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INORGANIC COMPOUNDS

Aspect ratio has no effect on genotoxicity of multi-wall carbon nanotubes

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Abstract Carbon nanotubes (CNTs) have specific physico-chemical and electrical properties that are useful for telecommunications, medicine, materials, manufacturing processes and the environmental and energy sectors. Yet, despite their many advantages, it is also important to determine whether CNTs may represent a hazard to the environment and human health. Like asbestos, the aspect ratio (length:diameter) and metal components of CNTs are known to have an effect on the toxicity of carbon nanotubes. Thus, to evaluate the toxic potential of CNTs in relation to their aspect ratio and metal contamination, in vivo and in vitro genotoxicity tests were conducted using high-aspect-ratio (diameter: 10–15 nm, length: ~10 μm) and low-aspect-ratio multi-wall carbon nanotubes (MWCNTs, diameter: 10–15 nm, length: ~150 nm) according to OECD test guidelines 471 (bacterial reverse mutation test), 473 (in vitro chromosome aberration test), and 474 (in vivo micronuclei test) with a good laboratory practice system. To determine the treatment concentration for all the tests, a solubility and dispersive test was performed, and a 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) solution found to be more suitable than distilled water. Neither the high- nor the low-aspect-ratio MWCNTs induced any genotoxicity in a bacterial reverse mutation test (~1,000 μg/plate), in vitro chromosome aberration test (without S9: ~6.25 μg/ml, with S9: ~50 μg/ml), or in vivo micronuclei test (~50 mg/kg). However, the high-aspect-ratio MWCNTs were found to be more toxic than the low-aspect-ratio MWCNTs. Thus, while high-aspect-ratio MWCNTs do not induce direct genotoxicity or metabolic activation–mediated genotoxicity, genotoxicity could still be induced indirectly through oxidative stress or inflammation.

Keywords Carbon nanotubes (CNTs) · Multi-wall carbon nanotubes (MWCNTs) · Genotoxicity · OECD test guidelines · Bacterial reverse mutation test · In vitro chromosome aberration test · In vivo micronuclei test · Good laboratory practice (GLP) · Cytotoxicity

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Introduction

Nanotechnology involves working with materials on a nanometric scale (1–100 nm) and engineering their properties based on controlling their size, thereby opening a multitude of potential uses for nanomaterials (Bonnemann and Richards 2001). Thus, the field of nanotechnology continues to advance rapidly and attract attention in many

scientific fields related to physical, chemical, biomedical, pharmaceutical, and mechanical applications (Murphy 2002). Nonetheless, despite the already widespread use of nanomaterials in modern technology, there is a serious lack of available information on the human health and environmental implications of manufactured nanomaterials (Braydich-Stolle et al. 2005; Hussain et al. 2005).

Carbon nanotubes (CNTs) are carbon-based nanomaterials that have specific physico-chemical and electronic properties (Lin et al. 2004), making them useful for lubricating oils, fuel cells, drug delivery systems, and next generation semiconductors (Gooding et al. 2003; Mattson et al. 2000; Hu et al. 2004; Bianco et al. 2005; Kam et al. 2005; Liu et al. 2007a, b; Peer et al. 2007). Therefore, with the ongoing commercialization of nanotechnology products, human and environmental exposure to CNTs will dramatically increase (Lewinski et al. 2008; Medina et al. 2007; Donaldson et al. 2006). However, despite the attractive properties of CNTs, a recent report suggested that these materials may pose problems for human health in the case of occupational and environmental exposure (Helland et al. 2007). The main reason for concern about CNTs is related to their fibrous structure, which is similar to that of asbestos, and a high-aspect-ratio nanoparticle theory has already been suggested for CNT toxicity (Tran et al. 2008). As seen with asbestos, high-aspect-ratio MWCNTs have more toxicity and potential to induce mesothelioma than low-aspect-ratio MWCNTs (Poland et al. 2008; Takagi et al. 2008). Exposure to asbestos fibers is already known to carry a high carcinogenic risk and be harmful to human health (IARC 1977). The carcinogenic effect of biopersistent fibers, such as asbestos, has also been associated with the local generation of reactive oxygen and nitrogen species and inflammatory reactions (Takagi et al. 2008), while genotoxic effects related to these phenomena or occurring independently may also be implicated (Lindberg et al. 2009). Yet, despite a large number of studies, the current understanding of the toxic effect of CNTs is still unclear and limited. Accordingly, to help clarify the health risks related to CNTs, this study examined the genotoxicity of commercially manufactured multi-wall carbon nanotubes using a genotoxicity battery test and the Organization for Economic Cooperation and Development (OECD) test guidelines 471 (OECD 1997a), 473 (OECD 1997b), and 474 (OECD 1997c) with good laboratory practice (GLP).

Materials and methods

Materials and dispersion

The multi-wall carbon nanotubes (MWCNTs) examined in this study were commercially available MWCNTs (product name: CM-95, diameter 10–15 nm, length ~20 microns) manufactured and supplied by Hanwha Nanotech (Incheon, Korea). The MWCNTs supplied by Hanwha Nanotech have also been designated as an alternative reference material for the sponsorship program for the safety testing of nanomaterials by the OECD WPMN (Working Party on Manufactured Nanomaterials). The purity of the MWCNTs was 95% carbon and approximately 5% iron.

After eliminating all the MWCNT impurities, low-aspect-ratio MWCNTs were manufactured by oxidizing 100 mg of the purified high-aspect-ratio MWCNTs with strong acid, consisting of 300 ml of nitric acid (DC Chemical, 70%) and 100 ml of sulfuric acid (Duksan Pure Chemicals, 95%). To help promote the oxidation and reduce the treatment time, the MWCNT and acid mixture was also placed in a bath sonicator (Hwashin Tech, 350 W) for 2 h. The mixture was then filtered using a membrane filter (Advantec, 0.2 μm) for neutralization, and the residual water MWCNTs dried in a vacuum oven overnight. The resulting low-aspect-ratio MWCNTs were then collected for the experiments.

The particle size distribution and morphology of the nanomaterials were determined using a scanning electron microscope (JEOL JSM-6700F) and dynamic light scattering (DLS). The DLS data were estimated using an ELS-8000 (Otsuka Electronics), and a 632.8-nm He–Ne laser was used for the DLS measurements. DLS measures the scattering intensity $I(q, t)$ of a sample's Brownian motion, and the autocorrelation function is acquired from $G_2(\tau) = \langle I(0)I(\tau)\rangle = 1/T \int I(t)I(t+\tau)dt$. When b is the experimental constant, the relationship of the normalized first-order autocorrelation function $G_1(\tau)$ is estimated by $G_2(\tau) = 1 + b|G_1(\tau)|^2$. $G_1(\tau)$ is then connected to the diffusion constant D, $G_1(\tau) = \exp(-2q^2Dt)$, where $q = (4pn/l_0)\sin(q/2)$. In this equation, n, l_0, and q are the refractive index of the solution, wavelength of the incident light, and scattering angle, respectively. The sample size was estimated using the Einstein–Stokes relation, $D = k_BT/6phr$, where k_B, T, and r are the Boltzmann factor, temperature, and hydrodynamic radius of the samples (Lee et al. 2005). Plus, a thermogravimetric analysis (TGA) and Brunauer–Emmett–Teller (BET) surface area analysis were also performed. For a precise thermogravimetric analysis (Q500, TA instruments), the MWCNTs were heated to 200°C for 2 h in a N_2 (99.999%) atmosphere. After eliminating any moisture from the MWCNTs, the flowing gas was changed to dry air and the measurements started at a rate of 5°C/min up to 1,000°C. The BET analysis was conducted as follows. To prevent any effect from moisture, the MWCNTs were dehydrated in an oven at 80°C for 1 day. Next, the MWCNTs (100 mg) were put in a sample tube and placed in the pretreatment port of the BET (ASAP 2020, Micromeritics). After being heated at 200°C for 1 day in a vacuum

($\sim 10^{-3}$ torr), the sample tube was then moved to the analysis port. When the vacuum reached 10^{-7} torr, the nitrogen gas was absorbed by the MWCNTs.

The dispersion medium (DM) was Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), pH 7.4, supplemented with 5.5 mM D-glucose, 0.6 mg/ml species-specific serum albumin, and 0.01 mg/ml 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). The DPPC was prepared fresh as a 10 mg/ml stock solution in absolute ethanol (Porter et al. 2008). The materials were dispersed in the DPPC solution and subjected to ultrasonication for 3 min at 37 kHz prior to treatment. The degree of macrodispersion of the MWCNTs was evaluated using the Korean Industrial Standards D 2717 method (Korean Agency for Technology and Standards 2009). After dispersing the MWCNTs, the light absorbance unit (A) was measured at 800 nm. The dispersed MWCNTs were then centrifuged at 6,000g for 10 min and the absorbance unit (B) measured at 800 nm. As such, the degree of macrodispersion (%) was $B/A \times 100$ (%).

Bacterial reverse mutation test (Ames test)

The MWCNTs were evaluated for mutagenic activity using the Ames test with four histidine-requiring strains of Salmonella typhimurium TA98, TA100, TA1535, and TA1537 and one tryptophan-requiring strain of Escherichia coli WP2uvrA in the presence and absence of a metabolic activation system consisting of the postmitochondrial fraction of liver homogenates from rats treated with Recolor 1254 (S9) (OECD 1997a; Maron and Ames 1983). The Salmonella typhimurium strains and Escherichia coli WP2uvrA were obtained from Molecular Toxicology Inc., USA, stored as frozen (−80°C) stock cultures, and their genotype checked regularly. Each dose was plated in triplicate. For the plating, the bacteria were suspended in a culture medium, then the MWCNTs suspended in the DPPC solution, an S9 mix, or a 0.1 mol/l phosphate buffer (pH 7.4) was added to the top agar supplemented with histidine and biotin (0.05 nmol each). The components were then mixed and spread evenly on minimal glucose agar plates. After the top agar hardened, the plates were incubated in the dark at 36 ± 1°C for 44–48 h. The number of revertant colonies was then determined using a colony counter (Suntex, USA). In all the experiments, negative and positive strain-specific and S9-specific control substances were assayed concurrently (dimethyl sulfoxide, daunomycin, 2-aminoanthracene, 2-aminofluorene, and methyl methanesulfonate).

In vitro chromosome aberration test

To evaluate the mutagenic ability of the MWCNTs, an in vitro chromosome aberration test was performed according to OECD test guideline No. 473 (OECD 1997b). The in vitro chromosome aberration test was assessed in the presence and absence of S9. Chinese hamster ovary cells (CHO-k1) were exposed to the test material for short term (6 h) and long term (24 h). The CHO-k1 cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in an F-12 medium supplemented with 10% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 mg/ml). The positive control substances used were mitomycin C (direct method) and cyclophosphamide (metabolic activation method). The cytotoxicity was determined using the trypan blue dye exclusion method. The cytotoxicity was expressed as GI_{50} (growth inhibition 50%) by calculating the probit. To make the chromosome slides, the harvested cultures were incubated with a hypotonic solution (0.075 M·KCl) for 30 min. The cells were then fixed in a methanol/glacial acetic acid solution [3:1(v/v)]. This fixation step was repeated twice, and the final cells dropped onto a clean slide. Following air drying, the cells were stained with a 3% Giemsa solution. At least two slides were generated per culture and 200 metaphases analyzed per concentration.

In vivo micronuclei assay

Seven-week-old male, specific pathogen-free (SPF) ICR mice were purchased from OrientBio (Korea) and acclimated for 1 week before starting the experiments. During the acclimation and experimental periods, the mice were housed in polycarbonate cages (no more than 3 mice per cage) in a room with controlled temperature (23 ± 2°C) and humidity (55 ± 7%) and a 12-h light/dark cycle. The mice were fed rodent chow (Harlan Teklab, Plaster International Co., Korea) and filtered water ad libitum. The mice were exposed to the MWCNTs based on intraperitoneal administration. A micronuclei assay was then conducted using a method based on OECD guideline 474 (OECD 1997c; MacGregor et al. 1987) Briefly, the femurs were removed and the bone marrow collected in 1.5-ml tubes containing 1 ml of fetal bovine serum and centrifuged at 1,000 rpm for 5 min. Two smears were prepared and allowed to air dry, prior to fixation with methanol and staining with an acridine orange solution. One drop of a 0.04 mM acridine orange solution in a phosphate buffer was placed on the fixed cells and covered with a coverslip. Observations were made within a day using a fluorescent microscope (Leica, Germany). The slides were coded and scored blind by an expert scorer; 2,000 polychromatic erythrocytes (PCEs) per animal were examined for the presence of micronuclei, which means 2,000 PCEs were scored per dose group. Since normochromatic erythrocytes (NCE) appear opaque when using a fluorescent stain, one more slide per animal was stained with May-Grünwald and

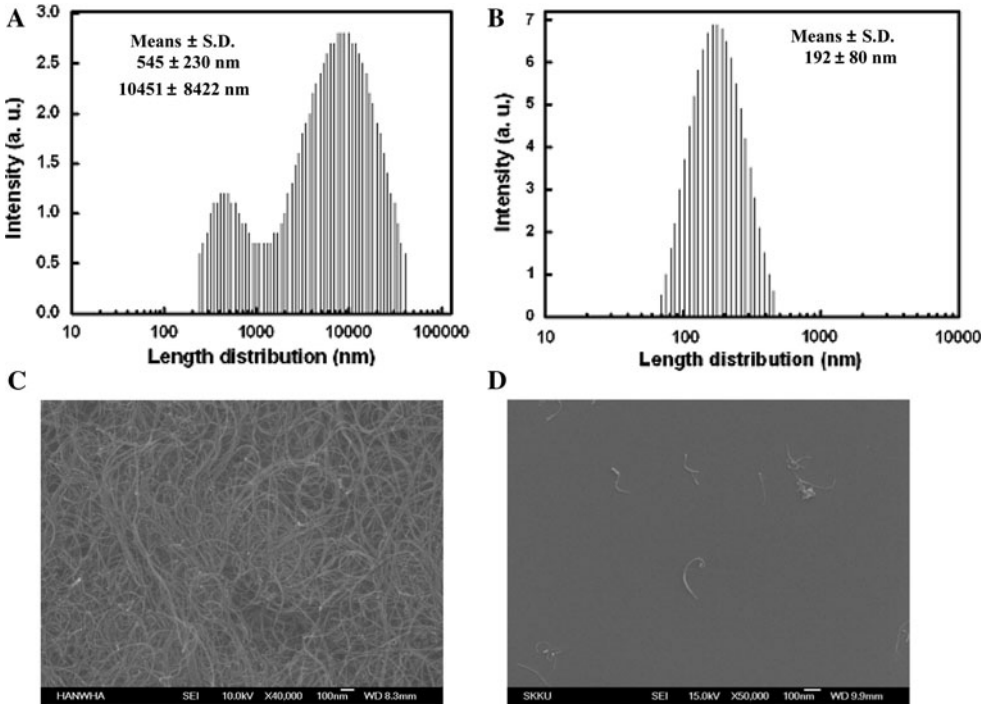

Fig. 1 Dynamic light scattering graphs of multi-wall carbon nanotubes (MWCNTs) (**a** high-aspect-ratio MWCNTs, **b** low-aspect-ratio MWCNTs) and scanning electron microscope (SEM) image of MWCNTs (**c** high-aspect-ratio MWCNTs, **d** low-aspect-ratio MWCNTs)

Giemsa solutions. To evaluate the bone marrow toxicity, the PCE/PCE + NCE ratio was calculated by counting a total of 200 erythrocytes using these slides (Schmid 1976).

Statistics

The statistical analyses were performed using SPSS 12.1, and the data expressed as the mean ± SD. An X^2 test and one-way analysis of variance (ANOVA) were applied to test all the data. A value of $P < 0.05$ indicated statistical significance.

Results

The multi-wall carbon nanotubes (MWCNTs) used in this study were obtained from Hanwha Nanotech and defined as high-aspect-ratio MWCNTs. Low-aspect-ratio MWCNTs were then manufactured using heat, acid, and ultrasonic wave treatment of the high-aspect-ratio MWCNTs. As a result, based on dynamic light scattering data and a scanning electron microscope, the MWCNTs were 10–15 nm in diameter, whereas the high-aspect-ratio MWCNTs were ~10 μm in length (Fig. 1a, c) and the low-aspect-ratio MWCNTs were ~150 nm in length (Fig. 1b, d). Meanwhile, the purity of the high-aspect-ratio MWCNTs and low-aspect-ratio MWCNTs was 95 and 99%, respectively (Fig. 2a, b), based on a thermogravimetric analysis. The TGA analysis and BET data also found that the low-aspect-ratio MWCNTs were more pure and had a greater surface area than the high-aspect-ratio MWCNTs (Fig. 2; Suppl. 1). To find the appropriate dispersion state, solubility and dispersive tests were performed, and based on the degree of macrodispersion, the low-aspect-ratio MWCNTs were found to disperse better than the high-aspect-ratio MWCNTs in distilled water and a 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) solution, which is a biocompatible dispersion agent (Suppl. 2, 3). Finally, since it was only possible to wet less than 0.5% MWCNTs in the dispersion agent, less than 0.5% MWCNTs in a DPPC solution was used to treat the cells and animals in this study.

To find the mutagenic potential of the MWCNTs, a battery genotoxicity test, consisting of a bacterial reverse mutation test, in vitro chromosome aberration test, and in vivo micronuclei test, was performed.

In the bacterial reverse mutation test, four histidine-requiring strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and one tryptophan-requiring strain of *Escherichia coli* (WP2uvrA) were used to evaluate the mutagenic potential of the high- and low-aspect-ratio MWCNTs in the presence and absence of a metabolic activation system (S9 mix). The test was performed using 1,000 μg/plate as the highest dose level, as well as subsequent threefold serial dilutions of 333, 111, 37, 12 μg/plate. The test MWCNTs did not exhibit any cytotoxicity at any of the dose levels in any of the strains without and with the metabolic activation system (Suppl. 4 and 5). The precipitation and aggregation of the MWCNTs

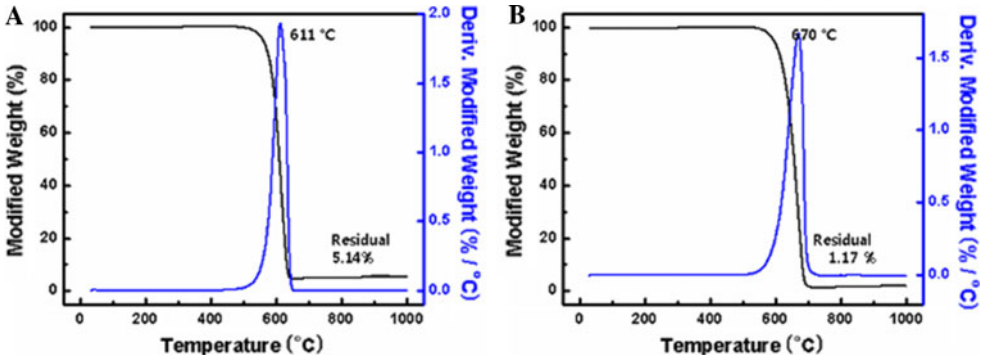

Fig. 2 Test results of thermogravimetric analysis for high-aspect-ratio (**a**) and low-aspect-ratio (**b**) multi-wall carbon nanotubes (MWNCTs)

was observed at a dose level of more than 333 µg/plate using a direct and metabolic activation method. When compared with the negative control, no significant number of revertant colonies was observed for any of the bacterial strains both with and without the metabolic activation system. Furthermore, no dose-dependent increase of revertant colonies was observed for any of the bacterial strains (Suppl. 4, 5). The number of revertant colonies in the positive and negative control groups was within the range of historical data (data not shown).

To assess the mammalian cell cytotoxicity of the MWCNTs towards cultured CHO-k1 cells, the relative cell count (RCC) was estimated for all the controls and cultures treated with the MWCNTs at 7 dose levels (3.125, 6.25, 12.5, 25, 50, 100, and 200 µg/ml) using the trypan blue dye exclusion method. For the groups treated for 24 h without the S9 mix, 6 h without the S9 mix, and 6 h with the S9 mix, there was a clear MWCNT dose-dependent decrease in cell growth at 3.125–50 µg/ml and cytotoxic effect at 100 and 200 µg/ml with the high-aspect-ratio MWCNTs (Figs. 3, 4, 5), whereas the low-aspect-ratio MWCNTs produced a dose-dependent decrease at all doses (3.125–200 µg/ml) (Figs. 3, 4, 5). Meanwhile, due to severe aggregation of the high-aspect-ratio MWNCTS at 100 µg/ml or above in the F-12 medium, the high-aspect-ratio MWCNTs showed low or no cytotoxic effect at the high doses (data not shown). However, the high-aspect-ratio MWCNTs (GI_{50} = 12.94, 12.94, and 41.90 µg/ml) were found to be more toxic than the low-aspect-ratio MWCNTs (60.20, 40.48, and 93.19 µg/ml) in the groups treated for 24 h without the S9 mix (Fig. 5), 6 h without the S9 mix (Fig. 3), and 6 h with the S9 mix (Fig. 4) based on a 50% growth inhibition (GI_{50}).

To elucidate whether the higher toxicity of the high-aspect-ratio MWCNTs was due to their high aspect ratio or impurities (iron oxide), the toxicity of iron oxide was tested in CHO-k1 cells. The test was performed using 12 µg/ml (6% of 200 µg/ml) as the highest dose level, as well as subsequent twofold serial dilutions of 6 dose levels (Suppl. 6). As a result, iron oxide was not found to induce any cyto-

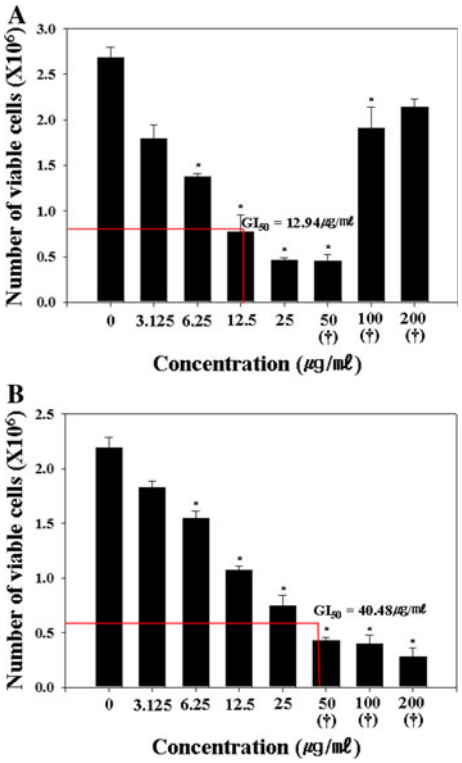

Fig. 3 The number of viable cells in the absence of S9 mix for high-aspect-ratio (**a**) and low-aspect-ratio (**b**) multi-wall carbon nanotubes (MWCNTs) (6-h exposure). †Aggregation and precipitation of test substance. *Significantly different from negative control at $P < 0.05$

toxic effect on mammalian cell growth, thereby highlighting the contribution of the high aspect ratio of the MWCNTs to the cytotoxic effect.

In addition, the degree of macrodispersion and length distribution for the various doses were also tested in F-12 media, where the lower treatment concentrations produced a higher degree of dispersion and the low-aspect-ratio MWCNTs were better dispersed than the high-aspect-ratio MWCNTs (Suppl. 7). Even when the high-aspect-ratio MWCNTs were shortened by ultrasonic treatment, they were still longer than the low-aspect-ratio MWCNTs (Table 1).

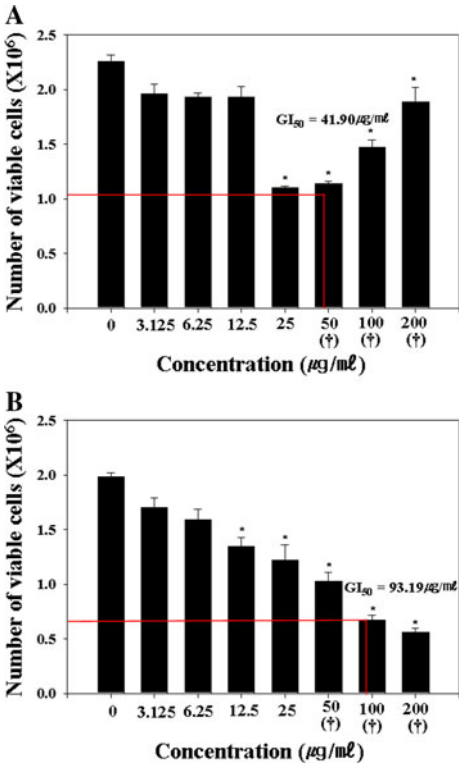

Fig. 4 The number of viable cells in the presence of S9 mix for high-aspect-ratio (**a**) and low-aspect-ratio (**b**) multi-wall carbon nanotubes (MWCNTs) (6-h exposure). †Aggregation and precipitation of test substance, *Significantly different from negative control at $P < 0.05$

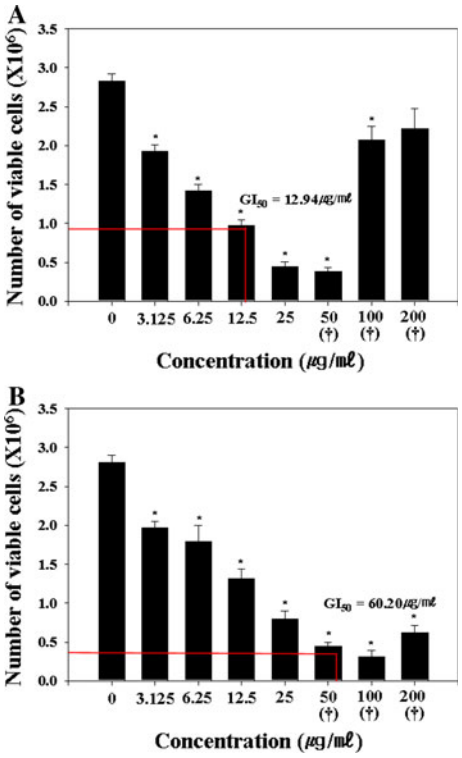

Fig. 5 The number of viable cells in the absence of S9 mix for high-aspect-ratio (**a**) and low-aspect-ratio (**b**) multi-wall carbon nanotubes (MWCNTs) (24-h exposure). †Aggregation and precipitation of test substance, *Significantly different from negative control at $P < 0.05$

Thus, on the basis of the cytotoxicity test, the treatment concentration for chromosome aberration was determined at a relative cell count (RCC) of around 50% (50 ± 5%). With the CHO-k1 cells, neither the high-nor the low-aspect-ratio MWCNTs produced a statistically significant increase in the number of cells with chromosome aberrations when compared with the negative control group at any of the dose levels tested, with or without metabolic activation (Tables 2, 3, 4). Furthermore, in the presence and absence of the S9 mix, neither the high- nor the low-aspect-ratio MWCNTs caused a statistically significant increase in the number of cells with polyploidy or endoreduplication when compared with the negative control group (Tables 2, 3, 4).

The in vivo genotoxic effect of the MWCNTs was examined using an in vivo micronuclei assay. No distinct effects were observed after treatment with the MWCNTs, and there were no significant differences in the body weights according to the dose of MWCNTs (Suppl. 8). Moreover, there was no statistically significant difference in the PCE/(PCE + NCE) ratio when compared with the control (Table 5), meaning that neither the high- nor the low-aspect-ratio MWCNTs were sufficiently absorbed to circulate, thereby having no cytotoxic effect on the mouse erythrocytes in the in vivo micronuclei study. The frequency of micronucleated polychromatic erythrocytes (MNPCEs) in 2,000 PCEs was 2.6, 3.0, and 2.2 (high-aspect-ratio MWCNTs) and 1.0, 2.6, and 4.0 (low-aspect-ratio MWCNTs) for the mice exposed to a concentration of 12.5, 25, 50 mg/kg, respectively, while that for the negative control was 1.6. (high-aspect-ratio MWCNTs) and 2.4 (low-aspect-ratio MWCNTs). Plus, no significant dose-related increase in MNPCEs was detected in the mice when compared to the corresponding negative controls for both the high- and low-aspect-ratio MWCNTs (Table 16). After killing, although high- and low-aspect-ratio MWCNTs were found in the abdominal cavity, none had penetrated into the organs or blood stream (Fig. 6).

Discussion

Carbon nanotubes (CNTs) have specific physico-chemical and electrical properties (Lin et al. 2004), which have allowed them to be applied in various industrial fields. However, despite their many advantages, CNTs are viewed

Table 1 Length distribution of multi-wall carbon nanotubes (MWCNTs) in F-12 nutrient mixture medium

Concentration (μg/ml)	High-aspect-ratio		Low-aspect-ratio	
	Mean ± standard deviation		Mean ± standard deviation	
50	277 ± 143 nm	2,280 ± 1,013 nm	53 ± 4 nm	284 ± 49 nm
25	349 ± 191 nm	2,671 ± 1,075 nm	284 ± 49 nm	358 ± 67 nm
12.5	214 ± 40 nm	2,794 ± 955 nm	138 ± 19 nm	424 ± 77 nm
6.25	194 ± 32 nm	2,415 ± 601 nm	60 ± 7 nm	386 ± 74 nm
3.125	160 ± 21 nm	4,173 ± 1,181 nm	142 ± 23 nm	683 ± 170 nm
1.5625	198 ± 27 nm	11,075 ± 2,638 nm	82 ± 10 nm	531 ± 108 nm

Table 2 Number of cells with chromosome aberrations in the absence of S9 mix for high-aspect-ratio (A) and low-aspect-ratio (B) MWCNTs (6-h exposure)

Dose (μg/ml)	No. of cells	Types of chromosome aberration						Total aberrations		Aberrant cells	
		ctb	cte	csb	cse	PP	Gap	(−)Gap	(+)Gap	(−)Gap	(+)Gap
A. High-aspect-ratio MWCNTs											
U.C.	100	0	0	0	0	0	1	0	1	0	1
	100	0	0	0	0	0	0	0	0	0	0
N.C.	100	0	0	0	0	0	0	0	0	0	0
	100	0	0	0	0	0	1	0	1	0	1
1.563	100	1	0	0	0	0	1	1	2	1	2
	100	1	0	0	0	0	1	1	2	1	2
3.125	100	1	0	0	0	0	1	1	2	1	2
	100	0	0	0	0	0	0	0	0	0	0
6.25	100	0	0	0	0	0	1	0	1	0	1
	100	1	0	0	0	0	0	1	1	1	1
MMC	100	5	19	0	0	0	1	24	25	24*	25
	100	5	22	1	0	0	2	28	30	27*	29
B. Low-aspect-ratio MWCNTs											
U.C.	100	0	0	0	0	0	0	0	0	0	0
	100	1	0	0	0	0	0	1	1	1	1
N.C.	100	0	0	0	0	0	0	0	0	0	0
	100	1	0	0	0	0	1	1	2	1	2
1.563	100	0	0	0	0	0	0	0	0	0	0
	100	0	1	0	0	0	0	1	1	1	1
3.125	100	1	0	0	0	0	0	1	1	1	1
	100	2	0	0	0	0	1	2	3	2	3
6.25	100	1	0	0	0	0	1	1	2	1	2
	100	1	0	0	0	0	0	1	1	1	1
12.5	100	1	1	0	0	0	0	2	2	2	2
	100	0	1	0	0	0	1	1	2	1	2
MMC	100	9	13	0	0	0	2	22	24	22*	23
	100	10	13	0	0	0	3	23	26	21*	24

U.C. untreated control, *N.C.* negative control, *MMC* mitomycin C (0.04 μg/ml), *ctb* chromatid-type breakage, *cte* chromatid-type exchange, *csb* chromosome-type breakage, *cse* chromosome-type exchange, *PP* polyploidy

* Significantly different from negative control at $P < 0.05$

by some as a potential health hazard. Therefore, various recent studies have investigated whether CNTs can have an adverse effect on humans and the environment.

As such, a high-aspect-ratio nanoparticle (HARN) theory has been introduced, as seen with asbestos fibers, where HARNs include carbon nanotubes, nanowires, and

Table 3 Number of cells with chromosome aberrations in the presence of S9 mix for high-aspect ratio (A) and low-aspect-ratio (B) MWCNTs (6-h exposure)

Dose (μg/ml)	No. of cells	Types of chromosome aberration						Total aberrations		Aberrant cells	
		ctb	cte	csb	cse	PP	Gap	(−)Gap	(+)Gap	(−)Gap	(+)Gap
A. High-aspect-ratio MWCNTs											
U.C.	100	0	0	0	0	0	0	0	0	0	0
	100	1	0	0	0	0	1	1	2	1	2
N.C.	100	0	0	0	0	0	0	0	0	0	0
	100	1	0	0	0	0	0	1	1	1	1
6.25	100	0	1	0	0	0	1	1	2	1	2
	100	1	0	0	0	0	0	1	1	1	1
12.5	100	1	1	0	0	0	0	2	2	2	2
	100	0	0	0	0	0	1	0	1	0	1
25	100	1	0	0	0	0	0	1	1	1	1
	100	0	0	0	0	0	0	0	0	0	0
CPP	100	5	27	0	0	0	2	32	34	32*	34
	100	2	32	0	0	0	1	34	35	31*	32
B. Low-aspect-ratio MWCNTs											
U.C.	100	0	0	0	0	0	0	0	0	0	0
	100	1	0	0	0	0	0	1	1	1	1
N.C.	100	0	0	0	0	0	1	0	1	0	1
	100	0	0	0	0	0	1	0	1	0	1
6.25	100	0	0	0	0	0	0	0	0	0	0
	100	1	1	0	0	0	1	2	3	2	3
12.5	100	0	1	0	0	0	0	1	1	1	1
	100	0	0	0	0	0	1	0	1	0	1
25	100	1	0	0	0	0	1	1	2	1	2
	100	0	2	0	0	0	0	2	2	2	2
50	100	1	1	0	0	0	0	2	2	2	2
	100	1	1	0	0	0	1	2	3	2	3
CPP	100	6	21	0	0	0	2	27	29	27*	29
	100	6	24	0	0	0	3	30	33	28*	31

U.C. untreated control, *N.C.* negative control, *CPA* cyclophosphamide H_2O (10 μg/ml), *ctb* chromatid-type breakage, *cte* chromatid-type exchange, *csb* chromosome-type breakage, *cse* chromosome-type exchange, *PP* polyploidy

* Significantly different from the negative control at $P < 0.05$

nanorods. The theory postulates that the similarities in shape and durability between HARNs and asbestos suggest that exposure to HARNs may cause similar adverse health effects (Tran et al. 2008). HARNs deposited in the lungs, due to their length, may be able to translocate to the pleura and cause mesothelioma, like asbestos (Tran et al. 2008). High-aspect-ratio MWCNTs have also shown more toxicity and potential to induce mesothelioma than low-aspect-ratio MWCNTs (Poland et al. 2008; Takagi et al. 2008). Therefore, to identify whether HARNs can indeed cause mesothelioma, other cancers, or fibrosis, this study compared the biologically relevant endpoints, such as the cytotoxicity and genotoxicity in vitro and in vivo, of high-aspect-ratio and low-aspect-ratio nanoparticles.

To assess and compare the genetic toxicity potential of high- and low-aspect-ratio MWCNTs, a battery genotoxicity test was performed, including a bacterial reverse mutation test, in vitro chromosome aberration test, and in vivo micronuclei test, using high- and low-aspect-ratio multiwall carbon nanotubes (MWCNTs) based on OECD test guidelines 471, 473, and 474 with good laboratory practice (GLP). The cytotoxicity of the high- and low-aspect-ratio MWCNTs was also evaluated in vitro.

Di Sotto et al. (2009) and Szendi and Varga (2008) previously reported that MWCNTs did not have any mutagenic effect in bacteria systems as they did not significantly increase the number of revertant colonies (Di Sotto et al. 2009; Szendi and Varga 2008). The present study also

Table 4 Number of cells with chromosome aberrations in the absence of S9 mix for high-aspect-ratio (A) and low-aspect-ratio (B) MWCNTs (24-h exposure)

Dose (μg/ml)	No. of cells	Types of chromosome aberration						Total aberrations		Aberrant cells	
		ctb	cte	csb	cse	PP	Gap	(−)Gap	(+)Gap	(−)Gap	(+)Gap
A. High-aspect-ratio MWCNTs											
U.C.	100	0	0	0	0	0	1	0	1	0	1
	100	0	0	0	0	0	0	0	0	0	0
N.C.	100	1	0	0	0	0	0	1	1	1	1
	100	0	0	0	0	0	1	0	1	0	1
1.563	100	0	0	0	0	0	0	0	0	0	0
	100	1	0	0	0	0	0	1	1	1	1
3.125	100	0	1	0	0	0	0	1	1	1	1
	100	0	0	0	0	0	0	0	0	0	0
6.25	100	2	0	0	0	0	1	2	3	2	3
	100	1	0	0	0	0	0	1	1	1	1
MMC	100	7	22	1	0	0	0	30	30	29*	29
	100	4	26	0	0	0	1	30	31	29*	30
B. Low-aspect-ratio MWCNTs											
U.C.	100	1	0	0	0	0	0	1	1	1	1
	100	0	0	0	0	0	0	0	0	0	0
N.C.	100	0	0	0	0	0	0	0	0	0	0
	100	1	0	0	0	0	0	1	1	1	1
1.563	100	1	0	0	0	0	0	1	1	1	1
	100	0	0	0	0	0	0	0	0	0	0
3.125	100	1	0	0	0	0	1	1	2	1	2
	100	0	0	0	0	0	1	0	1	0	1
6.25	100	1	0	0	0	0	1	1	2	1	2
	100	0	1	0	0	0	0	1	1	1	1
12.5	100	0	0	0	0	0	1	0	1	0	1
	100	0	0	0	0	0	0	0	0	0	0
MMC	100	5	23	0	0	0	3	28	31	28*	31
	100	9	21	0	0	0	1	30	31	28*	29

U.C. untreated control, *N.C.* negative control, *MMC* mitomycin C (0.04 μg/ml), *ctb* chromatid-type breakage, *cte* chromatid-type exchange, *csb* chromosome-type breakage, *cse* chromosome-type exchange, *PP* polyploidy

* Significantly different from negative control at $P < 0.05$

found that neither the high- nor the low-aspect-ratio MWCNTs induced DNA substitution or a frameshift in *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537) and *Escherichia coli* (WP2uvrA) at below 1,000 μg/plate, either with or without metabolic activation (S9 mix) (Suppl. 4, 5).

It has also been reported that CNTs can be harmful to mammalian cells, human T cells (Bottini et al. 2006), HEK293 kidney epithelial cells (Bottini et al. 2005), and skin epithelial cells (Cui et al. 2005) in a time- and dose-dependent manner. However, in Chinese hamster lung fibroblast V79 cells, MWCNT agglomerates have not been found to induce cytotoxicity or chromosomal aberration (Wirnitzer et al. 2009). In this study, an in vitro chromosome aberration test using Chinese hamster ovary cells (CHO-k1) found dose-dependent cell growth defects when the cells were treated with the MWCNTs at 3.12–50 μg/ml, and a statistically significant difference in cell proliferation at 3.125–100 μg/ml for the high-aspect-ratio MWCNTs and 3.125–200 μg/ml for the low-aspect-ratio MWCNTs when compared with the negative control after 6 and 24 h of treatment with or without metabolic activation (Figs. 3, 4, 5). Above 50 μg/ml, severe aggregation and precipitation phenomena were observed for the high-aspect-ratio MWCNTs, along with a decreased cytotoxicity. In the presence of the S9 mix (6-h exposure), a statistically significant difference in cell proliferation was observed at 25–100 μg/ml when compared with the negative control (Fig. 4). When comparing the GI_{50} for the high- and low-aspect-ratio MWCNTS, the high-aspect-ratio MWCNTs were more

Table 5 Frequency of PCE/(PCE + NCE) ratio in bone marrow of male mouse treated with indicated doses of high-aspect-ratio (*upper*) and low-aspect-ratio (*lower*) multi-wall carbon nanotubes (MWCNTs) for 24 h

Sampling time (hours)	Dose (mg/kg)	Animal no.	Frequency of MNPCE in 2,000 PCEs (Mean ± SD)	PCE/(PCE + NCE) (Mean ± SD)
24	U.C.	6	1.8 ± 1.2	0.53 ± 0.08
	V.C.	6	1.6 ± 1.6	0.54 ± 0.10
	12.5	6	2.6 ± 1.0	0.54 ± 0.05
	25	6	3.0 ± 1.6	0.49 ± 0.07
	50	6	2.2 ± 1.2	0.42 ± 0.08
	MMC	6	114 ± 55.8*	0.43 ± 0.05
Sampling time (hours)	Dose (mg/kg)	Animal no.	Frequency of MNPCE in 2,000 PCEs (Mean ± SD)	PCE/(PCE + NCE) (Mean ± SD)
24	U.C.	6	2.4 ± 1.6	0.38 ± 0.09
	V.C.	6	2.4 ± 1.2	0.35 ± 0.06
	12.5	6	1.0 ± 1.0	0.33 ± 0.08
	25	6	2.6 ± 1.4	0.33 ± 0.04
	50	6	4.0 ± 3.0	0.35 ± 0.03
	MMC	6	125.4 ± 48.8*	0.33 ± 0.06

U.C. untreated control, *V.C.* vehicle control, *MMC* mitomycin C (2.0 mg/ml), *PCE* polychromatic erythrocytes, *NCE* normochromatic erythrocyte, vehicle: 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC)

* Significantly different from vehicle control at $P < 0.05$ (one-way ANOVA)

toxic than the low-aspect-ratio MWCNTs in several of the experimental treatments. Thus, while the induction of mesothelioma observed in the mice treated with the high-aspect-ratio MWCNTs was not the direct result of genotoxic effects or metabolic activation, it may have been caused indirectly as a result of oxidative stress or inflammation.

Nanoparticles can exhibit unique optical, electronic, and magnetic properties. In particular, it is well known that multi-wall carbon nanotubes (MWCNTs) show high electrical conductivity and excellent mechanical strength (You et al. 2005). Such conditions for nanoparticles and linking them with protein side chains can be modulated by the pH or charge screening via controlling the ionic strength of the medium (Aubin-Tam and Hamad-schifferli 2008). In this study, the direct method (without the S9 mix) was demonstrated to be more cytotoxic than the metabolic activation method (with the S9 mix) in the case of MWCNT exposure (Figs. 3, 4, 5). Thus, it would appear that the weak toxicity in the presence of the S9 mix was due to the interaction of the MWCNTs with the S9 protein in the media, thereby preventing the MWCNTs from exerting a cytotoxic effect on the cells.

To estimate the in vivo genotoxic effect of MWCNTs, an in vivo micronuclei test that is widely used for the detection of cytogenic damage was also carried out. Several recent studies have already reported that the responses of CNTs are similar to the carcinogenic responses of asbestos fibers when injected into the peritoneal cavity, as the high aspect ratio of CNTs (>100) means they would be expected to

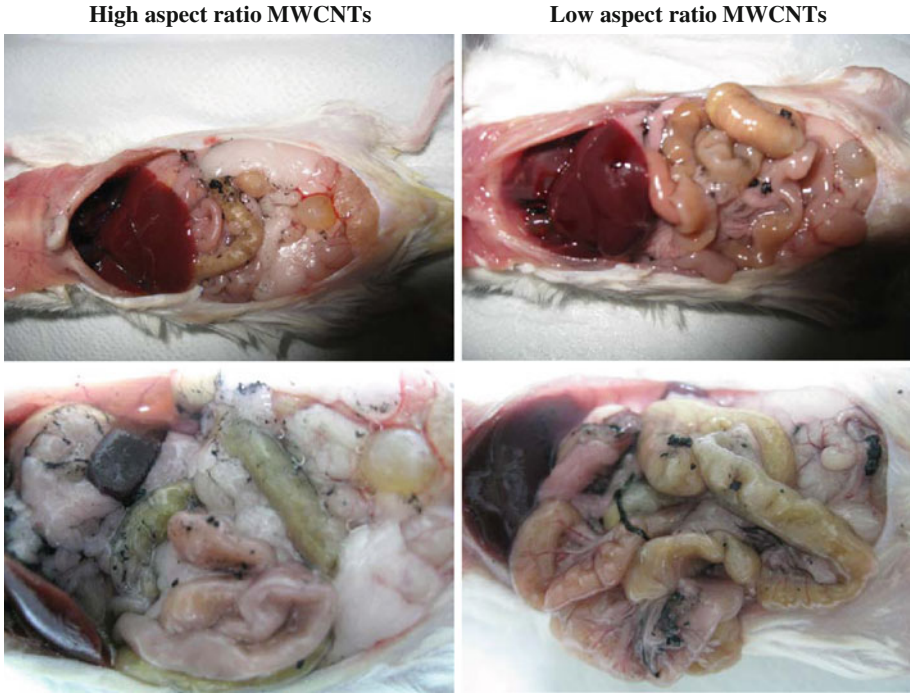

Fig. 6 Pictures of multi-wall carbon nanotubes (MWCNTs) in mouse abdominal cavity

behave like biopersistent fibers in vivo (Poland et al. 2008; Takagi et al. 2008; Aillon et al. 2009). For the in vivo micronuclei test in this study, ICR mice were treated with MWCNTs via intraperitoneal administration. The animals exhibited no weight change before and after the administration (Suppl. 8). Also, neither the high- nor the low-aspect-ratio MWCNTs had any affect on either the micronucleated polychromatic erythrocyte (MNPCE) generation, taken as an indicator of DNA damage, or the PCE/(PCE + NCE) ratio, an indicator of the cytotoxicity of bone marrow cells, in the mice (Table 5). Both the high- and the low-aspect-ratio MWCNTs remained in the abdominal cavity without any distribution or translocation to other organs (Fig. 6). Thus, neither the high- nor the low-aspect-ratio MWCNTs appeared to induce any cytotoxicity in the hematopoietic cells or genotoxicity in the mice due to their inability to translocate to the bone marrow of the femurs.

Concern has recently been increasing over the need for more data to provide a more extensive evaluation of the health and environmental effects of MWCNTs. The OECD WPMN has already recognized the importance of monitoring the potential harmful effects of MWCNTs and attempted to determine the health effects of MWCNTs following OECD test guidelines with good laboratory practice (GLP). Thus, to support this OCED activity, this study evaluated the genotoxicity of MWCNTs in accordance with OECD test guidelines 471, 473, and 474 and GLP.

In conclusion, no genotoxicity was exhibited by commercial MWCNTs in a bacterial reverse mutation test, in vitro chromosome aberration test, and in vivo micronuclei test. Nonetheless, MWCNT treatment was found to have an adverse effect on mammalian cell proliferation and cell viability.

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