bronchial mucus, either as single fibers or as loose aggregates

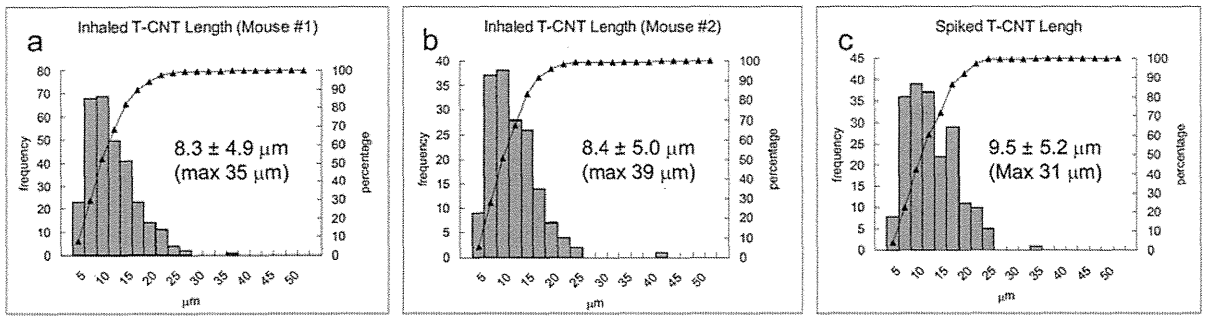

Fig. 9. T-CNT recovered from the mouse lung. a, b) Length distribution of T-CNT in the lung of two mice exposed 2 hr a day for 5 days at an average concentration of 1.8 mg/m³ (n = 306 and 166 each, mean ± s.d). c) Length distribution of T-CNT (1 μg) spiked to a non-exposed mouse lung (n = 198, mean ± s.d).

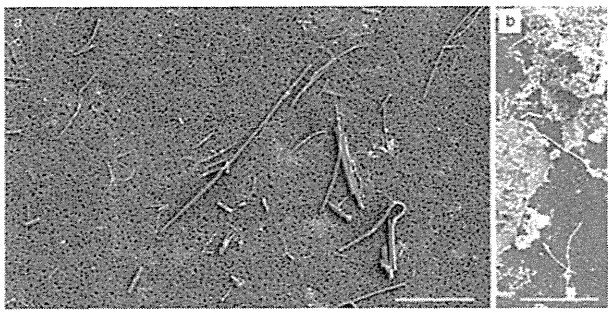

Fig. 10. T-CNT recovered from the mouse lung. a) SEM of the sediment of the dissolved lung of a mouse exposed to T-CNT in an inhalation chamber 2 hr a day for 5 days, x 2,000. Long and short single fibers are shown to be inhaled (treated with solution containing EDTA and ascorbic acid). b) SEM of a same sample treated without EDTA and ascorbic. The debris covering the fibers is considered to be iron-based amorphous substances soluble to EDTA, x 2,000. Ascorbic acid was found to be effective in keeping iron ions to be bivalent (ferrous) and soluble. (scale bars are 10 μm)

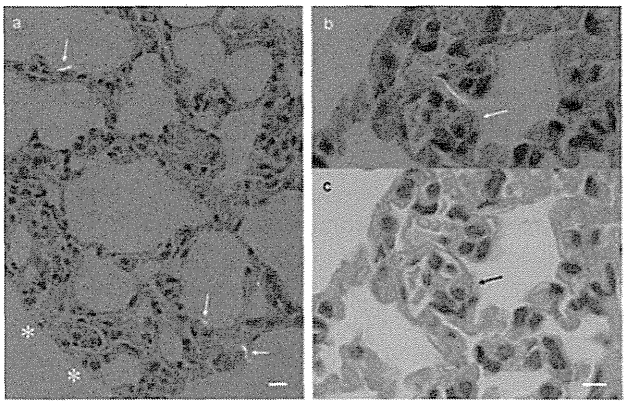

Fig. 11. a) A polarized microscopic view of the alveolar region of a lung exposed to 1 mg/m³ of T-CNT for 2 hr a day for 5 days. Arrows indicate single T-CNTs deposited in alveolar spaces phagocytized by alveolar macrophages. Asterisks indicates visceral pleural. (scale bar 10 μm) b,c) Another portion of alveolar region with a tadpole-shaped alveolar macrophage containing single long CNT in its cytoplasm shown in plain and polarized view. The lungs shown here are not inflated with formalin at fixation in order to avoid replacement of the CNTs. (scale bar 5 μm)

without inflammatory or granulomatous response, morphologically interpretable as a view of expectoration by the ciliary movement of the bronchial epithelium. There were no dense aggregates/agglomerates in the lungs so far as examined. In the peripheral alveolar space, single fibers are found phagocytized in alveolar macrophages as shown in Fig. 11. There were only mild inflammatory reactions such as neutrophilic migration against fibers in mucous blanket of the bronchial/bronchiolar segments and fibers in the alveolar space.

DISCUSSION

The MWCNT treated with the "Taquann" method (T-CNT) consisted of highly dispersed single fibers with marked reduction of aggregates/agglomerates, both in the aerosol and in the resuspended solution. The length and width distribution of the single fibers were not different between the T-CNT and the original U-CNT, indicating that this method is physically mild to the sample and does not shorten the fibers.

The Taquann method consists of two major steps, the

efficient filtration in liquid phase and the idea of critical point drying in order to prevent re-aggregation of the fibers by surface tension during drying. The latter step was inspired by the drying method for SEM samples. TB-sublimation technique used in this study is an alternative method used for SEM samples as well. Our trial-and-error added a few innovations such as gentle kneading of half-frozen TB suspension and a freeze-and-thaw process for a better dispersion (visible differences in fineness of suspension, data not shown), and vibration of the sieve for a faster and better yield of filtrate (approximately 7 fold increase in half the time). This Taquann method does not use high power sonication or other strong mechanical shearing, so that the length distribution of the single fibers did not change. The equipments and reagents used here are mostly available at regular biological or chemical laboratories. The new aerosol generating system by the direct injection of T-CNT had successfully generated highly dispersed aerosol of MWNT-7 and an exposure study confirmed the inhalation of MWNT-7 single fibers in mouse lung down to the peripheral alveolar spaces. In this condition, i.e. five consequtive days of 2 hr exposure, histologically, there were only mild neutrophilic infiltration. A long-term follow up study is underway.

It is highly plausible that the Taquann method can be applied to other types of particles as long as they are not soluble to TB (additional study in preparation). Well-dispersed samples generated by the Taquann method, together with the direct injection and the small scale inhalation chamber system, would facilitate the inhalation toxicity studies more relevant to human exposure not only at the big facilities but also at the small scaled laboratories.

Finally, this dispersion method may also be useful for industries where difficulty in dispersion of nanoparticles was a limiting process in developing new products. For a large scale manufacturing, carbon dioxide critical point drying may be suitable than TB sublimation.

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Short communication

In vitro clastogenicity and phototoxicity of fullerene (C_{60}) nanomaterials in mammalian cells

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ABSTRACT

Carbon nanomaterials such as carbon nanotubes, graphene, and fullerenes (C_{60}) are widely used in industry. Because of human health concerns, their toxic potential has been examined in vivo and in vitro. Here we used mammalian cells to examine the in vitro clastogenicity as well as the phototoxicity of C_{60}. While C_{60} induced no structural chromosome aberrations in CHL/IU cells at up to 5 mg/ml (the maximum concentration tested), it significantly induced polyploidy at 2.5 and 5 mg/ml with and without metabolic activation. In BALB 3T3 cells, C_{60} showed no phototoxic potential but the anatase form of titanium oxide did. Since insoluble nanomaterials cause polyploidy by blocking cytokinesis rather than by damaging DNA, we concluded that the polyploidy induced by C_{60} in CHL/IU cells was probably due to non-DNA interacting mechanisms.

1. Introduction

A variety of nanomaterials have been recently developed. They are used in industry and in medicine (for drug delivery) because of their unique physical and chemical properties [1,2]. But nanoparticles in diesel exhaust and nano-sized asbestos particles have toxic effects [3–5], with the latter being particularly hazardous because they lead to mesothelioma and bronchogenic carcinoma with high frequency [6,7]. This raises concerns about the health effects of newly developed nanomaterials. Here we report on the in vitro clastogenicity and phototoxicity of fullerene (C_{60}).

2. Materials and methods

2.1. Test chemicals

We obtained fullerene (C_{60}, Nanom purple SUH, purity >99.9%) from Frontier Carbon Corporation, Tokyo, and titanium dioxide (TiO_2) in the anatase form (lot # p-25, average size 21 nm) from Nippon-Aerosol Corporation, Tokyo, and in the rutile form (lot# LU175, average size 20 nm) from Ishihara Sangyo Kaisha, Ltd., Osaka, Japan. We purchased mitomycin C (MMC) from Kyowa-Kirin Co., Ltd., Tokyo, and cyclophosphamide monohydrate (CP) and chlorpromazine hydrochloride salt (CPZ) from Sigma–Aldrich Japan, Tokyo. For use, we prepared C_{60} sample by suspending them with 0.5% carboxymethylcellulose sodium solution (CMC-Na). TiO_2 samples were also prepared by suspending them with 0.5% CMC-Na. MMC, CP and CPZ were dissolved in distilled water. The state of C_{60} in the suspension was observed through a transmission electron microscope (TEM) (TEM-1010; JOEL Datum Ltd., Japan). The size distribution was measured by particle size distribution analyzer LA-920 (Horiba Ltd., Japan).

2.2. In vitro chromosome aberration test

We conducted the chromosomal aberration test according to OECD guideline TG 473 [9] and the procedure of Kusakabe and colleagues [10]. We grew CHL/IU cells obtained from the National Institute of Health Sciences, Tokyo, on culture medium which was Eagle's minimum essential medium (MEM) supplemented with 10% calf serum (Hyclone Inc., USA) and maintained them in culture plates at 37 °C in a humidified 5% CO_2 atmosphere. We treated the cells with the test chemicals for 6 h in culture medium with or without S9 mix and then cultured them for 18 h in fresh culture medium. We mixed S9 (Kikkoman Inc., Chiba, Japan) with co-factors immediately before use and added the mix to the cultures. We then treated the cells with 0.1 μg/ml colcemid for 2 h and prepared chromosome specimens by the air-dry method. We stained them with 3% Giemsa solution for 8 min and scored the number of cells with chromatid- and chromosome-type breaks and exchanges per 100 cells for each culture (200 cells at each concentration). We also scored polyploid cells per 800 cells at each concentration. We used the Fisher exact test to compare the frequency of aberrant cells in treated versus untreated cells. All tests were performed in duplicate.

2.3. In vitro phototoxicity test

We conducted the in vitro phototoxicity test according to OECD guideline TG 432 [11] and the procedure of the European Cosmetic Industry Association joint validation project [12]. We obtained BALB3T3 clone A31 cells from ATCC, grew them on Dulbecco's modified eagle medium (DMEM) supplemented with 10% heat-inactivated calf serum (Hyclone), and maintained them on culture plates at 37 °C in a humidified 5% CO_2 atmosphere. For the study, we seeded the cells into 96-well plates (10^4 cells/well), maintained them for 24 h, and then treated them with the test chemicals for 1 h in Earle's balanced salt solution (EBSS) at 37 °C in a

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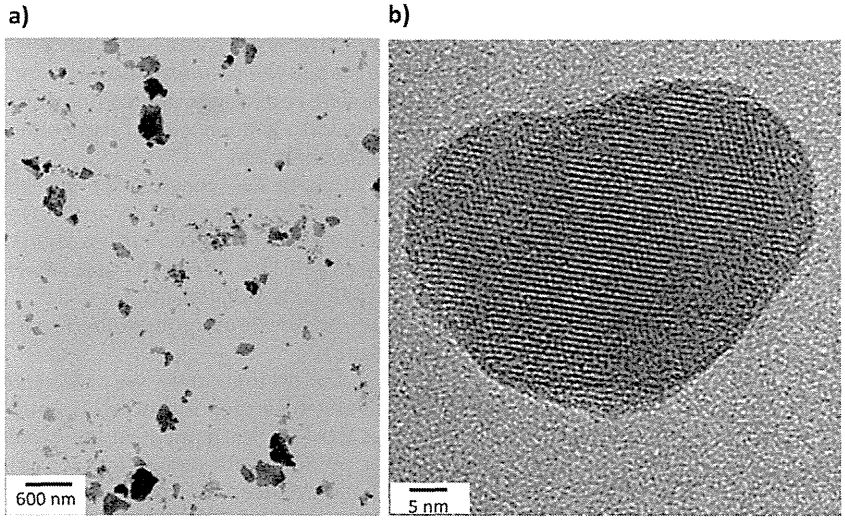

Fig. 1. TEM pictures of the C_{60} nanomaterial in the aqueous suspension used in this study. The left picture (a) shows the image of whole suspension (32,000×). The right picture (b) shows one of fine nanoparticle (210,000×).

humidified 5% CO_2 atmosphere. As they were being treated, we irradiated them with UVA (2.5 mW/cm^2) for 50 min using a sunlight simulator (SOL 500 Dr. Honle, Martinsried, Germany, or SLX 2500V, SERIC, Tokyo) (total, 7.5 J/cm^2) and measured the emitted energy with a UVA meter (UVR-3036/S, Topcon, Tokyo). After the treatment, we washed the cells, placed them in fresh medium, and cultured them for 17 h. We then placed them in fresh medium containing Neutral Red (NR) (final concentration, 50 μg/ml), cultured them for another 3 h, and fixed them with 1% $CaCl_2$/1% formaldehyde solution. We extracted NR with desorb solution (freshly prepared with 49 parts water, 50 parts ethanol, and 1 part acetic acid), measured its optical density at 540 nm with a spectrophotometer for the 96-well microplate, and determined the concentration responses obtained in the presence and absence of irradiation and the concentration required to reduce cell viability to 50% relative to the untreated controls (IC50).

3. Results

3.1. Characterization of the C_{60} nanomaterial suspension

The TEM pictures of C_{60} nanomaterial in the aqueous suspension are shown in Fig. 1. Most of C_{60} was agglomerated forming large particles (>100 nm in diameter) (Fig. 1a). Some fine particles (<50 nm in diameter) were also observed infrequently (Fig. 1b). The size of diameter was widely distributed ranging from 20 to 2000 nm (data not shown). The average diameter is approximately 300 nm.

3.2. In vitro cytotoxicity and chromosome aberration test

We conducted chromosomal aberration tests with CHL/IU cells to evaluate the clastogenicity of C_{60}, which was prepared by 0.5% CMC-Na. We treated the cells with C_{60} for 6 h in the presence and absence of rat S9-mix. Both with and without S9-mix, C_{60} exhibited moderate cytotoxicity in a concentration-dependent manner up to 5 mg/ml (Fig. 2), but it did not induce structural chromosome aberrations at any concentration although MMC (0.1 μg/ml) and CP (10 μg/ml), which are positive controls for the absence or presence of S9-mix, respectively, clearly produced structural chromosome aberrations (data not shown). On the other hand, it significantly increased the frequency of polyploid cells at 2.5 and 5 mg/ml treatments in both conditions.

3.3. In vitro phototoxicity test

We conducted in vitro phototoxicity tests with BALB3T3 cell for C_{60}, TiO_2 (rutile form), and TiO_2 (anatase form). We treated the cell with the chemicals prepared by 0.5% CMC-Na until 1 mg/ml. C_{60} and the rutile form of TiO_2 were not phototoxic at any concentration, but the anatase form of TiO_2 was phototoxic in a concentration-dependent manner starting at the lowest concentration (Fig. 3). The IC50 was calculated to be 0.09 mg/ml. CPZ, which is a positive control for phototoxicity tests, yielded clear phototoxicity with 0.46–0.67 μg/ml of IC50 (data not shown).

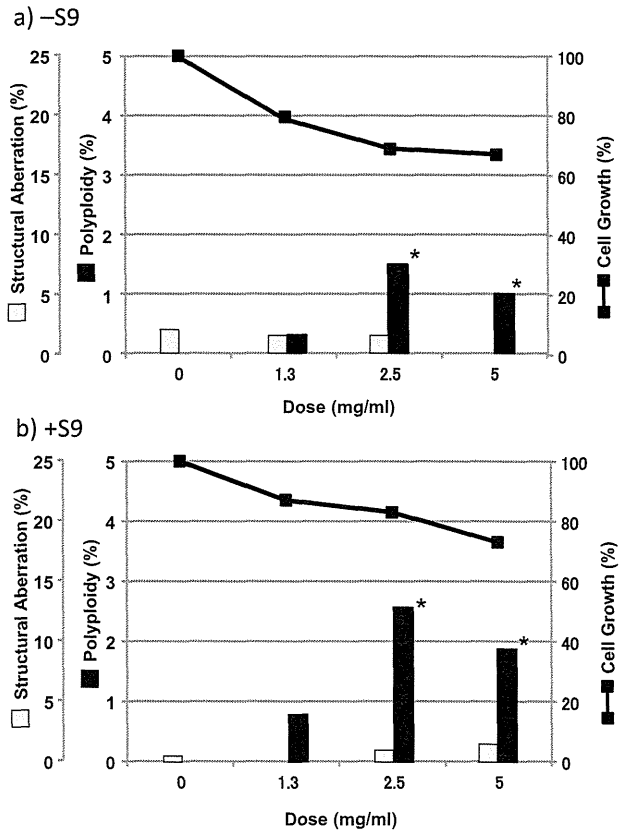

Fig. 2. Chromosome aberrations and cytotoxicity in CHL/IU cells exposed to C_{60} in (a) the absence of S-9 mix and (b) the presence of S-9 mix. Structural chromosome aberrations and polyploidy were examined independently. *$P < 0.01$, Fisher exact test.

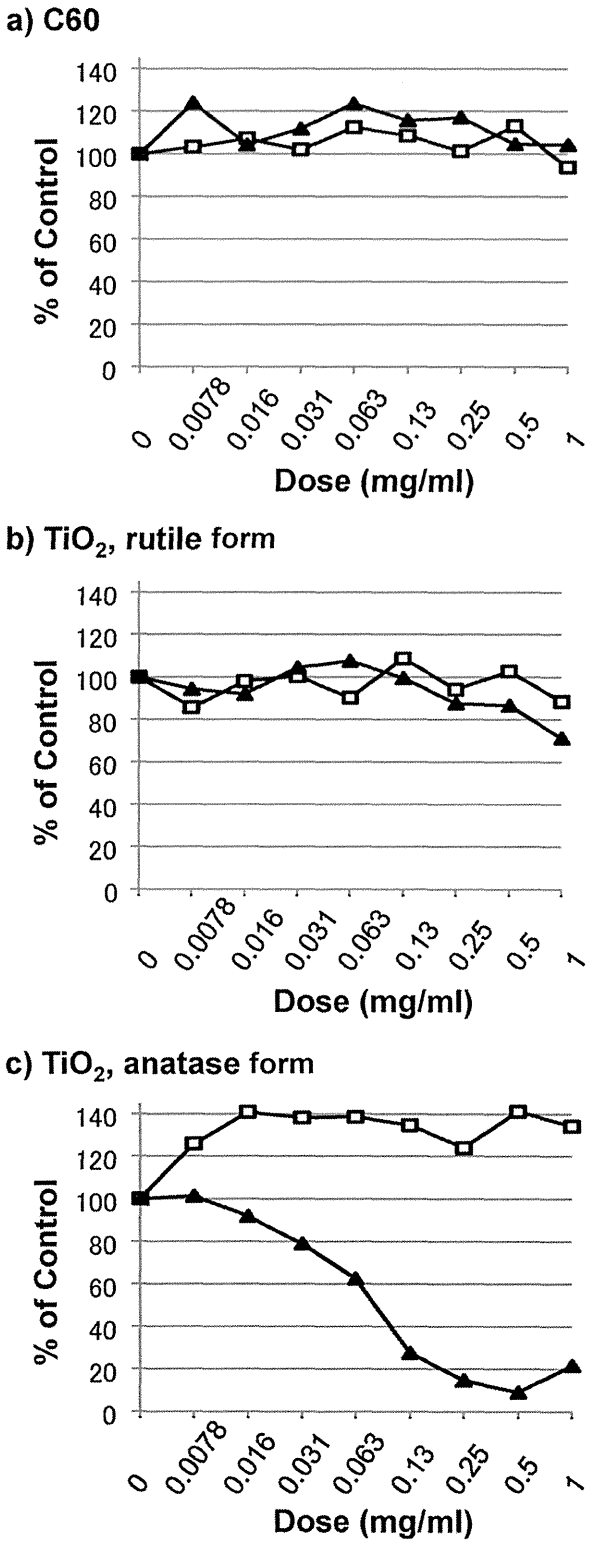

Fig. 3. Phototoxicity in BALB3T3 cells exposed to various concentrations of (a) C_{60}, (b) TiO_2 (rutile form), and (c) TiO_2 (anatase form). Open squares, without photoirradiation; closed triangles, with photoirradiation.

4. Discussion

After obtaining negative results in standard genotoxicity tests (Ames, chromosome aberration, and in vivo micronucleus tests) conducted according to OECD guidelines [9], Shinohara and colleagues concluded that C_{60} had no in vitro or in vivo genotoxic potential [8]. The Ames test, however, may not be appropriate for nanomaterials because bacterial cells lack the capacity for endocytosis and the nanomaterial may not be able to pass through the cell wall, resulting in misleading negative results [13]. Therefore, the in vitro genotoxicity of nanomaterials should be examined in mammalian cells.

Although C_{60} genotoxicity has been studied in mammalian cells in vitro, the results are conflicting. C_{60} significantly induced micronuclei in a concentration-dependent manner in human lung cancer cell line A549 [14] and weakly induced gene mutations in MEF cells isolated from gpt-delta mice [15], but it was negative in the chromosomal aberration test [8,16] and the comet assay [17].

In the present study, we conducted the chromosomal aberration test according to the OECD guideline [9], and demonstrated that the C_{60} clearly induced polyploidy. This result is contrast to that by Shinohara and colleagues [8]. We used the same lot of C_{60} and prepared the sample by the suspension with CMC-Na. The disparity is likely due to the size of the C_{60} in the treatment. Shinohara and colleagues suspended the C_{60} into 0.1% CMC-Na and extensively pounded in an agate mortar for 30 min, while we suspended the C_{60} into 0.5% CMC-Na in usual manners. As the results, the size of prepared C_{60} for the chromosomal aberration test was much different between them; 50 nm in Shinohara and colleagues vs. 300 nm in ours. Larger size of the C_{60} may specifically induce polyploidy. Totsuka et al. also demonstrated that C_{60} prepared by 0.05% Tween 80 induced micronuclei in human lung cancer cell line A549 [14]. The most abundant sizes were two peaks at 234 and 867 nm.

The induction of polyploidy may have been due to a physical interaction between C_{60} nanoparticles and the spindle apparatus during cytokinesis. Jensen and colleagues demonstrated that asbestos fiber induces polyploidy and aneuploidy by sterically blocking cytokinesis in monkey epithelial cells [18], and Asakura and colleagues recently reported that multiwall carbon nanotubes (MWCNTs) induce polyploidy but not structural chromosome aberrations in CHL cells [19]. Because asbestos and MWCNT did not induce micronuclei, but increase the number of bi-nucleated and multi-nucleated cells, the induction of polyploidy is considered to the result by the interference with components of the mitotic spindle during chromosome segregation or by blocking of cytokinesis. It is not clear whether C_{60} produces polyploidy by the similar mechanism. Because larger sizes of C_{60} only induced polyploidy, C_{60} heavily overlaying cell surface may physically block cell division. Anyway, these nanomaterials look not directly interact with DNA, and there may be a threshold for that activity [20]. C_{60} did not induce mesotheliomas in mice by intraperitoneal administration, while asbestos and MWCNT exposure cause mesotheliomas at high frequency [21,22].

Fullerene derivatives produce an excited triplet state by photoexcitation, yielding both singlet molecular oxygen and superoxide through energy and electron transfer to molecular oxygen [23]. They may thus cause oxidative DNA damage resulting in cytotoxicity and genotoxicity. In the present study in BALB3T3 cells, however, we did not observe any C_{60} phototoxicity, nor did Shinohara and colleagues find that UV-irradiation affected the in vitro cytotoxicity and genotoxicity of C_{60} [8]. On the other hand, we found that the anatase form (but not the rutile form) of TiO_2 was clearly phototoxic, which is in agreement with the finding of Nakagawa and colleagues who demonstrated in vitro photogenotoxicity of the anatase form of TiO_2 by the comet assay and the chromosomal aberration test [24]. The oxidative stress generated by the photocatalytic activity of anatase TiO_2 may contribute to cell killing and DNA damage [25].

In conclusion, C_{60} did not, by mutagenic or phototoxic activity, cause direct DNA damage in mammalian cells in vitro. It did,

however, induce polyploidy that was probably due to non-DNA interacting mechanisms.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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E 環境・衛生

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ナノマテリアルの曝露による次世代への影響について考える

「ナノマテリアル」とは，物質をナノメートル(10^{-9}メートル)の領域で制御する技術である「ナノテクノロジー」により製造された物質であり，物質を構成する3次元の長さのうち，少なくとも1次元の長さが100 nm以下のものを指す．金属や金属酸化物等の粒子状ナノマテリアルでは，3次元の長さがいずれもナノスケールであるが，カーボンナノチューブ(CNT)等の繊維状ナノマテリアルでは，直径はナノスケールだが長さはミクロンスケールとなっており，一概にナノマテリアルといっても様々な種類や形状の物質が含まれている．

近年の科学技術の進歩により，ナノマテリアルを様々な工業用品，消費者製品，医療機器・医薬品等に応用する研究が進められており，そのマーケットシェアも次第に拡大しつつあることから，安全性に関するデータの集積と評価が求められている．

通常の化学物質の場合は，その化学組成に基づいて規制と管理が行われているが，ナノマテリアルの場合は，化学組成よりもむしろ，サイズや形状等の物理化学的特性と，生体影響との関係に着目した評価が行われている．例えば，低溶解性—低毒性粒子がナノスケールになると生体影響がより大きくなることが報告されている.[1] この理由として，粒子サイズが小さくなると粒子重量当たりの表面積が大きくなるため，曝露部位において生体組織が粒子の活性表面に接触する面積が大きくなり，より強い影響が出るとする説が有力である．また，CNT等の繊維状ナノマテリアルについては，繊維の長さと毒性との関係について多くの議論がなされており，より長いCNTの方が排泄しにくく体内残留性が高いため，慢性毒性が発現しやすいことが報告されている.[2]

これらの報告に加えて，近年，ナノマテリアルの曝露による次世代への影響を示唆する報告が見られるようになった．一例を挙げると，妊娠期のマウスにおける多層CNTの曝露により，胎仔の奇形が誘発されることが報告されている.[3] この試験では，妊娠9日のICRマウスに，2%カルボキシメチルセルロースナトリウムで調製した多層CNT懸濁液を，最大5 mg/kgの投与量で腹腔内単回投与あるいは気管内単回投与し，妊娠18日に帝王切開して胎仔の検査を行った．その結果，腹腔内投与では2 mg/kg以上，気管内投与では4 mg/kg以上の用量で，欠指等の外表奇形に加えて肋骨の癒着等の骨格奇形が見られている．著者らは，奇形の発現メカニズムを解明するため，更なる実験が必要であると結論付けている．

Fujitaniらの報告において,[3] 気管内投与は，腹腔内投与と異なり現実に起こり得る曝露経路である吸入曝露を模した試験系であるため，現実の吸入曝露によっても同様の影響が起こるのかどうかについて，検証が望まれる．

また，同報告において,[3] 奇形の発現メカニズムを明らかにするためには，曝露したCNTの体内動態を明らかにすることが最も重要と考えられる．しかし，CNTについては生体組織内での定量分析法が未だ開発段階にあるため，分析法の確立が望まれる．

したがって，ナノマテリアルの曝露による次世代への影響について，より確かな結論を得るためにはナノマテリアルの種類，調製法，媒体，投与経路，投与時期，投与量を含む種々の条件について検討するとともに，生体組織内での体内動態の評価も併せた統合的なリスク評価を行うことが今後の課題と考える．

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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

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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 LD_{50} 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 observed 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

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administration period, blackish feces and black contents of the stomach and large intestine were observed. Fullerene C60 was not detected in the liver, spleen or kidney at the end of administration period. This study indicated that fullerene 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 tested 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.*, 2009; 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 Nikkiso SWCNT and MWCNT were set as principal samples in this Sponsorship Programme to examine mammalian toxicity.

Major current uses of MWCNTs are electronics applications such as super-capacitors and batteries and structural composite applications such as sporting equipments and conductive sheets (OECD, 2010a) and the future applications of MWCNTs include medical care and fabrics (Kobayashi *et al.*, 2009; MHLW, 2010). Major expecting uses of SWCNTs in future are super-capacitors, high speed transistor, fuel cells, super high strength wires (OECD, 2010b). 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 migration from food contact products or agricultural foods that uptake CNTs from environment (Magnuson *et al.*, 2011).

Many toxicity studies have been available for SWCNTs or MWCNTs dosed by pharyngeal aspiration (Erdely *et al.*, 2009; Shvedova *et al.*, 2008), intravenous injection (Yang *et al.*, 2008), intratracheal instillation (Inoue *et al.*, 2009; Elgrabli *et al.*, 2008; Inoue *et al.*, 2008) and inhalation (Shvedova *et al.*, 2008) 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 feature 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 Gotemba Laboratory, Bozo Research Center Inc. or the Safety Research Institute for Chemical Compounds Co., Ltd., respectively. These studies were conducted in compliance with the OECD Guideline 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 SWCNTs studies were conducted in compliance with the Act on Welfare and Management of Animals (Act No. 105 of October 1, 1973), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notice No.88 of the Ministry of Environment, dated April 28, 2006) 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

Crl:CD(SD) 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: 6 weeks old; MWCNT: 5 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.

Chemicals and dosing

Principal single-wall carbon nanotubes (SWCNT: purity > 95%; Lot No.: SW1859/SW1860/SW1865) and principal multi-wall carbon nanotubes (MWCNT: purity: > 98%; Lot No.: 04-12/10#1-(4)) supplied by Nikkiso 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 basic morphology is in sheet form consisting of entangle 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,

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 intervals), 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

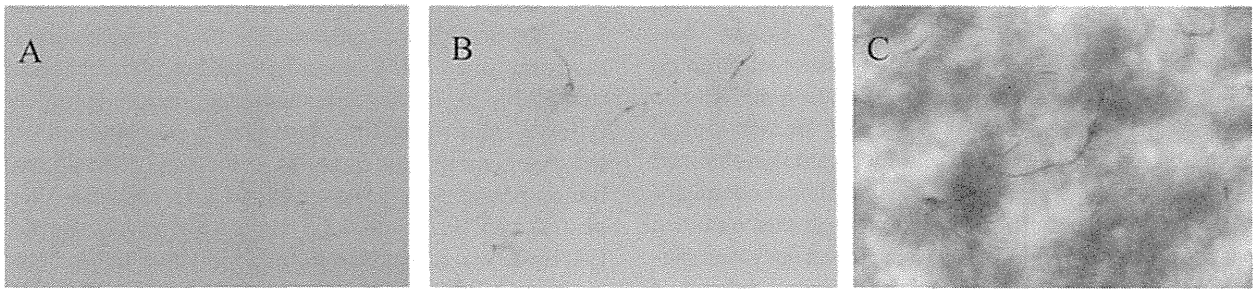

Fig. 1. Microscopic views of SWCNT suspensions (× 400). (A) 0.125 mg/kg bw/day (0.00625 mg/ml); (B) 1.25 mg/kg bw/day (0.0625 mg/ml); (C) 12.5 mg/kg bw/day (0.625 mg/ml).

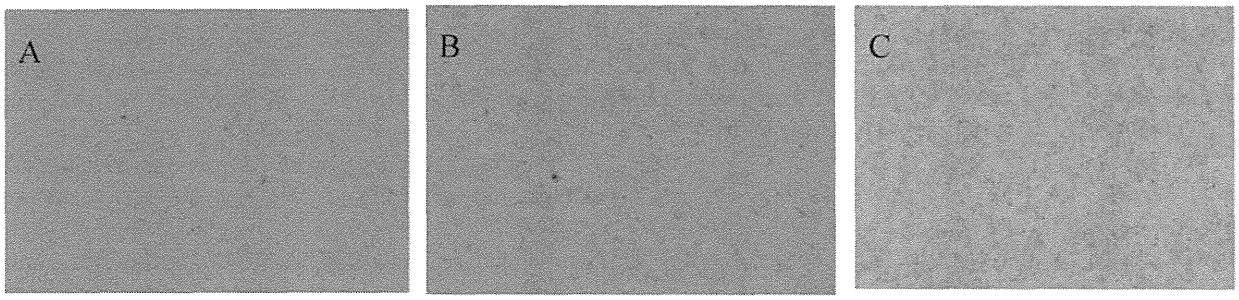

Fig. 2. Microscopic views of MWCNT suspensions (× 400). (A) 0.5 mg/kg bw/day (0.025 mg/ml); (B) 5.0 mg/kg bw/day (0.25 mg/ml); (C) 50 mg/kg bw/day (2.5 mg/ml).

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 LD_{50} 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 ± 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