Pharmacological and toxicological target organelles and safe use of single-walled carbon nanotubes as drug carriers in treating Alzheimer disease

Zhong Yang, PhD, Yingge Zhang, PhD, MD, Yanlian Yang, PhD, Lan Sun, PhD, Dong Han, PhD, Hong Li, PhD, Chen Wang, PhD, MD

Key Laboratory of Nanopharmacology and Nanotoxicology, Beijing Institute of Pharmacology and Toxicology, Beijing, China
National Center for Nanoscience and Nanotechnology, Beijing, China

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Abstract

Identification of pharmacological and toxicological profiles is of critical importance for the use of nanoparticles as drug carriers in nanomedicine and for the biosafety evaluation of environmental nanoparticles in nanotoxicology. Here we show that lysosomes are the pharmacological target organelles for single-walled carbon nanotubes (SWCNTs) and that mitochondria are the target organelles for their cytotoxicity. The gastrointestinally absorbed SWCNTs were lysosomotropic but also entered mitochondria at large doses. Genes encoding phosphoinositide-3-kinase and lysosomal-associated membrane protein 2 were involved in such an organelle preference. SWCNT administration resulted in collapse of mitochondrial membrane potentials, giving rise to overproduction of reactive oxygen species, leading to damage of mitochondria, which was followed by lysosomal and cellular injury. Based on the dosage differences in target organelles, SWCNTs were successfully used to deliver acetylcholine into brain for treatment of experimentally induced Alzheimer disease with a moderate safety range by precisely controlling the doses, ensuring that SWCNTs preferentially enter lysosomes, the target organelles, and not mitochondria, the target organelles for SWCNT cytotoxicity.

From the Clinical Editor: Single wall carbon nanotubes (SWCNT) could make excellent targeted delivery systems for pharmaceuticals. Inside the cells, lysosomes are the pharmacological target organelles of SWCNT, but in large doses mitochondria also take up SWCNT and mitochondrial toxicity becomes the reason for overall toxicity of this approach. In this paper, SWCNT were successfully used to deliver acetylcholine in Alzheimer’s disease brains with high safety range by controlling the doses to ensure lysosomal but not mitochondrial targeting.

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Key words: Single-walled carbon nanotubes; Lysosomes; Mitochondria; Organelles; Target treatment; Alzheimer disease

To successfully apply the new generation of nanomaterials as drug carriers in the treatment of diseases, it is essential to determine their pharmacological and toxicological profiles. Carbon nanotubes (CNTs) are nanoparticles with great promise in biomedicine as drug carriers, although their biosafety is of great concern. CNTs can interact with mammalian cells and enter cells via cytoplasmic translocation, they therefore can deliver a range of therapeutic reagents into the cell. For example, plasmid DNA has been internalized by the cell, and the expression of the plasmid-carried marker genes has been enhanced. Other macromolecules, including proteins, polymers, and single-stranded DNA have also been internalized by coating onto CNTs and through the interaction of CNTs with mammalian cells. Despite all of their proven potentials as drug carriers, CNTs have shown toxicity on cultured cells such as human keratinocytes, T lymphocytes, kidney cells, and alveolar macrophages as well as in animals. If inhaled into lungs, CNTs can be more toxic than carbon black or as toxic as quartz. The cytotoxicity of SWCNTs has been attributed to the cellular oxidative stresses induced by this material. However, other studies have found SWCNTs to have limited toxicity for endothelial cells in vitro.

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*Corresponding authors: 27 Taiping Road, Beijing 100850, China.
E-mail address: zhangyg@gmail.com (Y. Zhang).

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and to lead to no serious histopathological changes when implanted in the bodies of rats for 90 days.\textsuperscript{26} Obviously there is controversy about the toxic effects of CNTs, which must be resolved before CNTs can be acceptable for practical use as drug carriers. Unfortunately, the present data are far from sufficient to solve this controversy, although there have been some studies on the excretion of SWCNTs from the bodies of animals.\textsuperscript{27,28} The present study is designed to investigate the in vivo pharmacological and toxicological profiles of SWCNTs from basic mechanisms to experimental therapeutics, and to discover a method to use them as drug carriers.

**Methods**

*Single-walled carbon nanotubes*

SWCNTs were provided by Nachen Science and Technology Company (Beijing, China). A single tube has a diameter of 0.8–
1.2 nm and a length of several microns (Figure 1, A). Their Raman spectrum showed the chemical composition of 99.455% carbon, 0.439% oxygen, 0.106% silicon atoms, and no iron (Figure 1, C). To obtain a suspension for gastrogavage, SWCNTs were sheared in dimethyl formamide by a XHF-D hi-speed dispersator (Ningbo Scientific Biotechnology, Ningbo, China) at 20,000 rpm and were then centrifuged (1000 g, 10 minutes). The precipitate was suspended in normal saline by sonication²⁹ and allowed to stand for 10 minutes. The supernatant was then collected and recentrifuged (1000 g, 10 minutes), and shortened SWCNTs were obtained; these were dried, resuspended in normal saline by sonication, and incubated for 10–20 minutes before the gastrogavage. The shortened SWCNTs had a length of about 50–300 nm (Figure 1, B). Shearing and brief sonication did not change the chemical composition of SWCNTs, as shown by the similar Raman spectra before and after the shearing (Figure 1, D).²⁹ Samples were freshly prepared on the day of dosing. The composition of SWCNTs was determined by a RFS-100/S Raman spectrometer (Bruker, Fallenden, Switzerland) with excitation of 514 nm and power of 400 mW.

Handling of animals

Kunming mice obtained from the Animal Center of Beijing Academy of Medical Sciences were constantly monitored in the Rodent Health Monitoring Department of the center and fed with a fluid nutritional diet free of any pathogen, chemicals with potent to cause organelle diseases,³⁰ or particulate materials, a fluid nutritional diet free of any pathogen, chemicals with potent to cause organelle diseases,³⁰ or particulate materials. Rodent Health Monitoring Department of the center and fed with Academy of Medical Sciences were constantly monitored in the cycle for at least 1 week before being used in the study and were cared for and used humanely according to National Aeronautics and Space Administration (NASA) Animal Care and Use Program Guidelines. The use of the animals was approved by the Animal Subject Review Committee of the Beijing Academy of Medical Science.

SWCNT gastrogavage

A total of 120 Kunming strain male mice (20–22 g) were evenly and randomly divided into six groups, of which five were to receive gastrogavage of 5, 50, 100, 300, 400, and 500 mg/kg SWCNTs and one was to receive normal saline gastrogavage as control. To ensure that sufficient SWCNTs would enter into the body for ease of detection, these doses were given as a bolus once a day for 10 days. A 0.3-mL volume of freshly ultrasonicated SWCNTs suspension per 10 g body weight was gastrogavaged via a 24-gauge gastrogavage injector, and care was taken to avoid injury to the mouth, throat, and esophagus, or introduction of the suspension into the trachea. The entire operation was carried out under sterile conditions.

Transmission electron microscopy (TEM)

The intestinal tissues were sampled from two mice in each group at 30 minutes after gastrogavage every day for TEM observation. A 1-mm³ sample of internal organs was removed and immediately fixed in 3% glutaraldehyde, dehydrated in acetone, filtered, and embedded in epoxy resin. Ultrathin sections of 40 nm were made by a TY9334 ultramicrotome (Leica Microsystems, Weltzner, Germany) and stained in uranyl acetate according to the standard methods for the preparation of TEM samples. TEM was performed on a CM-120 microscope (Philips, Eindhoven, The Netherlands) at different accelerating voltages and magnifications, and images were obtained by a high-resolution charge-coupled device camera (AMT; NatureGene, Medford, New Jersey).

Collection and examination of SWCNTs in nucleus, lysosomes, and mitochondria of intestinal cells

Within 24 hours after the last gastrogavage, parts of the intestines were removed from two mice in each group, chopped into 2-mm³ pieces, and then homogenized with a XHF-D hi-speed dispersator (Ningbo Scientific Biotechnology). Nucleus, lysosomes, and mitochondria were isolated with corresponding Sigma isolation kits respectively, according to the method provided by Sigma (St Louis, Missouri) in the technical bulletin. The lysosomal and mitochondrial pellets were each weighed in a 1/100,000 balance and placed in a glass tube containing 10 mL 10 M nitric acid and digested at 90°C for 2 hours to remove the tissues and organic materials. The digested fluid was centrifuged, and the indigestible pellet was examined by Raman spectrometry (System RM2000; Renishaw, Hoffman Estates, Illinois) with 532-nm laser excitation. The SWCNTs collected from every mouse in a group were combined and scaled in a 1/100,000 balance. The SWCNT contents were expressed as the ratio between the weights of SWCNTs and those of corresponding organelles.

Enzymatic detection of the influences of mitochondria on lysosomal damage

The integrity of lysosomes was assessed by assaying released β-galactosidase.⁴⁹ One hundred milligrams of lysosomes (expressed as proteins) were incubated for 3 hours at 37°C in a solution of phospholipase A₂ (0.2 U mL⁻¹), 30 μM lysosomotropic detergent O-methyl-serine dodecylamide hydrochloride (MSDH), with 2.5 μg mL⁻¹ of either cathepsin B or cathepsin D (stock solutions of the cathepsins were in NaCl–inorganic phosphate pH 6.0, whereas MSDH and phospholipase A₂ were in NaCl–inorganic phosphate pH 7.4), and then centrifuged at 14,000 g for 10 minutes. The supernatants were removed, and 1 mL 0.1% Triton X-100 in distilled water (vol/vol) was added to lyse the remaining intact lysosomes. Activities of β-galactosidase in the ruptured lysosomal pellet and in the supernatant were respectively measured using Lysosomal β-Galactosidase Assay Kit (Beyotime, Haimen, China). The reaction was carried out in a 96-well plate. The reaction solution in each well contained 200 μL assay buffer, 10 μL lysosomal lysates, and 1.6 μg O-nitrophenyl-β-D-galactopyranoside, which were added into the well and incubated at 37°C for 20–60 minutes until a yellow color was observed. The optical density (OD) was measured at 420 nm in a UV-2800 ultraviolet-visible (UV-Vis) spectrometer (Unico, Shanghai, China) after 100 μL 1 M Na₂CO₃ had been added to stop the reaction. The β-galactosidase activity was calculated using the formula: β-gal (unit) = (OD 420 nm × 380 × 10)/
were determined in a 7020 automatic biochemistry analytical instrument (Hitachi, Tokyo, Japan). The data from six samples were statistically analyzed.

Mouse models of Alzheimer disease (AD)

Thirty Male Kunming strain mice (25–30 g, 9 weeks old) were trained for the learning and memory capability tests. Six were used as normal controls, which received normal saline injection. Twenty-four were used for drug treatment. The learning and memory capabilities were represented by the behavior merits of the trained mice in one trial avoidance test, shuttle test, and water maze test for 24 hours before the experiment. AD models were made by an intraperitoneal single-dose injection of 20 mg/kg kainic acid (KA). KA is a neurotoxic amino acid that can damage neurons to cause dementia and has been used as an agent in the preparation of animal models of AD. The learning and memory capabilities were retested 24 hours after KA injection. AD mice would have lower merits in comparison with themselves before KA injection as well as in comparison with the normal mice.

Therapeutic effects of SWCNT-acetylcholine on AD mice

SWCNTs were loaded with acetylcholine (ACh) by adding 100 mg SWCNTs into 10 mL of 1 M ACh solution. Then the mixture was placed in an ultrasonic field at 4°C for 30 minutes to allow the adsorption of ACh onto SWCNTs, followed by centrifuging at 3000 rpm, after which the pellet of ACh-loaded SWCNTs (SWCNT-ACh) was collected. The ACh loading of SWCNTs was confirmed by Raman spectrum in a RM2000 Raman spectrometer (Renishaw). The quantity of ACh adsorption was determined by measuring the ACh OD at 492 nm in the supernatant after adsorption with a UV-2800 UV-Vis spectrometer (Unico) and calculated by the formula: Q = (T_{ACh} - R_{ACh}) × V), where Q (mg/g) was the quantity of the adsorbed ACh on SWCNTs, T_{ACh} was the total quantity of ACh in 100 mL solution, R_{ACh} was the residual ACh at the end of the adsorption, and V was the total volume of the adsorption mixture. A Q of 200 mg ACh per gram SWCNTs could be obtained after the nonspecifically adsorbed ACh was washed off by centrifuging SWCNT-ACh three times in pH 7.4 phosphate buffer solution. The dependence of the release of ACh from SWCNT-ACh complex on pH was confirmed by a Q value of 5 mg ACh per gram SWCNTs when the washing was carried out in pH 4.5 phosphate buffer solution.

Coefficient of the safety range of SWCNTs in treating AD mice

One hundred-twenty AD male mice (25–30 g) were divided into two groups of 60 mice each for the calculation of the 99% effective dose (ED99) for SWCNTs in the treatment of AD, and for the toxic dose of SWCNTs expressed as the damage of 1% of the mitochondria (TD1). The safety range was expressed as the ratio of ED99 to TD1.

Measurement of mitochondrial membrane potential

Mitochondria (50 μg) were incubated with SWCNTs for 20 minutes in a respiratory buffer (0.07 M sucrose, 0.23 M mannitol, 30 mM Tris HCl, 4 mM MgCl2, 5 mM KH2PO4, 1 mM ethylenediamine tetraacetic acid, 0.5% bovine serum albumin, pH 7.4) in a spectrofluorophotometer cuvette at 37°C. The fluorescence was observed under a TCS-SP2 laser-scanning confocal microscope (Leica Microsystems) after addition of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazo-
zolyl-carbocyanide iodine (JC-1; Molecular Probes) in a final concentration of 3 μM. JC-1 is a unique cationic dye to signal the loss of mitochondrial membrane potential (MMP). The negative charge established by the intact MMP allows JC-1 to aggregate in the mitochondrial matrix and become fluorescent red. When MMP collapses the JC-1 cannot aggregate within the mitochondria, remaining in a green fluorescent monomeric form. To quantitatively measure the influences of SWCNTs on MMP, 50 μg mitochondrial membranes were added into 0.9 mL five times–diluted JC-1 staining working solution (50 μL JC-1 + 8 mL ultrapure water + 2 mL staining buffer) for each well of the six-well plate, mixed evenly, and incubated at 37°C for 20 minutes. The red and green fluorescence intensity were determined separately at 600 nm with an exciting wavelength of 550 nm and at 535 nm with an excitation of 485 nm in a FL500 fluorimeter (BioTek, Winooski, Vermont). MMPs were expressed as the ratio of red to green fluorescence intensity.

Blood biochemical measurements

Sera were collected by centrifuging the blood samples collected from the carotid artery for a series of biochemical tests. The activities of serum enzymes, total proteins, albumin, blood urea nitrogen, creatine, and creatine kinase in the serum were determined in a 7020 automatic biochemistry analytical instrument (Hitachi, Tokyo, Japan).
Statistical analysis

The results are reported as means ± standard deviation. Groups were analyzed by analysis of variance, and pairwise comparison was performed using the Tukey test with significance defined by a \( P \) value of less than 0.05.

Results

Confirmation of SWCNTs in tissues

On different days of SWCNT gastrogavage treatment with 50–300 nm SWCNTs, fiberlike structures (FLSs) were found in the absorption cells in the intestinal tissues (Figure 2, A) of the mice under TEM. No FLSs can be found in normal saline-gastrogavaged mice, excluding the possibility of non-SWCNT materials forming FLSs in the intestinal tissues and suggesting that the FLSs are SWCNTs from the intestinal cavity of SWCNT-gastrogavaged mice. Most of the FLSs had a diameter of 2–10 nm (Figure 2, D–G), with some distinguishable 1-nm individuals, and a length of 50–300 nm (Figure 2, C), which was identical with the diameters and the lengths of the SWCNTs in the gastrogavage suspension. There were good positive correlations between FLS incidence (FLS-I) and SWCNT doses (SWCNT-D), and between FLS-I and SWCNT gastrogavage times (SWCNT-GT) (Figure 2, H). FLS-I–SWCNT-GT curves shifted toward the upper left with an increase in SWCNT-D (Figure 2, H), further showing that FLSs are SWCNTs. The SWCNTs recollected from in vivo cells were also fiberlike, with diameters of 0.8–1.6 nm and lengths of 5–300 nm. The chemical examination demonstrated a Raman spectrum quite similar to the original ones (Figure 1, E), further indicating that the FLSs in in vivo cells are SWCNTs.

Tissue and cell distribution of SWCNTs absorbed via the gastrointestinal route

SWCNTs were detected only in the absorption cells of the mucous membrane (Figure 2, A, arrow a) and the macrophage cells (Figure 2, A, arrows b, c) in the submucous layer of intestinal wall, but not in any other cells. Absorption cells were confirmed by the microvilli in their cell margin (Figure 2, B) and their position in the absorptive mucous membranes (Figure 2, A, arrow a). The macrophages were identified by their multiple nuclei, rich in lysosomes and dense-stained chromatins along the inner margin of the nuclear membrane (Figure 2, D–G), and their position in the submucous-layer tissue space (Figure 2, A). SWCNTs in the absorption cells formed vesicles (Figure 2, B),

***\( P < 0.001 \) compared with jejunum. (J) Comparison of numbers of SWCNT-containing cells (SCC) in jejunum with those in ileum. ***\( P < 0.001 \) compared with jejunum.

Figure 2. Single-walled carbon nanotubes (SWCNTs) in live cells and the content differences in different intestinal segments examined by transmission electron microscopy. (A) Intestinal wall tissues. An absorption cell with SWCNTs in the intestinal mucous layer (arrow a); two macrophages with SWCNTs in the submucous layer (arrows b and c). (B) Magnification of the absorption cell indicated by arrow a in A. Ve, absorption vesicle; Vi, microvilli. (C) The double-layer membrane (arrow) enclosing the Ve. (D) Magnification of macrophage indicated by arrow b in A. Many SWCNTs can be seen (arrows). (E) Magnification of macrophage indicated by arrow c in A. Many SWCNTs can be seen (arrows). (F) SWCNT-containing lysosomes in one cell (SCLCs) from jejunum; (G) SCLCs from ileum. (H) The relationship between the ratio taken by the lysosomes containing SWCNTs (as FLSs) in the total number of the observed lysosomes and the times of gastrogavage. (I) Comparison of the average number of SWCNTs in one cell (ANOSC) from jejunum with that from ileum. ***\( P < 0.001 \) compared with jejunum. (J) Comparison of numbers of SWCNT-containing cells (SCC) in jejunum with those in ileum. ***\( P < 0.001 \) compared with jejunum.
the transportation organelles in the absorption cells. SWCNTs inside the vesicles aggregated into bundles, whereas some single SWCNT fibers were recognizable (Figure 2, C) and those in the macrophages aggregated into one or two bundles (Figure 2, F, G). The numbers of SWCNTs in the intestinal cells increased dose- and time-dependently (Figure 2, H). SWCNT contents in different segments of intestines were different. None were found in duodenum, some in jejunum (Figure 2, F, G), and most in ileum (Figure 2, D, E). Both the average number of SWCNTs in one cell (Figure 2, I) and the percentages of cells containing SWCNTs (Figure 2, J) showed that the distribution of SWCNTs in ileum was richest and that in jejunum secondary.

SWCNTs were also detected in other internal organs including liver, brain, and heart. Interestingly, SWCNTs were seen only in neurons (Figure 3, A, B) and neurites (Figure 3, E, F), but not in glial cells (Figure 3, C). In neurons, SWCNTs localized in lysosomes (Figure 3, A, B). In neurites SWCNTs manifested as rodlike in the sections parallel to neurites (Figure 3, D) and as dots in the cross sections of the neurites (Figure 3, E).

Subcellular distribution of SWCNTs

At doses of 5–300 mg/kg, SWCNTs were detected exclusively in lysosomes (Figures 2, D–G, 3, B, and 4, A), but at doses above 400 mg/kg SWCNTs began to appear in mitochondria (Figure 4, C). SWCNTs in lysosomes were parallel to the longitudinal axis of the organelles (Figure 4, A), but those in mitochondria had no determined orientation (Figure 4, C). There may have been one or two bundles of SWCNTs in one lysosome or mitochondrion. Figure 4, D quantitatively illustrates the relationships between doses and percentages of organelles containing SWCNTs in lysosomes and mitochondria, which revealed the lysosomotropic property of SWCNTs by two curve features: the first is the dose-dependent increase SWCNT-containing rate only in lysosomes at doses less than 300 mg/kg; the second is that the curve relating dose to the SWCNT-containing rate of lysosomes was considerably higher than that of mitochondria when doses exceeded 400 mg/kg.

The influences of autophagy inhibitor on subcellular distribution of SWCNTs

3-MA, an inhibitor of autophagosome formation, in an intraperitoneal injection of 20 mg/kg, decreased the number of SWCNTs in lysosomes significantly and increased that in mitochondria significantly (Figure 4, E). Similar results were obtained by intraperitoneal injection of 20 mg/kg small interfering RNA (siRNA) (the sequences of the regions targeted by the siRNA in the exon 8a of the gene encoding lysosomal-associated membrane protein 2 were 5′-GACTGCAGTGCA-GATGAAG-3′, 5′-CTGCAATCTGATTGATTA-3′, and 5′-TAAACACTGCTTGACCACC-3′, corresponding to bases 1198–1216, 1331–1359, and 1678–1700) that was specific to inhibit the function of chaperone-mediated autophagy (CMA) (Figure 4, E).

Under energy deprivation by subtracting energy-producing components from the fluid diet, the distribution of SWCNTs increased in lysosomes but decreased in mitochondria (Figure 4, E). These results revealed a dynamic equilibrium between the distribution in lysosomes and that in mitochondria.

The different damage effects of SWCNTs on lysosomes and mitochondria

At doses of 5–300 mg/kg, SWCNTs did not induce any significant damage, even to the SWCNT-containing lysosomes. At doses of 400–500 mg/kg they caused different degrees of damage to the mouse organelles. The damage was mainly localized in the lysosomes (Figure 5, A–E) and mitochondria.
(Figure 5, F–H). Damaged lysosomes dilated with a decrease in their contents (Figure 5, B–D), destruction of membranes (Figure 5, D), excretion or dissolution of contents (Figure 5, D), and cavity formation (Figure 5, E). The damage rate can reach more than 30% of the total numbers of SWCNT-containing lysosomes (Figure 5, I). Almost all of the SWCNT-containing mitochondria were injured (Figures 4, C, 5, F–H), so that the damage rate was nearly equal to the percentage containing SWCNTs (Figure 5, J). The injured mitochondria manifested swelling, decrease or disappearance of their cristae, and complete destruction (Figures 4, C and 5, F–H). At 400–500 mg/kg the proportion of mitochondria containing SWCNTs and the proportion showing damage may become higher than 30% (Figure 5, J). No damage effects were observed in other organelles.

The target organelles for SWCNT damage

SWCNTs did not induce ROS production in lysosomes (Figure 6, A) but did so in mitochondria (Figure 6, B), demonstrating that mitochondria are the direct-target organelles of SWCNT damage. In another experiment measuring the release of β-galactosidase, without the presence of mitochondria SWCNTs did not induce significant release of β-galactosidase from lysosomes, but the addition of mitochondria homogenate greatly increased the β-galactosidase activities by three to eight times dose-dependently (Figure 6, C), demonstrating that lysosomal damage was secondary to mitochondria damage. Confocal laser-scanning microscopy with JC-1 fluorescent dye demonstrated that SWCNTs significantly decreased MMP (Figure 6, D, E), which was followed by the increase of ROS production (Figure 6, F), demonstrating that ROS overproduction was caused by collapse of MMP. These results in combination revealed that mitochondria are the target organelles of SWCNT toxicity.

The target organelles of SWCNTs as drug carriers

Because SWCNTs can enter the brain (Figure 3, A–C), preferentially enter lysosomes (Figure 3, A, B), and are distributed specifically in neurons and neurites (Figure 3, D, E), they present great potential for use as drug carriers to deliver drugs inside the brain for the treatment of neurodegenerative diseases. The therapeutic effects of SWCNT-ACh on experiments with AD mice revealed that lysosomes are the target organelles of SWCNT toxicity.

ACh was successfully loaded onto SWCNTs, as verified by Raman spectrum (Figure 7, A). Using SWCNT-ACh, AD mice recovered their learning ability to the normal levels as indicated by the results of the step test (Figure 7, B), shuttle test (Figure 7, C), and Morris water maze test (Figure 7, D). In these experiments neither the free-ACh group nor the free-SWCNT alone group (Figure 7, B–D) achieved such effects. The effects of SWCNT-ACh on recovery of learning and
memory ability of AD mice showed good dose-effect relations in all three of the tests.

**Effects of SWCNTs on the overall physiology and health status of mice**

SWCNTs administered at doses under 300–500 mg/kg caused no significant changes in the activities of serum enzymes including glutamic-pyruvic transaminases, glutamic oxaloacetic transaminases, creatine kinase, and lactate dehydrogenase (Figure 8, A); neither did they have significant influences on the blood cell counts (Figure 8, B), total protein, or blood urea nitrogen (Figure 8, A). At the tested range of doses, SWCNTs also did not show any adverse effects on the overall growth status of mice, in that mice in all treatment groups had body weight increments similar to those of the control group during the 10-day experiment (Figure 8, C). These results reflected the insidiousness of SWCNT damage, because there were still no
manifestations of disease although considerable pathological changes had taken place in the ultrastructure of cells at doses of 400–500 mg/kg. The safe range for SWCNT dosing in treatment of AD mice

Because of the lack of symptoms or systematic signs of disease, it is difficult to determine a safe range for SWCNT dosage in the treatment of diseases in biomedicine and for the exposure limit in nanotoxicology. Here we propose to evaluate the safe range or the exposure limit by virtue of the pathological changes we observed in organelle ultrastructures.

Figure 6. Relationships between lysosomal damage and mitochondrial damage studied in vitro. (A) Effects of SWCNTs on the production of reactive oxygen species (ROS) in lysosomes. (B) Effects of SWCNTs on the production of ROS in mitochondria. NAC, N-acetylcysteine (10 μmol L⁻¹); Vit E, vitamin E (10 μmol L⁻¹). (C) The influence of mitochondria on the lysosomal damage by SWCNTs. Mitochondria were added at the indicated concentrations, and the activities of β-galactosidase were measured. Lysosomal damage was expressed as the activity of the β-galactosidase leaked from lysosomes. (D) Fluorescence images showing the influences of SWCNTs on the mitochondrial membrane potential (MMP). Green fluorescence increased and red fluorescence decreased with incubation time. (E) Changes in the red-to-green fluorescence ratios in the mitochondrial membranes over 25 minutes of incubation with SWCNTs. SWCNTs significantly decreased the ratio time-dependently. (F) The time courses for the changes of both MMP and mitochondrial ROS production. ***p < 0.001 compared with control. ##p < 0.001 compared with SWCNT.
Based on the experiments on the treatment of AD mice, the effective doses of SWCNT-ACh were determined to be in the range of 20–50 mg/kg, corresponding to the doses of ACh at 4–10 mg/kg. The ED$_{90}$ value of SWCNTs was determined to be 25 mg/kg, corresponding to that of ACh at 5 mg/kg. Based on ED$_{90}$ and the maximal safe dose of 300 mg/kg for mitochondria and lysosomes, the safe dose range for SWCNTs was proposed as 12 mg/kg in the treatment of AD in mice.

**Discussion**

The significance of gastrointestinal absorption of SWCNTs

The gastrointestinal tract is the most convenient avenue for drug administration. The distribution of SWCNTs in internal
SWCNTs demonstrated the importance of gastrointestinal exposure, which should be avoided if at all possible. Although some studies have dealt with the effect of exposure to modified CNTs, there have been no studies on gastrointestinal exposure to pristine SWCNTs. Because most environmental SWCNTs are pristine ones, our results are of more practical importance in comparison with results with the modified CNTs.

Mechanisms for gastrointestinal SWCNT absorption

There have been many studies reporting the lymphatic absorption of nanoparticles, but our studies have shown, to our knowledge for the first time, that SWCNTs can be absorbed through the columnar epithelial cells of intestines, for which the selective distribution of SWCNTs in transport vesicles of the intestinal columnar epithelial cells provides strong evidence. The lack of SWCNT distribution in other intestinal cells besides absorption cells and macrophages excluded the possibilities of other absorption mechanisms such as lymphatic absorption and the passive absorption resulting from the syringe effects of SWCNTs.

The lysosomotropic property of SWCNTs, its mechanism and meaning

Several studies have reported the distribution of SWCNTs in lysosomes, but few have addressed their distribution in mitochondria and the relations between organelles in SWCNT distribution. The present study demonstrated that lysosomes are the preferred organelles for SWCNT distribution. The mitochondria are organelles for SWCNT distribution only at high doses that are beyond the capacity of lysosomes. The lysosomotropic property of SWCNTs may arise because SWCNT-containing vesicles can enter lysosomes by simple membrane fusion. However, we found that the genes encoding phosphoinositide-3-kinase and lysosomal-associated membrane protein 2 were involved in the process of SWCNT entry, as demonstrated by the experiments with 3-MA and siRNA. More importantly, there is equilibrium between lysosomes and mitochondria in SWCNT distribution. Blockage of the autophagy of lysosomes can decrease the quantity of SWCNTs in lysosomes and increase that in mitochondria, and vice versa. These results mean that the quantity of SWCNTs in lysosomes and mitochondria can be controlled or regulated with the aid of autophagy regulators used deliberately according to the therapeutic need. This offers hope for the design of clinical protocols in the treatment of diseases and SWCNT overexposure.

Figure 8. Influences of SWCNTs on the blood biochemistry, blood cell counts, and body weight of mice. (A) Changes in enzyme activities and concentrations of metabolites in the serum. ALB, albumin; BUN, blood urea nitrogen; CK, creatine kinase; CRE, creatine; GOT, glutamic oxaloacetic transaminases; GPT, glutamic-pyruvic transaminases; LDH, lactate dehydrogenase; TP, total protein. (B) Influence of SWCNTs on the numbers of red blood cells, white blood cells, and platelets. (C) Influence of SWCNTs on the body weight of mice during the experiment.
The target organelles for SWCNTs as drug carriers and as toxic agents

We found that SWCNTs did not damage lysosomes, although lysosomes are their preferred organelles in distribution, meaning that lysosomes may be employed as the pharmacological target organelles of SWCNTs. Lysosomes, with an interior pH lower than that in cytoplasm, are favorable targets for drug delivery, especially for pH-sensitive target drug delivery systems, in which the drug release can be triggered by a mild acidic environment, such as that occurring in solid tumors and inflammatory tissues. \(^{45-47}\) SWCNT-ACh was such a pH-sensitive drug delivery system that successfully delivered ACh into brain with good curative effects on experimental AD, providing good evidence for lysosomes as the pharmacological target organelles of SWCNTs. The selective distribution of SWCNTs in the lysosomes in neurons and neurites is favorable for the release of ACh to develop transmitter function.

Many studies have reported the cytotoxicity of SWCNTs, \(^{13,20,23-27}\) but none of them investigated the relations between different organelles, and we still do not know which organelles are responsible for the initiation of SWCNT damage. On TEM examination it was noted that lysosomes were never damaged unless mitochondria were damaged also, implying that lysosomal damage was secondary to that of mitochondria. Experiments with isolated lysosomes and mitochondria found that SWCNTs failed to induce the production of ROS in isolated lysosomes but significantly induced overproduction of ROS in isolated mitochondria, indicating that SWCNTs can directly damage mitochondria but not lysosomes. Further experiments demonstrated that SWCNTs significantly induced the production of ROS in lysosomes when mitochondria homogenate was added into the isolated lysosomal experimental system. These results indicate that mitochondria are the original organelles for ultrastructural damage of the cells. These isolated organelle experiments in combination with the in vivo TEM examination revealed that mitochondria are the target organelles of SWCNT toxicity. The significance of the elucidation of the target organelles for toxicity and for pharmaceutical delivery is that we can direct SWCNTs (based on the target mechanisms) to selectively enter lysosomes but not mitochondria, so as to obtain the ideal therapeutic effects while avoiding the toxic effects.

Mechanism for the damaging effects of SWCNTs on mitochondria and lysosomes

There have been no available data on the mechanism by which SWCNTs injure mitochondria. Our experiments have answered this question primarily, although further study is required. SWCNTs induced the collapse of MMP, which was followed by the overproduction of ROS, indicating that the influence of SWCNTs on MMP is the cause for mitochondrial damage. Mitochondria are the power station of cells, where biological oxidation develops. ROS can be produced by electrons leaking from the electron transport chains and may be eliminated by the enzyme system in cells under normal conditions. \(^{48,49}\) SWCNTs have many \(\pi\) electrons in their macromolecules that interact with one or some of the oxidation-reduction pairs such as NAD/NADH and NADP/NADPH in the electron transport chain, blocking the electron transportation and increasing the leaking of electrons, leading to the collapse of MMP and further to the increment of ROS. \(^{50}\) ROS would damage mitochondria by peroxidation of lipids, proteins, and DNA. The damaged mitochondrial membranes allow ROS to diffuse out to attack lysosomes as well as the other cell structures. The damaged lysosomes release various digestive enzymes, finally leading to the cell’s destruction.

The insidiousness of SWCNT damage on animals

Interestingly, one of the features of SWCNT toxicity is their insidiousness, which may be the reason some experiments found toxic effects of SWCNTs whereas others did not. At certain dose levels they caused significant subcellular damage revealed as ultrastructural pathological changes but did not affect overall physiology and well-being of the animals, in that the animals showed no signs of clinical abnormality by symptom observation and growth assessment. Traditionally, drug toxicity in the clinic is largely evaluated by symptoms, histopathological observations, and blood panels including hematology. In animal experiments toxicity is often evaluated as the dosage for the death of half of the animals (LD\(_{50}\)), the symptoms, and the organ injuries. Obviously these indexes cannot accurately evaluate the toxicity of SWCNTs because of the insidiousness of their toxicity. Based on the unique pathological feature of SWCNTs, the safety range of some nanomaterials should be re-evaluated by the ultrastructural pathological changes at the subcellular level in preference to using traditional histopathological and symptomatic indexes.

The mechanism for the insidiousness of SWCNT damage may be related to SWCNTs’ giant molecules. Individual molecules of SWCNTs may be several hundreds or thousands times larger than the molecules of substances in general, so that they cannot distribute as evenly as do usual chemicals in tissues and cells. This uneven distribution leads to their presence in only a few organelles in cells or only a few cells in tissues, which results in high concentrations in single organelles or single cells, although the SWCNT-containing organelles and cells may account for only a very small proportion of the total organelles and cells. The high concentration in single organelles or cells causes considerable ultrastructural pathological changes to develop in single organelles and single cells, but is not enough to cause systematic symptoms. Although systematic symptoms are lacking, the effects of damage by SWCNTs on individual organelles and cells should be avoided, because such damage may be the cause of those diseases that develop from a single or a few cells, such as tumors. The relations between the damage of mitochondria and the tumor development are being established. \(^{51}\)

The use of SWCNTs as drug carriers in the treatment of AD

All nanomaterials may be toxic in a strict sense, but their toxicity does not necessarily militate against their application in nanomedicine. The key is to control the dosage. Our study showed that only at high dosages did SWCNTs cause the pathological changes in the ultrastructures of lysosomes and mitochondria; they are highly safe at low doses, providing the basis for their use as drug carriers. In the present study SWCNTs
were successfully used to deliver drugs for the treatment of experimental AD and achieved satisfactory results with the doses precisely controlled. These results exemplified the importance of understanding the therapeutic and toxicological profiles of nanomaterials for their application in modern biomedicine.

AD is a neurodegenerative disease caused by a decrease in neurotransmitter ACh of the cholinergic nervous system due to the inability of neurons to synthesize it. Administration of ACh into the brain can improve the dementia of patients, but free ACh cannot enter the brain because of its strong polarities and ease of decomposition in blood. Therefore, currently brain free ACh cannot enter the brain because of its strong polarities. ACh into the brain can improve the dementia of patients, but the central nervous system.

Results showed, to our knowledge for the first time, that lysosomes, the pharmacological target organelles, whereas few toxicological effects can be avoided by precisely maintaining the ability of nanomaterials to deliver drugs, it is important to compare their pharmacological and toxicological effects, to evaluate the structural changes in SWCNT, whereas SWCNT should be evaluated by the structural changes in SWCNTs are insidious, so we propose that the safe range of SWCNT toxicity. There demonstrated that SWCNT are excellent drug carriers but have insidious damage effects on cells by causing the collapse of mitochondria are the target organelles of SWCNT toxicity. There are differences in doses for SWCNTs to target these two kinds of organelles, which is the key for the safe use of SWCNT as drug carriers. As a toxicological feature, the damage effects of SWCNTs are insidious, so we propose that the safe range of SWCNT should be evaluated by the structural changes in organelles instead of by traditional parameters. To evaluate the ability of nanomaterials to deliver drugs, it is important to compare their pharmacological and toxicological effects, to determine whether their negative effects can be avoided by any method. SWCNTs can carry ACh into the lysosomes of the neurons and achieve excellent therapeutic effects, whereas their toxicological effects can be avoided by precisely maintaining the doses under 300 mg/kg, thus ensuring that SWCNTs enter only lysosomes, the pharmacological target organelles, whereas few or none enter mitochondria, the target organelles for toxicity. Our results showed, to our knowledge for the first time, that SWCNTs are good drug carriers in the treatment of diseases of the central nervous system.

References


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