Journal of Toxicology and Environmental Health, Part A: Current Issues
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/uteh20

Potential Toxicity of Differential Functionalized Multiwalled Carbon Nanotubes (MWCNT) in Human Cell Line (BEAS2B) and Caenorhabditis elegans

Nivedita Chatterjee[a], JiSu Yang[a], Hyun-Mi Kim[b], Eunhye Jo[b], Phil-Je Kim[b], Kyunghee Choi[b] & Jinhee Choi[a]

[a] School of Environmental Engineering, Graduate School of Energy and Environmental System Engineering, University of Seoul, Seoul, Korea

[b] Risk Assessment Divisions, National Institute of Environmental Research, Incheon, Korea

Published online: 24 Oct 2014.

To cite this article: Nivedita Chatterjee, JiSu Yang, Hyun-Mi Kim, Eunhye Jo, Phil-Je Kim, Kyunghee Choi & Jinhee Choi (2014) Potential Toxicity of Differential Functionalized Multiwalled Carbon Nanotubes (MWCNT) in Human Cell Line (BEAS2B) and Caenorhabditis elegans, Journal of Toxicology and Environmental Health, Part A: Current Issues, 77:22-24, 1399-1408, DOI: 10.1080/15287394.2014.951756

To link to this article: http://dx.doi.org/10.1080/15287394.2014.951756

Journal of Toxicology and Environmental Health, Part A, 77:1399–1408, 2014

ISSN: 1528-7394 print / 1087-2620 online
DOI: 10.1080/15287394.2014.951756

Taylor & Francis
Taylor & Francis Group

POTENTIAL TOXICITY OF DIFFERENTIAL FUNCTIONALIZED MULTIWALLED CARBON NANOTUBES (MWCNT) IN HUMAN CELL LINE (BEAS2B) AND *Caenorhabditis elegans*

Nivedita Chatterjee[1], JiSu Yang[1], Hyun-Mi Kim[2], Eunhye Jo[2], Phil-Je Kim[2], Kyunghee Choi[2], Jinhee Choi[1]

[1]School of Environmental Engineering, Graduate School of Energy and Environmental System Engineering, University of Seoul, Seoul, Korea
[2]Risk Assessment Divisions, National Institute of Environmental Research, Incheon, Korea

The aim of this study was to evaluate in vitro (human bronchial epithelial cells, BEAS2B cells) and in vivo (the nematode *Caenorhabditis elegans, C. elegans*) toxicity outcomes following exposure to pristine as well as surface-functionalized multiwalled carbon nanotubes (MWCNT) following hydroxylation-oxygenation (O^+), amination (NH_2), or carboxylation (COOH) of the carbon nanotubes (CNT). Cell viability and proliferation were measured by Ez-Cytox, trypan blue exclusion, and colony formation assays. The genotoxic potential of the MWCNT was determined by using the alkaline comet assay. In addition, survival and reproduction were used as endpoints for detection of toxicity of MWCNT in *C. elegans*. The carboxylated (COOH)-MWCNT was found most toxic as evidenced by cytotoxic and genotoxic among all tested compounds. The order of sensitivity was COOH > O^+ > NH_2 > pristine. There were almost no marked changes in survival following exposure of *C. elegans* to MWCNT. It is of interest that only pristine MWCNT exerted significant reduction in reproductive capacity of *C. elegans*. Surface functionalization significantly influenced the bioactivity of MWCNT, which displayed species as well as target-organ specificity. The mechanisms underlying these specific modes of nano-biological interactions need to be elucidated.

Carbon nanotubes (CNT) are engineered graphene tubes produced as either as single-walled carbon nanotubes (SWCNT) or multiwalled carbon nanotubes (MWCNT). Structurally, MWCNT consist of concentric layers of graphene sheets rolled up, where smaller diameter tubes are encased into larger diameter ones. The unique properties of MWCNT, such as small size, light weight, large surface area, stability, rigidity, extraordinary tensile strength, and efficient heat conduction, have been applied in supercapacitors, batteries, and structural materials in automotive and aerospace, and in electronics industries (Pacurari et al., 2012; Guo et al., 2012). In addition, CNT, particularly MWCNT, have shown great potential in biomedical and pharmaceutical applications, including protein and peptide transport, drug and gene delivery, biosensors, medical imaging, diagnosis, and targeted therapeutics (Pacurari et al., 2010; Zhao and Castranova, 2011; Jiang et al., 2013; Di Sotto et al., 2009). However, low solubility and dispersibility in both organic and inorganic solutions limited the applications of pristine MWCNT. Functionalization with covalent and noncovalent approaches of MWCNT is an effective way to overcome this difficulty, making them easier to process for commercial applications (Li et al., 2013; Yu et al., 2013).

The anticipated enormous growth in production and use of CNT, including MWCNT, has raised concern regarding exposure, health, and safety issues. Therefore, comprehensive

Address correspondence to Jinhee Choi, School of Environmental Engineering, Graduate School of Energy and Environmental System Engineering, University of Seoul, 163 Siripdaero, Dongdaemun-gu, Seoul 130-743, Korea. E-mail: jinhchoi@uos.ac.kr
Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/uteh

toxicity profiling of MWCNT is a critical factor in determining utility and medical use (Snyder-Talkington et al., 2012). Similar to other nanomaterials, the extent of biological reactivity and toxicity of CNT depends on numerous physicochemical characteristics, including structure (single- or multiwalled), length and aspect ratios, diameter, surface area, tendency to agglomerate, extent of oxidation, bound functional group(s), method of manufacturing, concentration, and types of model systems used to evaluate toxicity (Bussy et al., 2012; Kayat et al., 2011; Snyder-Talkington et al., 2012). Several reports related to CNT (particularly MWCNT) toxicity at the molecular, cellular, and whole-animal levels were published (Clark et al., 2012; Tabet et al., 2009; Kim et al., 2010a; 2010b; Liang et al., 2010), but data obtained and information are still controversial (Liu et al., 2013; 2014; Jiang et al., 2013; Li et al., 2013; Coccini et al., 2010; Cavallo et al., 2012).

The aim of the present study was to assess the potential toxicological effects of functionalized MWCNT in vitro using human bronchial epithelial cells and in vivo employing the nematode *Caenorhabditis elegans* (*C. elegans*). On the basis of a functionalization paradigm and the hypothesis that type of functionalization is an important parameter to consider when evaluating cytotoxic effects of MWCNT, a comprehensive in vitro–in vivo study was designed that aimed to compare the biological effects, on BEAS2B cells and *C. elegans*, with four types of MWCNT that differed in their surface functionalization (pristine, hydroxylated-oxygenation O^+, carboxylated-COOH, and aminated-NH_2) with similar length and diameter.

MATERIALS AND METHODS

Multiwalled Carbon Nanotubes (MWCNT)

The powdered MWCNT of similar size and tube length but with different surface functionalization (pristine, $-O^+$, $-NH_2$, and $-COOH$) were purchased from Cheap Tubes, Inc. (Brattleboro, VT) and prepared as stock in distilled water at 1000 mg/L with sonication.

Cell Culture and MWCNTs Treatment

BEAS2B cells (human bronchial epithelial cells) were cultured in DMEM/F12 (GIBCO), supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics, at 37°C in a 5% CO_2 atmosphere. The MWCNT was freshly prepared in cell culture medium (DMEM/F12) at the desired concentrations with appropriate amount from the stock (1000 mg/L in distilled water) and sonicated for 15 min prior to exposure.

Cytotoxicity and Cell Viability Assessment

Cytotoxicity of each MWCNT was determined by EZ-Cytox cell viability assay kit (Daeil Lab Service, Korea) based on the cleavage of the tetrazolium salt to water-soluble formazan by succinate-tetrazolium reductase as described previously (Chatterjee et al., 2013). Approximately 5×10^3 cells/well were seeded in 96-well plates 24 h prior to treatment and exposed to a range of concentrations (from 5 to 200 mg/L) of 4 different MWCNT for the next 24 h. In addition, cells (in 96-well plates) were exposed to all MWCNT (20 mg/L) for different time periods from 4 to 72 h. After completion of exposure time, 10 µl of EZ-Cytox reagent was added to each well, including treated and control (without MWCNT). Absorbance (OD450) was read at 450 nm after 2 h of incubation at 37°C. Appropriate blanks were used for each concentration to validate the absorbance.

In addition, cell viabilities due to MWCNT exposure at concentration from 25 to 200 mg/L were measured using the standard trypan blue (Invitrogene, USA) exclusion method, and total numbers of stained and unstained cells were counted using a hemocytometer. Three separate experiments were performed for all concentrations in triplicate, and effective concentration (EC) values including EC_{10}, EC_{20}, EC_{50},

and EC_{90} were calculated by using 4 parametric logistic equations.

Colony Formation and Morphology Changes

The colony formation assays were carried out as described by Herzog et al. (2007). Exponentially growing cells were harvested and seeded in 6-well culture plates at a density of 250 cells/well. Each well contained 2 ml cell culture medium. Cells were allowed to attach for approximately 24 h. Cells were then washed with 2 ml of phosphate-buffered saline (PBS) and treated with 2 ml nanoparticles prepared in cell culture medium to final concentrations of 20 mg/L. Cells were exposed to nanoparticles over the time period needed to form colonies, with a colony being defined as at least 50 clones of one cell and incubated for 10 d. Before colonies were counted, particle solutions were removed, and cells were washed with PBS and finally fixed and stained using a 0.1% Giemsa stain. Colony shape and cell morphology were detected under microscopy.

Comet Assay

BEAS2B cells (5×10^4 cells/ml) were seeded in 6-well plates 24 h prior to treatment and subsequently treated with MWCNT at 20 mg/L for the next 24 h. After treatments, alkaline (pH > 13) comet assay was performed according to the method of Singh et al. (1988). Briefly, after exposure cells were trypsinized and centrifuged at 1200 × g for 3 min. Approximately 3×10^4 cells were resuspended in 0.5% low-melting-point agarose (LMPA, Bio-Rad Laboratories, Hercules) at a 1:2 ratio. The resuspended cells in agarose were placed onto slides pre-coated with 1% normal-melting agarose. After solidification, slides were immersed in cold lysing solution (2.5 *M* NaCl, 100 m*M* ethylenediamine tetraacetic acid [EDTA], 10 m*M* Tris-base, 1% *N*-laurolsarcosinate, 1% Triton X-100) for 1.5 h at 4°C, after which they were transferred to an electrophoresis tank containing freshly made electrophoresis buffer (1 m*M* EDTA, 300 m*M* NaOH, pH > 13) and kept for 20 min at room temperature to allow DNA unwinding. Electrophoresis was performed in the same buffer at room temperature for 20 min at 25 V and 300 mA. The slides were then neutralized thrice with 0.4 *M* Tris-chloride buffer (pH 7.5), air-dried, and fixed in 70% ethanol. The slides were stained with 50 μl ethidium bromide (5 μg/ml) and analyzed using a fluorescence microscope (Leica DM IL) at 40× magnification. Approximately, 50 cells per slide (3 slides per treatment) were examined. DNA damage was expressed as the tail extent moment using an image analysis by the Komet 5.5 software (Kinetic Imaging Ltd).

Maintenance of *C. elegans* and Treatment

Caenorhabditis elegans were grown in petri dishes on nematode growth medium (NGM) and fed with *Escherichia coli* (OP50) according to standard protocol (Brenner, 1974). Worms were incubated at 20°C and young adults (3 d old) from an age-synchronized culture were used in all the experiments. Wild-type (N2) *C. elegans* was exposed to MWCNT at concentrations of 100 mg/L for 24-h survival assay and 50 mg/L of MWCNT for 72-h reproduction assays in K-medium.

Survival (24-h Assay) and Reproduction (72-h Assay) of *C. elegans*

The survival of the wild-type worms was determined after 24 h of exposure to 100 mg/L MWCNT as described previously (Roh et al., 2009). Briefly, survival was measured by counting the number of live and dead individuals under a dissecting microscope while probing the worms with a platinum wire. Five replicates were conducted for survival assays.

The effects of MWCNT on reproduction of wild-type worms were investigated as described by Roh et al. (2009). After a young adult was exposed to MWCNT at 50 mg/L for 72 h, the offspring at all stages beyond the egg were counted. Five replicates were conducted for reproduction assays.

Statistical Analysis

Statistical significance of differences between groups was determined using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test. The criterion for significance was set at $p < .05$. All statistical analyses were carried out using SPSS 12.0KO (SPSS, Inc.), and graphs were prepared in SigmaPlot (Version 12.0).

RESULTS

MWCNT Characterization

The details of size, tube length, diameter, functionalization, and methods of preparations supplied by the manufacturer are summarized in Table 1. Pristine and three other functionalized (carboxylated, −COOH, aminated $-NH_2$, and hydroylated/oxygenated O^+) versions with similar tube length and diameter were used.

Cell Viability and Cytotoxicity

The Ez-Cytox and trypan blue exclusion assays demonstrated a significant decrease in cell viability of BEAS2B cells in a concentration-dependent (Figures 1A and 1B) as well as time-dependent (Figure 1C) manner due to MWCNT exposure. The carboxylated (-COOH) functionalized MWCNT was found to be the most toxic one among the four types of tested MWCNT. The order of sensitivity of BEAS2B cells toward MWCNT was found to be COOH > O^+ > NH_2 > pristine. The O^+ and NH_2 functionalization showed similar cytotoxic effects on BEAS2B cells. The calculated 24-h effective concentrations (EC_{10}, EC_{20}, EC_{50}, and EC_{90}) of all MWCNT to BEAS2B cell are summarized in Table 2.

Colony Formation and Morphology Changes

Significant decreases in colony numbers were evident after 10 d of exposure to MWCNT (Figure 2A) with respect to controls (without MWCNT),but the differences between effects of the functionalization were not as significant as with Ez-Cytox acute cytotoxicity assays. All three functionalized and pristine MWCNT showed a similar number of colonies with the lowest number in the −COOH functionalized group. The order of colony formation decrease of MWCNT was found as pristine > O^+ > NH_2 > COOH. Although colony size was not determined quantitatively, smaller colonies were found when cells were exposed to COOH-MWCNT than to other compounds (Figure 2B). In addition, a significant deformation in morphological characteristics was observed in −COOH functionalized MWCNT-exposed BEAS2B cells (Figure 2B).

Genotoxic Potential of MWCNT

In agreement with cytotoxicity results, −COOH functionalized MWCNT displayed

TABLE 1. Characterization of MWCNT (as Supplied by the Manufacturer) by Name of MWCNT and Catalogue Number

Parameter	NH_2 Functionalized (Sku-030114)	Pristine (Sku-030111)	O^+ functionalized (Sku-030112)	COOH functionalized (Sku-030113)
Outer diameter	13–18 nm	13–18 nm	13–18 nm	13–18 nm
Inside diameter	4 nm	4 nm	4 nm	4 nm
Length	1–15 μm	1–15 μm	1–15 μm	1–15 μm
Purity	>99%	>99%	>99%	>99%
Impurity	0 %	0 %	0 %	0 %
Plasma process gas	Nitrogen	Argon	Oxygen	Proprietary oxygen blend
Primary functionality	N=H	Not functionalized	COH	COOH
Other functionalities	N-H, O=C-N-H2, C≡N	Atmospheric gas	C=O, COOH, other oxygen	COH, C=O, other oxygen groups
Source material	CCVD[a] MWCNT			
Process	Split plasma			

[a]CCVD: combustion chemical vapor deposition.

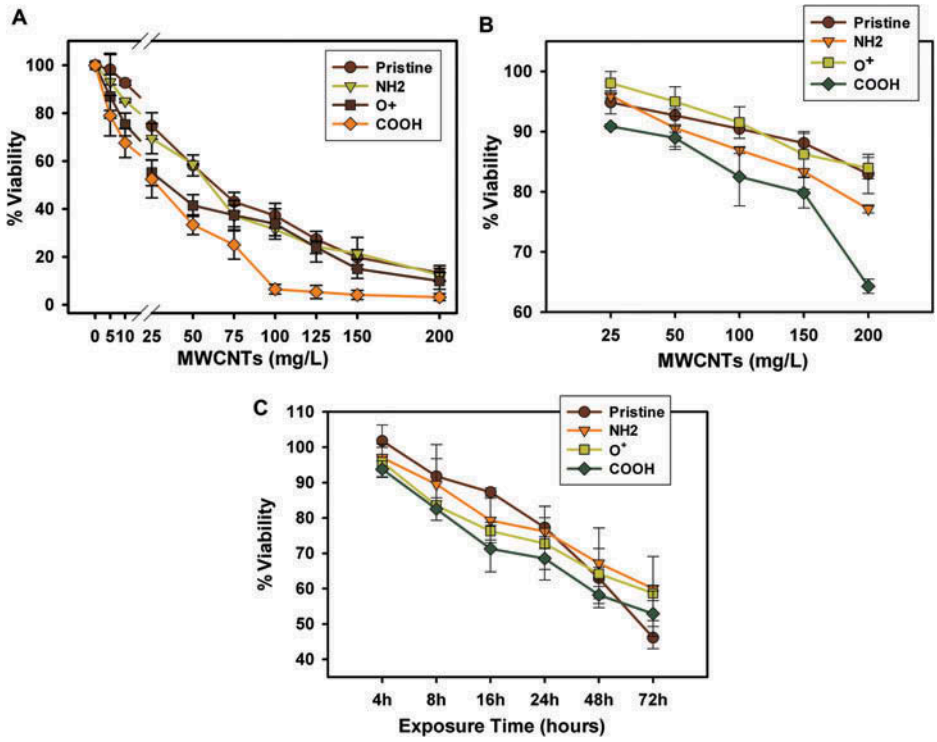

FIGURE 1. Cytotoxicity of MWCNT to BEAS2B cells. (A) Percent viability determined by Ez-Cytox assay after 24 h of exposure to MWCNT in complete DMEM F/12 medium. (B) Percent viability determined by trypan blue exclusion method after 24 h of exposure to MWCNT in complete DMEM F/12 medium. (C) Cells exposed to MWCNT at 20 mg/L at different time points and cytotoxicity determined by Ez-Cytox assay. Data presented as mean ± SEM.

TABLE 2. The 24-h Effective Concentrations (ECs) (Calculated From Percent Viability) of MWCNT to Beas2B Cells

Name	EC_{10} (mg/L)	EC_{20} (mg/L)	EC_{50} (mg/L)	EC_{90} (mg/L)
MWCNT-O^+	2.24	6.89	39.54	197.27
MWCNT-NH_2	7.05	15.54	55.27	223.17
MWCNT-COOH	2.15	5.39	23.67	123.23
Pristine	11.41	21.78	63.17	236.42

highest amount of DNA damage (olive tail moment, a comet parameter) followed by O^+ and NH_2-MWCNT, whereas pristine-MWCNT did not show any significant DNA damage in BEAS2B cells exposed to similar concentrations (20 mg/L) for 24 h (Figure 3).

Effects of MWCNTs on Survival and Reproduction of *C. elegans*

No marked change in survival of wild-type (N2) *C.elegans* occurred following 24 h of exposure to all 4 types of MWCNT (Figure 4A) except for a moderate decrease in reproduction (Figure 4B) without displaying any specificity toward MWCNT functionalization effects. Interestingly, only a significant reduction in reproduction potential was found in pristine-MWCNT-exposed worms (Figure 4B).

DISCUSSION

Since, carbon nanomaterials are known to interact with colorimetric indicator dyes frequently used in cytotoxicity assays (Liu et al., 2013; Mishra et al., 2012; Herzog et al., 2007), more than one assay to analyze MWCNT toxicity was employed, including EZ-Cytox, trypan

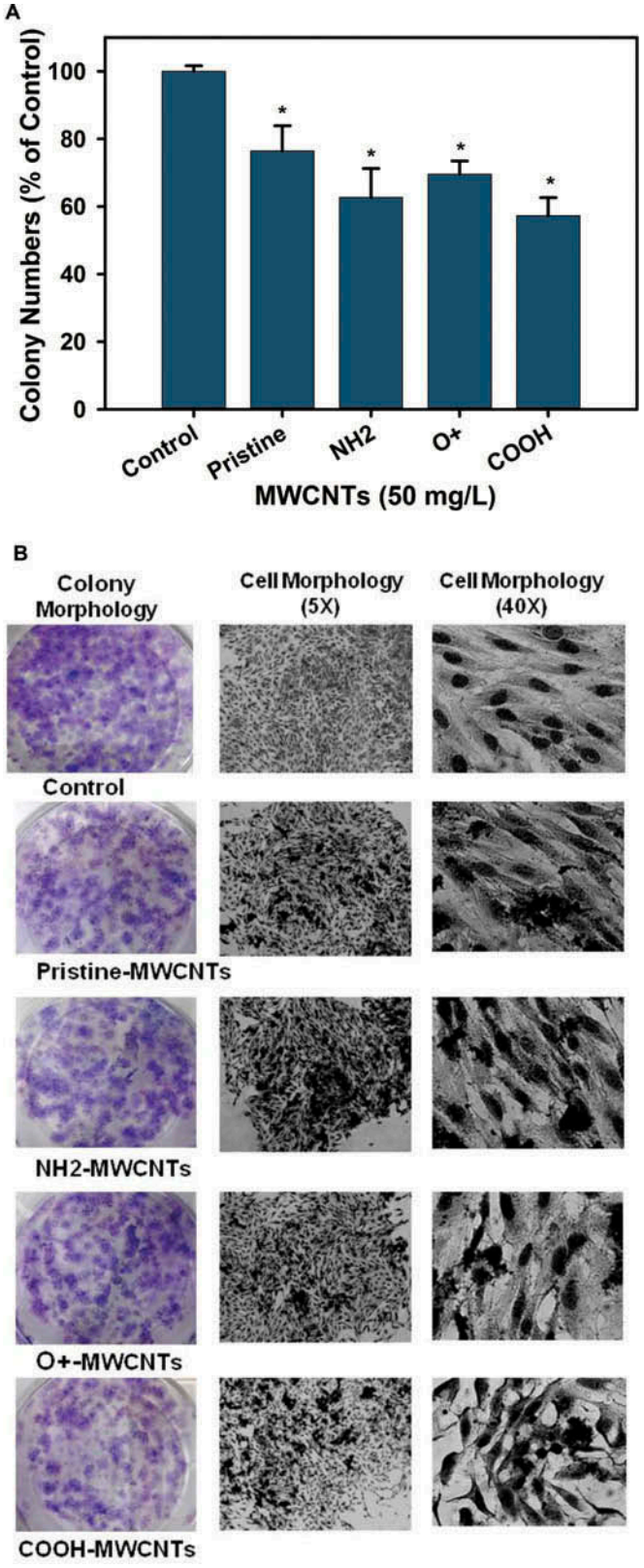

FIGURE 2. Effects of MWCNT exposure on colony formation in BEAS2B cells for 10 d. (A) Effects on colony number and data presented as percent of control, mean ± SEM. Asterisk indicates significant at $p < .05$. (B) Colonies and cell morphology of BEAS2B cells after 10-d exposure to 20 mg/L MWCNT at 5× and 40× magnification.

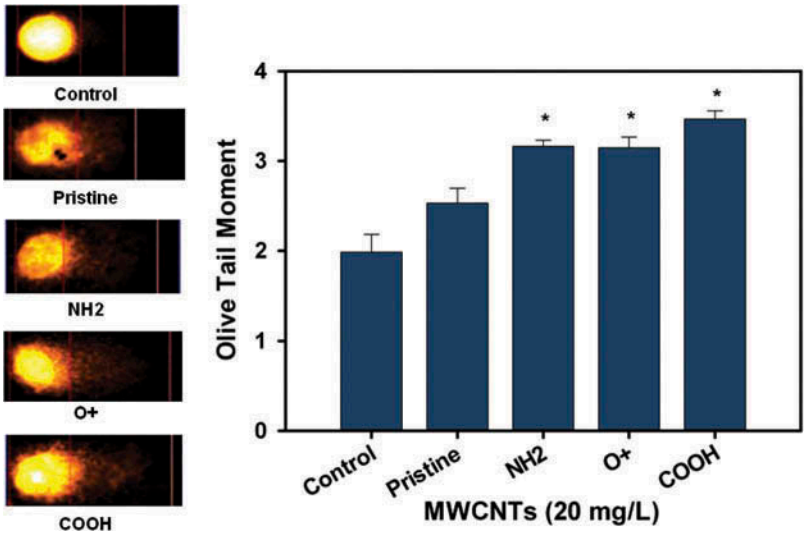

FIGURE 3. DNA damage potentiality of MWCNTs exposed BEAS2B cells at 20 mg/L. Single-stranded DNA damage measured with alkaline comet assay (presented with olive tail moment). Data presented as mean ± SEM. Asterisk indicates significant at $p < .05$.

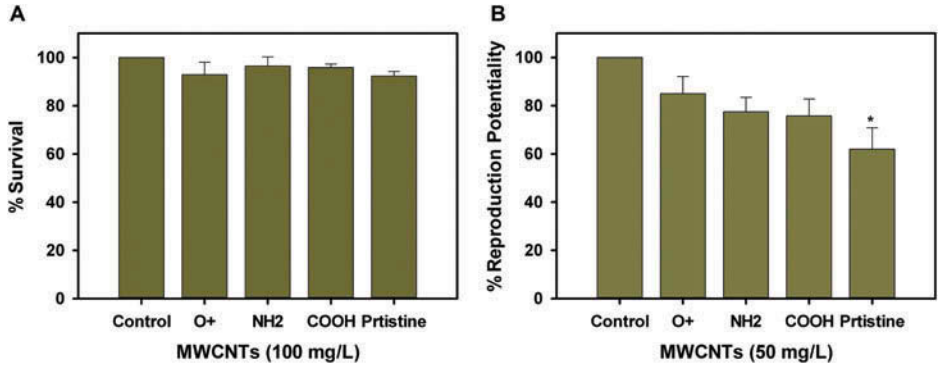

FIGURE 4. Effect of MWCNTs treatment on *C. elegans*. (A) Survival percentage of wild-type *C. elegans* due to MWCNT exposure at 100 mg/L for 24 h. Data presented as mean ± SEM. (B) Percent reproductive potential of wild-type *C. elegans* due to MWCNT exposure at 50 mg/L for 72 h. Data presented as mean ± SEM. Asterisk indicates significant at $p < .05$.

blue, and colonogenic assays. All performed assays displayed a similar trend of toxicity, where COOH-MWCNT was found most toxic (Figures 1A and 1C). The differential response between EZ-Cytox and trypan blue exclusion assay might be because the assays do not work on the same principle (Figures 1A and 1C). In general, the trypan blue exclusion assay is based on membrane integrity whereas the EZ-Cytox assay exploits metabolic activity and mitochondrial function (Chatterjee et al., 2013). In previous studies, BEAS2B cells were found most sensitive among WI38-VA13 and A549 cells (He et al., 2011; Mishra et al., 2012).

Further, the colonogenic assay was used as a more reliable method for in vitro toxicity assessment of CNT (Herzog et al., 2007). Although the colony number was not markedly different between functionalized MWCNT, the reduction in colony sizes was markedly significant in the case of COOH-MWCNT (Figure 2B), which indicates that the COOH-MWCNT exposure compromised cellular capacity to grow and proliferate. Using colony size and number as endpoints for carbon nanomaterials (SWCNT and Printex 90 carbon black nanoparticles) toxicity assessment, significant effects were observed by Herzog et al. (2007).

DNA damage (single- and double-strand breakage, DNA–DNA crosslinks, DNA–protein crosslinks, and DNA adducts) might result in a mutation, which in turn leads to malignant transformation (cancer), and the DNA strand break, especially double strand break, is considered the most detrimental one among all the types of DNA lesions. Hence, detection of DNA strand breaks is a sensitive marker of genotoxic damage potential and premutagenic lesions. Among the available DNA strand break and subsequent genotoxicity indicator tests, the comet assay is capable of detecting single as well as double DNA strand breaks and alkali-labile sites even at low levels of DNA damage (Collins, 2004). In the current study, MWCNT, mainly the functionalized ones (COOH > O^+ and NH_2), were found to be genotoxic to BEAS2B cells. A similar trend of genotoxicity was found in BEAS2B cell due to CNT exposure by Lindberg et al., (2009). In addition, Muller et al. (2008) suggested that MWCNT induced micronuclei in MCF-7 cells by both clastogenic and aneugenic mechanisms.

The variable response of BEAS2B cells to MWCNT exposure observed in the present study was likely due to differences in agglomerate size or contact surface area with the cells. Moreover, the COOH-MWCNT compound is more hydrophilic and stable in aqueous buffers than other functionalized as well as pristine compounds (Wang et al., 2011; Li et al., 2013), accounting for greater toxicity.

A few reports are available regarding MWCNT toxicity in *C. elegans*. No significant changes in 24-h survival (Figure 4A) and decrease of reproduction potential (Figure 4B) in MWCNT-exposed worms suggested that MWCNT may damage the reproductive organ. In addition, the significant decrease of reproduction due to pristine-MWCNT exposure may shed light on the fact that functionalization (O^+/NH_2/COOH) prevented the adverse effects of MWCNT on the *C. elegans* reproductive system. In particular, carboxyl (-COOH) modification was reported to prevent the toxicity of MWCNT on the targeted organs in *C. elegans* at concentrations less than 100 mg/L (Nouara et al., 2013). This is possibly because of greater elimination of functionalized MWCNT than pristine, which might accumulate in reproductive organs, producing reduced reproductive capacity. Burke et al. (2011) suggested that covalent functionalization might lower the overall toxicity profile of CNT by enhancing clearance. However, it is not possible to suggest that functionalization effectively prevented the adverse effects of MWCNT, as carboxyl modification exerted highest toxicity as evidenced by viability and DNA damage in BEAS2B cells.

CONCLUSION

The adverse effects of MWCNT depend upon surface functionalization as well as model organisms tested. Future study is necessary to evaluate the underlying mechanism of differential mode of toxicity exerted by surface functionalization of MWCNT, which consequently would help for better biocompatibility assessment and use of MWCNT.

FUNDING

This work was supported by the Korean Ministry of Environment through National Institute of Environmental Science (NIES) and "Environmental Health R&D Program" (2012001370009) and also by the Mid-career Researcher Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning (2013R1A2A2A03010980). The authors thank Qaisra Naheed Choudhry for technical support.

REFERENCES

Bhattacharaya, K., Andon, F.T., El-Sayed, R., and Fadeel, B. 2013. Mechanisms of carbon nanotube-induced toxicity: Focus on pulmonary inflammation. *Adv. Drug Deliv. Rev.* 65: 2087–2097.

Brenner, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71–94.

Burke, A. R., Singh, R. N., Carroll, D. L., Owen, J. D., Kock, N. D., D'Agostino,

R., Jr., Torti, F. M., and Torti, S. V. 2011. Determinants of the thrombogenic potential of multiwalled carbon nanotubes. *Biomaterials* 32: 5970–5978.

Bussy, C., Pinault, M., Cambedouzou, J., Landry, M. J., Jegou, P., Mayne-L'hermite, M., Launois, P., Boczkowski, J., and Lanone, S.2012. Critical role of surface chemical modifications induced by length shortening on multi-walled carbon nanotubes-induced toxicity. *Part. Fibre Toxicol*. 9: 46.

Cavallo, D., Fanizza, C., Ursini, C. L., Casciardi, S., Paba, E., Ciervo, A., Fresegna, A. M., Maiello, R., Marcelloni, A. M., Buresti, G., Tombolini, F., Bellucci, S., and Iavicoli, S. 2012. Multi-walled carbon nanotubes induce cytotoxicity and genotoxicity in human lung epithelial cells. *J. Appl. Toxicol*. 32: 454–464.

Chatterjee, N., Eom, H. J., Jung, S. H., Kim, J. S., and Choi, J. 2013. Toxic potentiality of bio-oils, from biomass pyrolysis, in cultured cells and *Caenorhabditis elegans*. *Environ. Toxicol*. doi:10.1002/tox.21871.

Clark, K. A., O'Driscoll, C., Cooke, C. A., Smith, B. A., Wepasnick, K., Fairbrother, D. H., Lees, P. S., and Bressler, J. P. 2012. Evaluation of the interactions between multiwalled carbon nanotubes and Caco-2 cells. *J. Toxicol. Environ. Health A* 75: 25–35.

Collins, A. 2004. The comet assay for DNA damage and repair. *Mol. Biotechnol*. 26: 249–261.

Coccini, T., Roda, E., Sarigiannis, D. A., Mustarelli, P., Quartarone, E., Profumo, A., and Manzo, L. 2010. Effects of water-soluble functionalized multi-walled carbon nanotubes examined by different cytotoxicity methods in human astrocyte D384 and lung A549 cells. *Toxicology* 269: 41–53.

Di Sotto, A., Chiaretti, M., Carru, G. A., Bellucci, S., and Mazzanti, G. 2009. Multi-walled carbon nanotubes: Lack of mutagenic activity in the bacterial reverse mutation assay. *Toxicol. Lett*. 184: 192–197.

Guo, N. L., Wan, Y. W., Denvir, J., Porter, D. W., Pacurari, M., Wolfarth, M. G., Castranova, V., and Qian, Y. 2012. Multiwalled carbon nanotube-induced gene signatures in the mouse lung: potential predictive value for human lung cancer risk and prognosis. *J. Toxicol. Environ. Health A* 75: 1129–1153.

He, X., Young, S. H., Schwegler-Berry, D., Chisholm, W. P., Fernback, J. E., and Ma,Q. 2011. Multiwalled carbon nanotubes induce a fibrogenic response by stimulating reactive oxygen species production, activating NF-kappaB signaling, and promoting fibroblast-to-myofibroblast transformation. *Chem. Res. Toxicol*. 24: 2237–2248.

Herzog, E., Casey, A., Lyng, F. M., Chambers, G., Byrne, H. J., and Davoren, M. 2007. A new approach to the toxicity testing of carbon-based nanomaterials-the clonogenic assay. *Toxicol. Lett*. 174: 49–60.

Jiang, Y., Zhang, H., Wang, Y., Chen, M., Ye, S., Hou, Z., and Ren, L. 2013. Modulation of apoptotic pathways of macrophages by surface-functionalized multi-walled carbon nanotubes. *PLoS One* 8: e65756.

Kayat, J., Gajbhiye, V., Tekade, R. K., and Jain, N. K. 2011. Pulmonary toxicity of carbon nanotubes: A systematic report. *Nanomedicine* 7: 40–49.

Kim, J. E., Lim, H. T., Minai-Tehrani, A., Kwon, J. T., Shin, J. Y., Woo, C. G., Choi, M., Baek, J., Jeong, D. H., Ha, Y. C., Chae, C. H., Song, K. S., Ahn, K. H., Lee, J. H., Sung, H.J., Yu, I. J., Beck, G. R., Jr., and Cho, M. H. 2010a. Toxicity and clearance of intratracheally administered multiwalled carbon nanotubes from murine lung. *J. Toxicol. Environ. Health A* 73: 1530–1543.

Kim, J. S., Song, K. S., Joo, H. J., Lee, J. H., and Yu, I. J. 2010b. Determination of cytotoxicity attributed to multiwall carbon nanotubes (MWCNT) in normal human embryonic lung cell (WI-38) line. *J. Toxicol. Environ. Health A* 73: 1521–1529.

Li, R., Wang, X., Ji, Z., Sun, B., Zhang, H., Chang, C. H., Lin, S., Meng, H., Liao, Y. P., Wang, M., Li, Z., Hwang, A. A., Song, T. B., Xu, R., Yang, Y., Zink, J. I., Nel, A. E., and Xia, T. 2013. Surface charge and cellular processing of covalently functionalized multi-wall carbon nanotubes determine pulmonary toxicity. *ACS Nano* 7: 2352–2368.

Liang, G., Yin, L., Zhang, J., Liu, R., Zhang, T., Ye, B., and Pu,Y. 2010. Effects of subchronic exposure to multi-walled carbon nanotubes on mice. *J. Toxicol. Environ. Health A* 73: 463–470.

Lindberg, H. K., Falck, G. C., Suhonen, S., Vippola, M., Vanhala, E., Catalan, J., Savolainen, K., and Norppa, H. 2009. Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells in vitro. *Toxicol. Lett*. 186: 166–173.

Liu, Y., Zhao, Y., Sun, B., and Chen, C. 2013. Understanding the toxicity of carbon nanotubes. *Acc. Chem. Res*. 46: 702–713.

Liu, Z., Dong, X., Song, L., Zhang, H., Liu, L., Zhu, D., Song, C., and Leng, X. 2014. Carboxylation of multiwalled carbon nanotube enhanced its biocompatibility with L02 cells through decreased activation of mitochondrial apoptotic pathway. *J. Biomed. Mater. Res. A* 102: 665–673.

Mishra, A., Rojanasakul, Y., Chen, B. T., Castranova, V., Mercer, R. R., and Wang, L. Y. 2012. Assessment of pulmonary fibrogenic potential of multiwalled carbon nanotubes in human lung cells. *J. Nanomater*. 2012: 1–11.

Muller, J., Decordier, I., Hoet, P. H., Lombaert, N., Thomassen, L., Huaux, F., Lison, D., and Kirsch-Volders, M. 2008. Clastogenic and aneugenic effects of multi-wall carbon nanotubes in epithelial cells. *Carcinogenesis* 29: 427–433.

Nouara, A., Wu, Q., Li, Y., Tang, M., Wang, H., Zhao, Y., and Wang, D. 2013. Carboxylic acid functionalization prevents the translocation of multi-walled carbon nanotubes at predicted environmentally relevant concentrations into targeted organs of nematode *Caenorhabditis elegans*. *Nanoscale* 5: 6088–6096.

Pacurari, M., Castranova, V., and Vallyathan, V. 2010. Single- and multi-wall carbon nanotubes versus asbestos: are the carbon nanotubes a new health risk to humans? *J. Toxicol. Environ. Health A* 73: 378–395.

Pacurari, M., Qian, Y., Fu, W., Schwegler-Berry, D., Ding, M., Castranova, V., and Guo, N. L. 2012. Cell permeability, migration, and reactive oxygen species induced by multiwalled carbon nanotubes in human microvascular endothelial cells. *J. Toxicol. Environ. Health A* 75: 112–128.

Roh, J. Y., Sim, S. J., Yi, J., Park, K., Chung, K. H., Ryu, D. Y., and Choi, J. 2009. Ecotoxicity of silver nanoparticles on the soil nematode-Caenorhabditis elegansusing functional ecotoxicogenomics. *Environ. Sci. Technol*. 43: 3933–3940.

Singh, N. P., McCoy, M. T., Tice, R. R., and Schneider, E. L. 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res*. 175: 184–191.

Snyder-Talkington, B. N., Qian, Y., Castranova, V., and Guo, N. L. 2012. New perspectives for in vitro risk assessment of multiwalled carbon nanotubes: Application of coculture and bioinformatics. *J. Toxicol. Environ. Health B* 15: 468–492.

Tabet, L., Bussy, C., Amara, N., Setyan, A., Grodet, A., Rossi, M. J., Pairon, J. C., Boczkowski, J., and Lanone, S. 2009. Adverse effects of industrial multiwalled carbon nanotubes on human pulmonary cells. *J. Toxicol. Environ. Health A* 72: 60–73.

Wang, X., Xia, T., Ntim, S. A., Ji, Z., Lin, S., Meng, H., Chung, C. H., George, S., Zhang, H., Wang, M., Li, N., Yang, Y., Castranova, V., Mitra, S., Bonner, J. C., and Nel, A. E. 2011. Dispersal state of multiwalled carbon nanotubes elicits profibrogenic cellular responses that correlate with fibrogenesis biomarkers and fibrosis in the murine lung. *ACS Nano* 5: 9772–9787.

Yu, K. N., Kim, J. E., Seo, H. W., Chae, C., and Cho, M. H. 2013. Differential toxic responses between pristine and functionalized multiwall nanotubes involve induction of autophagy accumulation in murine lung. *J. Toxicol. Environ. Health A* 76: 1281–1292.

Zhao, J., and Castranova, V. 2011. Toxicology of nanomaterials used in nanomedicine. *J. Toxicol. Environ. Health B* 14: 593–632.