Long-term accumulation and low toxicity of single-walled carbon nanotubes in intravenously exposed mice

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\textbf{Abstract}

The biomedical application of single-walled carbon nanotubes (SWCNTs), such as drug delivery and cancer treatment, requires a clear understanding of their fate and toxicological profile after intravenous administration. In this study, the long-term accumulation and toxicity of intravenously injected SWCNTs in the main organs (such as liver, lung and spleen) in mice were carefully studied. Although SWCNTs stayed in mice over 3 months, they showed low toxicity to mice. The long-term accumulation of SWCNTs in the main organs was evidenced by using Raman spectroscopy and TEM technique. Statistically significant changes in organ indices and serum biochemical parameters (LDH, ALT and AST) were observed. The histological observations demonstrate that slight inflammation and inflammatory cell infiltration occurred in lung, but the serum immunological indicators (CH 50 level and TNF-\textalpha level) remained unchanged. No apoptosis was induced in the main organs. The decreasing glutathione (GSH) level and increasing malondialdehyde (MDA) level suggest that the toxicity of SWCNTs might be due to the oxidative stress.

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\section{1. Introduction}

With the development of nanotechnology, numerous nanomaterials are created for various applications. Among these nanomaterials, single-walled carbon nanotube (SWCNT) is one of the most concerned, for its unique physicochemical properties and promises in technological applications (Endo et al., 2008). SWCNTs can be used in sensor, electronic device, wastewater treatment and many other industrial applications. Importantly, broad biomedical uses of SWCNTs, such as in drug delivery systems (Lacerda et al., 2006), bone cell growth (Saito et al., 2008) and cancer treatment (Gannon et al., 2007), have been investigated. With the rapid advances in SWCNT-based new materials and technologies, there is a growing recognition that a fundamental understanding of the toxicological properties of SWCNTs is imperative (Warheit, 2006).

However, the toxicity of SWCNTs is barely known when they are introduced into the blood circulation, which is especially vital for their biomedical applications. Previous studies have reported the cytotoxicity, pulmonary and skin toxicity of SWCNTs (Smart et al., 2006). Pristine SWCNTs have been proven to be cytotoxic, inducing the cell viability loss (Jia et al., 2005), oxidative damage (Pulskamp et al., 2007), inflammation (Brown et al., 2007) and apoptosis (Cui et al., 2005). The cytotoxicity depends on the aggregation degree and pretreatment of SWCNTs samples (Wick et al., 2007). It is also suggested that the cytotoxicity of pristine SWCNTs could be reduced via chemical functionalization (Sayes et al., 2006). In the pulmonary and skin toxicity studies, pristine SWCNTs also show considerable toxicity, including animal death, inflammation and other clinical signals (Smart et al., 2006). Only a very recent pilot study shows polyethylene glycol (PEG) modified SWCNTs are non-toxic after intravenous injection by using very limited animals (Schipper et al., 2008).

In fact, most of i.v. exposure studies just focus on the biodistribution and pharmacokinetics of SWCNTs (Lacerda et al., 2006). We have reported that the surfactant suspended SWCNTs accumulate in liver, lung, and spleen and retain for at least one month without any sign of degradation after mice are i.v. exposed (Yang et al., 2007). Except for lung, the clearance of SWCNTs in body is rather...
low, manifesting that the long-term fate and bio-effect of SWCNTs should be concerned. The experience of pulmonary toxicity studies, in which SWCNTs retain in lungs for 90 days and lead to the formation of granuloma (Lam et al., 2004; Warheit et al., 2004), also suggests that the study of long-term consequence is of prime importance.

Herein, we focus on the toxicity of SWCNTs to main organs, including liver, lung and spleen, to provide a general toxicological profile. Liver, lung and spleen are selected here mainly because of the high accumulation of SWCNTs in these organs (Yang et al., 2007). After i.v. administration, the long-term accumulation of SWCNTs was proved by Raman spectroscopy and transmission electron microscopy (TEM). Organ indices, serum biochemical parameters, histological observations, apoptosis, immunological indicators, and oxidative stress indicators were recorded to evaluate the toxicity.

2. Materials and methods

2.1. Synthesis, purification and characterization of SWCNTs

SWCNTs were synthesized by arc-discharge method and purified to a carbonaceous purity of 95% following the reported method with minor modifications (Wang et al., 2004). Briefly, graphite powder was mixed with FeS and Ni/Y at a mass ratio of 1:5:8.6. The obtained raw SWCNTs were refluxed in 15% H2O2 for 2 h, then in 6 mol/L HCl for 12 h and finally in 2.6 mol/L HNO3 for another 12 h. The resulting residues were heated under air at 400 °C for 1 h and annealed under N2 at 1000 °C for 2 h. The purified SWCNTs were characterized by TEM (JEM-200CX, Japan), Brunauer–Emmett–Teller (BET) technique (ASAP2010, Micromeritics, USA), thermogravimetric analysis (TGA) (SDT 2900, Thermal, USA), Raman spectroscopy (Renishaw micro-Raman instrument, England), inductively coupled plasma-mass spectrometry (ICP-MS) (Thermo Elemental X7, Thermo Electron Co., USA) and infrared spectroscopy (IR) (Magna-IR 750, Nicolet, USA). The purified SWCNTs were suspended in 1.0 wt% Tween® 80 aqueous solution by sonication for 30 min for the following animal exposure.

2.2. Animal administration and sampling

All animal experiments were performed in compliance with the institutional ethics committee regulations and guidelines on animal welfare (Animal Care and Use Program Guidelines of Peking University). Male CD-ICR mice (∼25 g) were obtained from Peking University Animal Centre, Beijing, China. They were housed in plastic cages (5 mice/cage) and kept on a 12 h light/dark cycle. Food and water were provided ad libitum. Following acclimation, mice were randomly divided into four groups (5 mice/group).

Fig. 1. A representative TEM image of the purified SWCNTs sample.

Fig. 2. Representative Raman spectra of SWCNTs suspended in Tween® 80 aqueous solution (a) and Raman spectra of tissue homogenates of liver (b), lung (c) and spleen (d) of both SWCNTs exposed mice at 90 days post-exposure and control mice.
Mice of each group were single i.v. (tail vein) injected with SWCNTs in 1.0 wt% Tween® 80 aqueous solution at a dose of 40 μg/mouse, 200 μg/mouse and 1.0 mg/mouse, respectively. For 1.0 mg/mouse group, each mouse was exposed to SWCNTs by three times of i.v. injection of 1.0 ml solution once every 4 h (330 μg SWCNTs/0.33 mL). Mice i.v. exposed to 1.0 wt% Tween® 80 aqueous solution were taken as the control group. Body weight and behaviors were recorded once per 3 days post-exposure.

At 90 days post-exposure, mice were sacrificed and blood/organ samples were collected for accumulation determination and toxicological assays. Serum samples were obtained from blood by centrifugation (3000 rpm for 10 min). Liver, lung and spleen were collected and weighed for organ indices (organ weight/body weight) calculation. A piece of each organ sample was cut off and fixed in 4% formaldehyde solution. All the rest were stored at −80°C before assays.

2.3. Raman spectra of SWCNTs in organ homogenates

To determine the accumulation of SWCNTs in mice. Raman spectra of homogenates of liver, lung and spleen were recorded. Organs of 1.0 mg/mouse group and the control group were homogenized in water (0.1 g tissue in 1.0 mL water) with homogenizers. The homogenate was dropped on glass slip and dried under infrared lamp. Raman spectra of these samples were recorded upon a Renishaw micro-Raman instrument (laser excitation wavelength 785 nm, 50 mW power, 50× objective, laser spot size ~2 μm × 2 μm, 10 s collection time). The Raman spectra of SWCNTs suspended in Tween® 80 were also recorded.

2.4. TEM investigation of SWCNTs in digested organs

For TEM analyses, liver and lung samples of 1.0 mg/mouse group were digested with 10 mL mixture of 65% HClO4 and 30% H2O2 (1:1 in volume) at 90°C for 30 min. After centrifugation (12,000 rpm for 15 min), the residue was collected and washed with deionized water twice and alcohol once. The final residue was dispersed in 20 mL nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) solution for 20 min. The resulting slides were washed twice with buffer B and stained with nuclear fast red solution. Finally the slides were dehydrated and observed under the light microscopy.

2.7. TNF-α assay

To determine the plasma tumor necrosis factor α (TNF-α) levels, enzyme linked immunosorbent assay (ELISA) was performed by using commercial kits that are selective for mouse TNF-α (BD Biosciences, USA). Manufacturer’s instruction was followed. The absorbance was measured on a microplate reader at 450 nm (Varioskan Flash, Thermo Electron, Finland) and the TNF-α concentration of samples was calculated from a standard curve.

2.8. Cell apoptosis assay

Cell apoptosis was evaluated by using terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling (TUNEL) staining on the organ sections. All the reagents used were purchased from Dingguo Biotechnology Co., Beijing, China and the instruction was followed. Briefly, the slides were predigested to get rid of paraffin. Then, the slides were digested in 20 μg/mL of proteinase K at 37°C for 10 min and rinsed in PBS. After that the slides were incubated in 0.3% H2O2 methanol solution and rinsed twice for 2 min in PBS. After adding 0.1% Triton X-100, the slides were incubated at −20°C for 10 min. The slides were washed twice with PBS and dried at room temperature. Then, 10μL terminal deoxynucleotidyl transferase (TDT) solution was placed on the slides, followed by incubation at 37°C for 2 h. The reaction was stopped by washing slides twice with buffer A (pH 9.0, containing 0.85% NaCl and 1.21% Tris). After blocking with 3% BSA solution for 30 min, the slides were incubated for 60 min with streptavidin–alkaline phosphatase conjugate. The slides were washed twice with buffer B (pH 9.0, containing 0.85% NaCl, 1.0165% MgCl2 and 1.21% Tris). And the slides were further developed with 40 μL nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (BCIP/NBT) solution for 20 min. The resulting slides were washed twice with buffer B and stained with nuclear fast red solution. Finally the slides were dehydrated and observed under the light microscopy.

2.9. Oxidative stress assay

For the assays of reduced GSH level and MDA level, each organ sample was minced and homogenized in 4°C water for three times (10 s/time, intermittent for 30 s) to yield 10% (w/v) homogenate. The homogenates were centrifuged at 2000 rpm for 10 min to obtain the supernatants. Protein concentrations in the supernatants were determined according to the method of Bradford, 1976. The reduced GSH level of the supernatant was examined by using spectrophotometric diagnostic kits (Nanjing Jiancheng Biotechnology Institute, China) based on the method of Jollow et al. (1974). Results of GSH were expressed as mg GSH/g protein. The lipid peroxidation indicator malondialdehyde (MDA) was determined using the standard histopathological techniques. The mounted sections were stained with hematoxylin–eosin (H&E) and examined by light microscopy.

Table 1

<table>
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<tr>
<th>Organ indices of the control mice and the SWCNTs exposed mice at 90 days post-i.v. exposure. Data represent mean ± S.D. (n = 5)</th>
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* p < 0.05 compared with control group.
Fig. 4. Serum biochemical parameters of the control mice and the SWCNTs exposed mice at 90 days post-i.v. exposure. Data represent mean ± S.D. (n = 5). *p < 0.05 compared with control group.

method of thiobarbituric acid reactive species (Nanjing Jiancheng Biotechnology Institute, China). The level of MDA was expressed as nmol MDA/mg protein, using 1,1,3,3-tetraethoxypropane (TEP) as the standard (Dahle et al., 1962). The measurements of GSH and MDA were performed following the manufacturer’s instruction.

2.10. Statistical analysis

All data are presented as the mean of five individual observations with the standard deviation. Significance has been calculated using Student’s t-test. Difference was considered significant if p < 0.05.

3. Results

3.1. Characterization of the purified SWCNTs

The purified SWCNTs were characterized by TEM, BET, TGA, ICP-MS and IR. A representative TEM image is shown in Fig. 1. The SWCNTs are generally pure, with limited carbon particles and catalyst. The SWCNTs form bundles with 10–30 nm in diameter and lengths of 2–3 μm in length in the suspension. The specific surface area (SSA) of SWCNTs is 267.8 m²/g according to the BET analysis. TGA analysis suggests that the carbonaceous purity of SWCNTs is higher than 95.4%. ICP-MS further confirms the high purity. The metal impurities are 0.4 wt% Fe, 3.0 wt% Ni and 1.3 wt% Y. The remaining metals can be mainly attributed to the encapsulation by carbon, which prevents the solubilization of the metals during the purification process and also makes them non-bioavailable (Liu et al., 2008a). An IR was recorded to show the surface properties of SWCNTs. Only a tiny peak is observed at around 1744 cm⁻¹, suggesting few carbonyls or carboxyls are on the surface of sidewall. There is no signal of carbon–hydrogen bond observed. The nearly intact surface of SWCNTs should be due to the final anneal process. The purified SWCNTs were finally suspended in 1.0 wt% Tween® 80 aqueous solution for animal exposure.

3.2. Raman spectra of SWCNTs in organ homogenates

To determine the long-term accumulation of SWCNTs in main organs (liver, lung and spleen), Raman spectra of the SWCNTs Tween® 80 suspension and the homogenized tissue samples were recorded. Fig. 2a shows the typical G-band of SWCNTs at around

Fig. 5. Histopathological observation of the control mice and the SWCNTs exposed mice at 90 days post-i.v. exposure (400×). White arrows point to SWCNT aggregates.
anorexia, vomiting, or diarrhea, during the 90 days post-exposure. 3.4. Clinical symptoms and organ indices as well as the subsequent chemical digestion process. stable against the biotransformation during the 90 days exposure otube structures observed in TEM images indicate that SWCNTs are identified in the digested liver and lung samples. The intact nan- et al., 2004; Warheit et al., 2004). of SWCNTs, in which SWCNTs also retain in lung over 90 days (Lam consistent with the results of pulmonary intratracheal instillation light microscopic images). The long-term accumulation in lung is at 90 days post-exposure (a lot of black spots formed in lung in the remarkable amount of SWCNTs are still accumulated in lung even exposed group, although rather weak, G-band in liver and spleen band was observed in all control tissue samples (Fig. 2b–d). In the exposed group, although rather weak, G-band in liver and spleen is observed, suggesting there are still a few of SWCNTs at 90 days post-exposure. But in lung, the Raman intensity is still pretty high, which refers to the high concentration of SWCNTs in lung. Although a remarkable clearance in lung was observed previously during the first 28 days post-exposure, the Raman spectra demonstrate that a remarkable amount of SWCNTs are still accumulated in lung even at 90 days post-exposure (a lot of black spots formed in lung in the light microscopic images). The long-term accumulation in lung is consistent with the results of pulmonary intratracheal instillation of SWCNTs, in which SWCNTs also retain in lung over 90 days (Lam et al., 2004; Warheit et al., 2004). 3.3. TEM images of SWCNTs in digested organs The high accumulation level in liver and lung allow the direct electron microscopic imaging of residue from the digested tissues. Fig. 3 shows the characteristic structures of SWCNTs could be clearly identified in the digested liver and lung samples. The intact nanotube structures observed in TEM images indicate that SWCNTs are stable against the biotransformation during the 90 days exposure as well as the subsequent chemical digestion process. 3.4. Clinical symptoms and organ indices All mice showed no symptoms of abnormality, such as lethargy, anorexia, vomiting, or diarrhea, during the 90 days post-exposure. The body weight increase is similar among the four groups, except 1.0 mg/mouse group, in which the body weight increase is a little lower than that of the control group at initial 3 days (data not shown). The organ indices were recorded to provide a general impression of the toxicity. As shown in Table 1, no significant difference is observed in the liver indices. The lung index of 1.0 mg/mouse group is significantly higher than that of the control group, indicating the potential pulmonary injury. The spleen indices of 40 μg/mouse group and 200 μg/mouse group increase, too. This suggests that SWCNTs may induce the immunological response. 3.5. Serum biochemical parameters The serum biochemical parameters were assayed to further evaluate the toxicity of SWCNTs. Fig. 4 shows that TBIL, TBA and ALP levels of all groups are similar after mice were exposed to SWCNTs. But ALT and AST levels of 200 μg/mouse group and 1.0 mg/mouse group are higher than those of the control group. The increase of ALT and AST, which are important indicators of the hepatic injury, demonstrates that SWCNTs induced hepatic injury still remains even at 90 days post-exposure. Furthermore, the increase of ALT and AST is dose-dependent, inferring that the induced hepatic injury is dose-dependent. The LDH levels of all exposed group are nearly two folds of those of the control group. LDH is a cytoplasmic enzyme and is often used as an indicator of alveolar macrophage (AM) injury in the pulmonary toxicity study. LDH is also a general indicator of hepatic injury. The high level of LDH activity manifests that serious pulmonary or hepatic injury is aroused by SWCNTs. 3.6. Histopathological observations The histopathological observations were carried out to determine the organic damage induced by SWCNTs (Fig. 5). The black or brown aggregates observed in liver, lung and spleen sections are SWCNTs, which is consistent with the Raman spectra measurements. No obvious hepatic damage is observed in the histopathological study among all the exposed groups, though serum biochemistry indicates a considerable hepatic toxicity. There is no histopathological change in spleen either. The SWCNTs are generally trapped in capillary and aggregate to different sizes in lung. Some inflammatory cells are observed surrounding the trapped SWCNTs in lung. When the dose increases to 1.0 mg/mouse, the inflammation becomes more serious and inflammatory cell infiltration is observed. This is much different with that of intratracheally instilled SWCNTs, which induce granulomas within 90 days post-exposure (Lam et al., 2004; Warheit et al., 2004). 3.7. Serum immunological indicators The inflammation happened in lung according to the histopathological observations. To evaluate the immune response, CH 50 level and TNF-α level in serum were measured. The CH 50 level is a typical indicator of complement activation. Complement activation could regulate the phagocytosis ability and the immunological activity. As shown in Fig. 6a, the CH 50 level is quite similar among all groups. Although SWCNTs were reported to activate the complement system via the classical pathway (Salvador-Morales et al., 2006), in this study the complement level may increase and then decrease to the normal level within 90 days exposure. Besides being an important indicator of immunological activity, TNF-α level is also an indicator of inflammation and fibrosis. Like-wise, SWCNTs do not induce the change of TNF-α level (Fig. 6b), which is coincident with that no fibrosis is observed in the exposed mice. The slight inflammation is unable to induce an increase of TNF-α level.
3.8. Cell apoptosis

Apoptosis is regarded as a general adaptive response when cells are exposed to SWCNTs in vitro. Here, the apoptosis of the cells in organ sections was assayed by TUNEL method. As shown in Fig. 7, white arrows point to the accumulated SWCNTs in the main organs. The TUNEL assay at 90 days post-exposure, indicating little apoptosis occurred. But the apoptosis levels are similar among all exposed and control groups. Similar to the histopathological observations, black or brown aggregates of SWCNTs are observed in the exposed groups. Interestingly, the dye molecules are adsorbed onto SWCNTs slightly during the staining process, resulting in the blue-black SWCNTs. This adsorption should be due to the stacking interaction between SWCNTs and the tetrazolium structure of the dye molecules.

3.9. Oxidative stress

The oxidative stress aroused by SWCNTs in main organs was measured to reveal the possible toxicological pathway. As shown in Fig. 8, the reduced GSH level in liver and lung of all exposed groups decreases statistically, and 1.0 mg/mouse group shows the most serious oxidative damage. Contrast to the GSH results, MDA level of liver and lung in 1.0 mg/mouse group shows a statistical increase, indicating that SWCNTs induce oxidative damage. The reduced GSH level and MDA level in spleen remain unchanged in all groups. Namely, there is no observed oxidative damage to spleen.

4. Discussion

In this study, we find that pristine SWCNTs retain in mice for 3 months after i.v. exposure and induce low toxicity. The long-term accumulation seems to be a bio-property of most SWCNTs. Except for DTPA-SWCNTs and hydroxylated SWCNTs (Singh et al., 2006; Wang et al., 2004), other SWCNTs retain in body for more than one month (Yang et al., 2007, 2008; Liu et al., 2008b; Schipper et al., 2008). All the available data imply that the accumulation of SWCNTs depends on the functional groups and the functionalization degree. Lacerda et al. also reported that functionalized multi-walled carbon nanotubes (MWCNTs) possessed a much lower accumulation than that of pristine MWCNTs and that was attributed to the high individualization of functionalized MWCNTs (Lacerda et al., 2008b). The long-term accumulation of SWCNTs suggests that the study of the corresponding long-term toxicity is valuable and vital. The accumulation also demands the proper chemical functionalization of SWCNTs to achieve a lower accumulation in reticuloendothelial system (RES) for the sake of the drug delivery and related biomedical applications.

Compared with PEGylated SWCNTs, pristine SWCNTs show higher toxicity in the serum biochemical parameters assessments. The ALT and AST levels of mice exposed to pristine SWCNTs showed a dose-dependent increase, while those of mice exposed to PEGylated SWCNTs remained unchanged (Schipper et al., 2008). Since the toxicity of SWCNTs is dependent on their functionalization degree (Sayes et al., 2006), such hepatic toxicity might be reduced when proper chemical functionalization is adopted to obtain a high...
functionalization degree of SWCNTs with a higher in vivo stability. In our previous study of taurine functionalized MWCNTs, we did not find the increase of the ALT and AST levels and the hepatic toxicity (Deng et al., 2007). Lacerda et al. also found that serum did not find the increase of the ALT and AST levels and the hepatic toxicity. In our previous study of taurine functionalized MWCNTs, we exposed cells to SWCNTs (Lewinski et al., 2008). Although the oxidative stress of the control mice and the SWCNT exposed mice at 90 days post-i.v. exposure. (a) GSH level of main organs. (b) MDA level of main organs. *p < 0.05 compared with control group. Data represent mean ± S.D. (n = 5).

The low toxicity of intravenously exposed SWCNTs might be due to the active protecting mechanism (restriction of the SWCNTs by certain cells). RES uptake of nanoparticles post-i.v. injection is the main adaptive mechanism here. The opsonin regulated RES capture avoids the interaction of SWCNTs with parenchyma cells and restricts the toxicity to certain cells, such as Kupffer cells (KC) and AM (Owens and Peppas, 2006). SWCNTs could be secreted by AM and leave the lung via mucus through mucociliary transport (Yang et al., 2007). SWCNTs trapped in KC could be excreted via bile, though the process is very slow (Liu et al., 2008b). As for intratracheally instilled SWCNTs, they interacted with all cells they encountered, so a more serious injury was induced (Lam et al., 2004; Warheit et al., 2004). When SWCNTs were injected to peritoneal cavity of mice, inflammation and the formation of granuloma occurred (Poland et al., 2008).

According to our results, the dominant toxicological mechanism of the intravenously exposed SWCNTs would be the oxidative stress. Oxidative stress is a broadly existent phenomenon when cells are exposed to SWCNTs (Lewinski et al., 2008). Although the cytotoxicity of SWCNTs is not always observed in the cell culture studies, oxidative stress is regarded as the cause of the cytotoxicity (Pulskamp et al., 2007). In fact, oxidative stress is taken as an important pathway of toxicity of SWCNTs and other nanomaterials (Stern and McNeil, 2008).

It is very hard to eliminate the metal residues from SWCNT samples, even though most of the metals can be removed by refluxing SWCNTs sample in H2O2 and acid. Traditional purification is helpful to remove the majority of metals loaded on the outside and internal surface of tubes, but this process is in vain for metals packed inside the surrounding carbon fragments. A few studies reported that metal contents in SWCNTs were partly responsible for the serious oxidative stress (Guo et al., 2007; Liu et al., 2007, 2008a; Pulskamp et al., 2007). But a very recent study by Liu et al. focused on the bioavailability of metals in SWCNTs suggest that the encapsulated metals are non-bioavailable for at least two months (Liu et al., 2008a). Our purification procedure is similar (even stricter) to Liu et al.’s study (Liu et al., 2008a). The metal impurities left in our SWCNT samples should be inside SWCNTs or encapsulated in carbon fragments, therefore they are hardly attributed to the toxicity of SWCNTs. In other words, the oxidative stress comes from SWCNTs per se.

In summary, the long-term accumulation and low toxicity in mice i.v. exposed to SWCNTs are reported. Only serum biochemical changes and pulmonary inflammation were observed. No obvious cell apoptosis or changes of immunological indicators were induced. The proposed main toxicological mechanism is oxidative stress aroused in liver and lungs. The low toxicity of pristine SWCNTs implicates that SWCNTs could be used as the safe materials for biomedical applications. It also suggests that further chemical functionalization should be taken to improve the dispersion and excretion of SWCNTs.

**Conflict of interest**

The authors declare that there are no conflicts of interest.

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