Applied Microscopy
Korean Society of Microscopy

pISSN 2287-5123 · eISSN 2287-4445
http://dx.doi.org/10.9729/AM.2014.44.2.68

Multi-walled Carbon Nanotubes Affect the Morphology and Membrane Potential of Mitochondria in HeLa Cell

Wonwoo Lee[†], Hyo Min Cho[1,†], Chung Seok Oh, Eun Hae Kim, Woong Sun[1,*]

Gyeonggi Science High School for the Gifted, Suwon 440-800, Korea
[1]Department of Anatomy, Korea University College of Medicine, Brain Korea 21, Seoul 136-705, Korea

[†]These authors contributed equally to this work.

*Correspondence to:
Sun W,
Tel: +82-2-920-6404
Fax: +82-2-929-5696
E-mail: woongsun@korea.ac.kr

Received June 13, 2014
Revised June 29, 2014
Accepted June 29, 2014

With wide use of nano-materials, it is increasingly important to address their potential toxicity to mammalian cells. However, toxic effects of these materials have been mainly assessed by the cell survival assays. Considering that mitochondrial morphology and quality are highly sensitive to the condition of the cells, and the impairment of mitochondrial function greatly affect the survival of cells, here we tested the impact of multi-walled carbon nanotubes (MWNT) on the survival, mitochondrial morphology, and their membrane potential in HeLa cells. Interestingly, although MWNT did not induce cell death until 24 hours as assessed by pyknotic cell assay, mitochondrial length was elongated and the mitochondrial membrane potential was significantly reduced by exposure of HeLa cells to MWNT. These results suggest that MWNT exposure is potentially harmful to the cell, and the mechanism how MWNT alters mitochondrial quality should be further explored to assess the safety of MWNT use.

Key Words: Carbon nanotubes, Mitochondria, HeLa cell, Mitochondrial morphology, Mitochondrial membrane potential

INTRODUCTION

Carbon nanotubes (CNTs) have unique physical features with high strength, and they could be first used as field-emission electron sources (Deheer & Ugarte, 1995), which could be applied to transistors (Bachtold et al., 2001). With these and related characteristics, the use of CNTs is rapidly increasing in wide variety of fields, such as clothes' fibers, unique materials for extreme conditions, and parts of nano-machines. However, the safety of CNT's is one of the important and emerging concerns. Several researches have shown that a specific type and concentrations of CNT's are toxic to mammalian cells (Jia et al., 2005; Monteiro-Riviere et al., 2005; Muller et al., 2005; Kostarelos, 2008; Firme & Bandaru, 2010). For example, high concentration of multi-walled carbon nanotubes (MWNT) induced apoptosis of T lymphocyte (Bottini et al., 2006). Although relatively low concentration of MWNT, and CNTs in general, is believed to be safe, duration of the CNT exposure, and the cellular accumulation of these synthetic materials are yet to be explored. In addition, development of more sensitive tools to assess cellular quality after CNTs is desirable.

Mitochondria are one of the most essential organelles for the life of cells. They produce adenosine triphosphate (ATP) during the electron chain transfer cycles occurring in the inner mitochondrial membrane. For the ATP production, mitochondrial membrane potentials are required. Morphologically, mitochondria are highly dynamic in cells, and continuously fuse and divide (Kane & Youle, 2010; Galloway et al., 2012; Kim et al., 2013; Hoppins, 2014). More recently, it has been demonstrated that mitochondrial function/membrane potential is closely associated with mitochondrial morphology (Galloway et al., 2012; Liesa & Shirihai, 2013; Liu et al., 2014). In fact, many cell proliferation/

This research was supported by Gyeonggi Science High School for the Gifted.

survival assays such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide are based on the quality of the mitochondria (Gerlier & Thomasset, 1986).

In this study, we tested whether CNTs affected mitochondrial morphology and the quality and assessed whether these changes are accompany by the changes in the cell viability. Our study demonstrated that CNTs modified several aspects mitochondrial morphology/function without affect cell survival. These results suggest that CNTs are potentially harmful to the cells even they did not immediately reduce the cell viability. In addition, we propose that mitochondria may be important and sensitive targets to assess the quality of the cells.

MATERIALS AND METHODS

Carbon Nanotubes

We used two CNTs; multi walled carbon nanotube, MWNT and MWNT-OH which is a MWNT having-OH at the end of the nanotube (Skyspring Nanomaterial Inc., USA).

Cell Culture, Transfection

HeLa cells were maintained in 5% CO_2 at 37°C with Dulbecco modified Eagle medium (DMEM) (Wellgene, Korea) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Gibco BRL, USA). Prior to experiments, we trypsinized the cell using 0.25% Trypsin-EDTA (Gibco BRL) and plate onto coverslip at 0.5×10^5 cells/mL. To analyze the effects of CNTs on mitochondria, we labeled mitochondria using Dsred-Mito construct expressing mitochondrial targeted red fluorescence protein (Knott et al., 2008). For transfection into HeLa cell, lipofectamine 2000 (Invitrogen, USA) used according to manufacturer instruction. After 24 hours, either MWNT or MWNT-OH was added to the media without FBS. The final concentrations of CNTs were 40 μg/mL and 400 μg/mL.

Nuclear Staining

To label nuclei of the cells, cells were fixed by 4% paraformaldehyde for 15 min, washed twice with phosphate buffer saline (PBS), and incubated with PBS containing Hoechst 3342 (1:5000, Molecular probe, USA). Fluorescence signals were observed using a confocal microscope (LSM510; Carl Zeiss, Germany).

Measurement of Mitochondrial Membrane Potential

To analyze mitochondrial membrane potential, cells were incubated with tetramethyl rhodamine methyl ester (TMRM; Invitrogen) according to the manufacturer instruction. Briefly, Hela cells were plated onto 18-mm glass coverslip, and were treated with both MWNT and MWNT-OH. After 24 hours of treatments, cells were washed with PBS, and incubated with 1 mL DMEM containing 20 nM TMRM for 40 minutes at 37°C in 5% CO_2 incubator. TMRM signals were acquired using an inverted fluorescence microscopy (Observer Z1; Carl Zeiss, Germany) equipped with CoolLED (pE-2; Roper Scientific, France) as the light source, CCD camera (CoolSNAP fx; Photometrics, USA) and a humidified chamber maintaining cells at 37°C in 5% CO_2 condition.

RESULTS

Cell Survival Rate on CNT-included Medium

First, we tested whether survival rates of HeLa cells were affected by the 24 hour treatment with multi walled carbon nanotubes, MWNT or MWNT-OH. Fig. 1 shows the nuclei after grown on different concentrations of CNTs (40 or 400 μg/mL). However, virtually all nuclei exhibited normal morphology, and we failed to identify any morphological hallmarks of pyknotic cells (e.g., nuclear condensation and fragmentation) in all conditions. Therefore, it appeared that these range of CNTs did not affect the viability of the HeLa cells.

Mitochondria's Morphological Changes on CNT-included Medium

The mitochondria exhibit diverse shapes in the cell, and they continuously change their morphology in response to the environment-dependent intracellular context changes (Chen & Chan, 2004; Kim et al., 2013). Cells can be divided into three categories based on their mitochondrial morphology: (1) Fragment: most mitochondria exhibited fragmented and short morphology in a cell; (2) Intermediate: a mixture of fragmented and long mitochondria; and (3) Elongate: most mitochondria are inter-connected as a network form in a cell (Fig. 2). By these three criteria, we quantified the number of cells in each category (Fig. 3). Interestingly, in all conditions treated with CNTs exhibited the reduction in the number of cells in 'Fragment' category, and the compensatory increases in the number of 'Intermediate' and 'Elongate' groups.

Mitochondria Membrane Potential Changes on CNT-included Medium

In the cells, mitochondrial generate cellular energy, ATP using electron transfer chain complex, and directional proton movement between inner mitochondrial membrane generates mitochondrial membrane potential. Mitochondrial membrane potential is essential for the action of complex V ATP synthase, changes in the mitochondrial membrane potential is closely related with the potential to produce ATP (Chaturvedi & Flint Beal, 2013). Therefore, to assess whether CNTs altered mitochondrial function, we next measured mitochondrial membrane potential using fluorescent dye. Notably, treatment of low concentration of CNTs sufficiently reduced mitochondrial membrane potential in a statistically

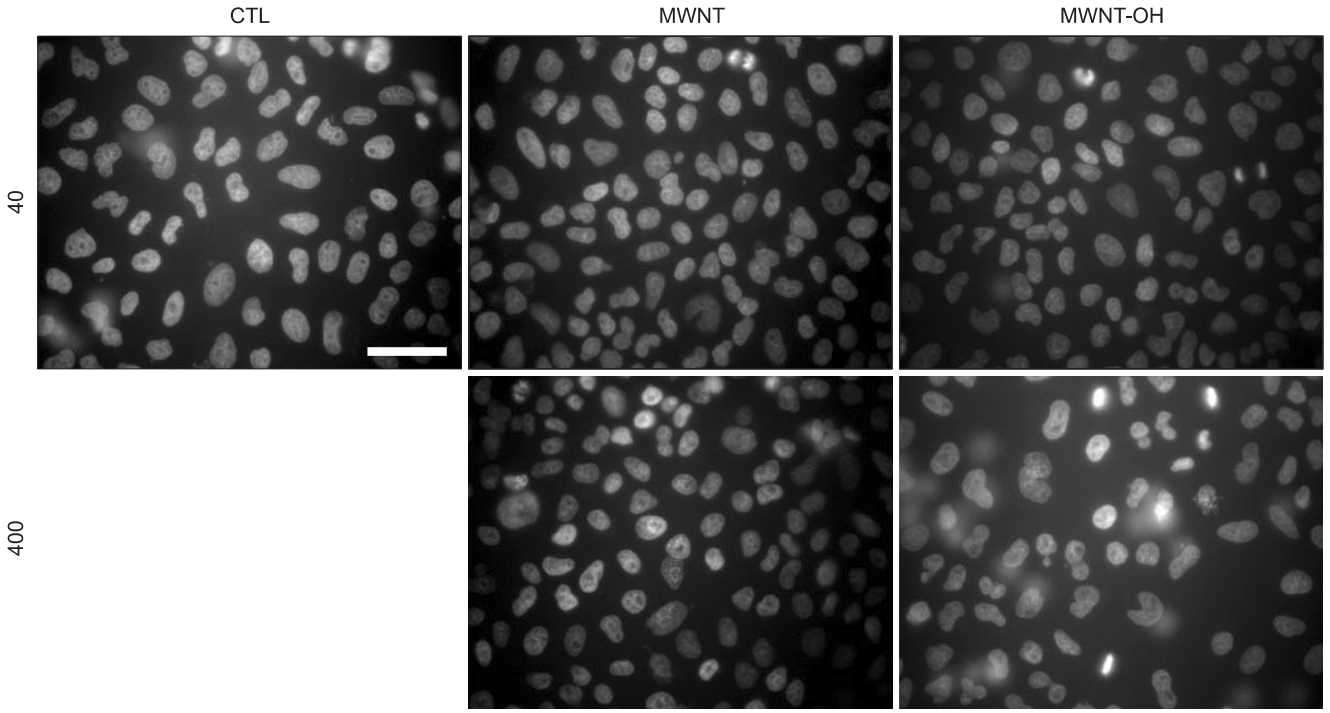

Fig. 1. Pyknotic cell assay in carbon nanotubes treated condition. Twenty-four hours after multi-walled carbon nanotubes (MWNT) or MWNT-OH treatments, HeLa cells were stained with to visualize nuclear morphology. The concentrations of nanoparticle (40 or 400 μg/mL) to the media are described on the left side of the images. Control group (CTL) did not receive nanoparticles. Scale bar=40 μm.

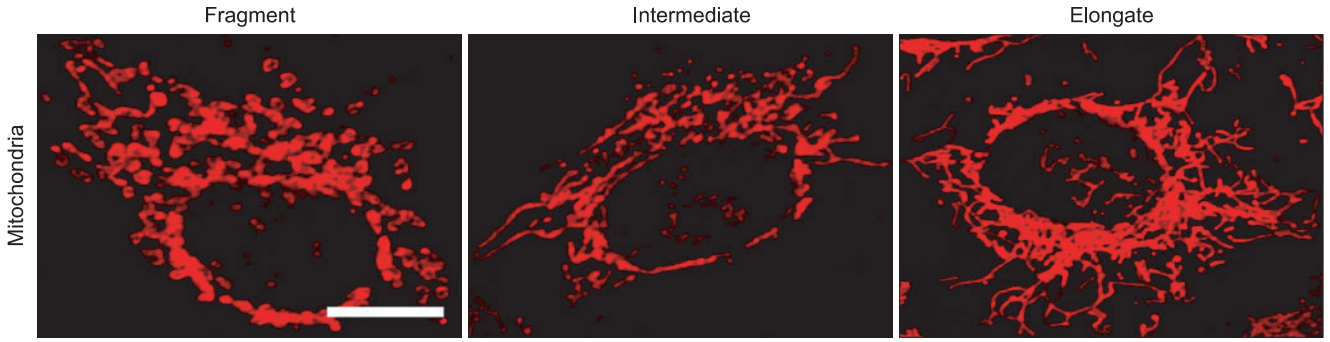

Fig. 2. Classification of mitochondrial morphology in HeLa cells. The images show the red fluorescence signals derived from mitochondria of transfected cells. Based on the mitochondrial morphology, cells are classified to 3 categories. In 'Fragment' cells, mitochondria are short and individually separated. In 'Intermediate' cells, their mitochondria are inter-connected in some parts while in some area fragmented mitochondria are seen. In 'Elongate' cells, mitochondria show tubular network morphology. Scale bar=10 μm.

significant manner, and high concentration of CNTs markedly impaired membrane potential (Fig. 4).

DISCUSSION

CNTs are increasingly utilized in many fields for researches and manufacturing. Because these materials are also applied for the drug delivery, gene therapy, and long-term-tracing of the cells, there are many potential that cells are exposed to the CNTs (Al Faraj et al., 2010; Meng et al., 2012; Bates & Kostarelos, 2013; Bhatnagar et al., 2014; Che Abdullah et al., 2014). However, the safety of CNTs is less understood. In this study we found that at least two types of CNTs, MWNT and MWNT-OH, do not induce apoptosis within 24 hours of exposure period.

Although above results suggest that these CNTs are not toxic, we found the changes in the mitochondrial morphology and the function. Morphological analysis showed that mitochondrial length was increased by MWNT and MWNT-OH treatment, compared to the control group. It has been

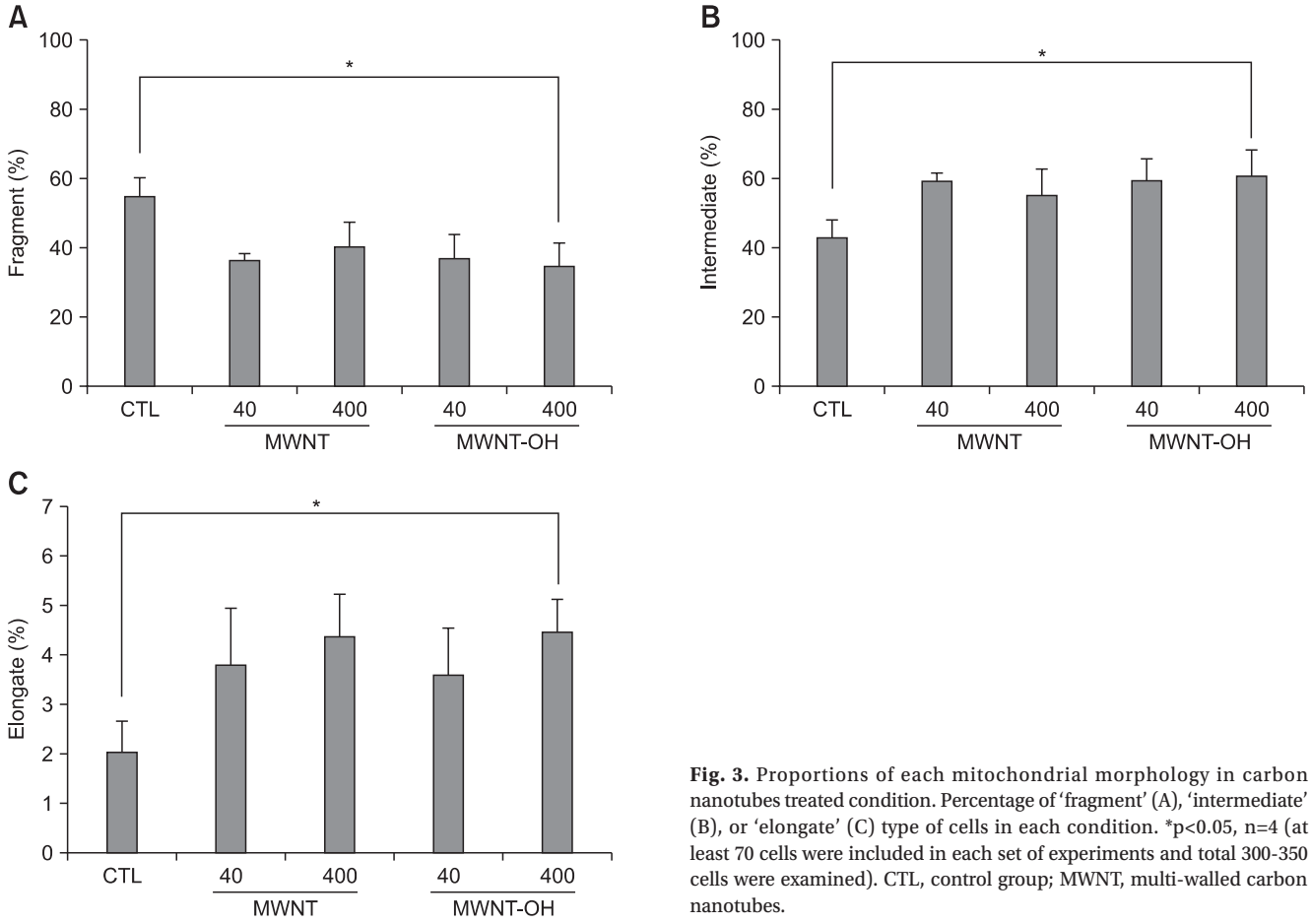

Fig. 3. Proportions of each mitochondrial morphology in carbon nanotubes treated condition. Percentage of 'fragment' (A), 'intermediate' (B), or 'elongate' (C) type of cells in each condition. *p<0.05, n=4 (at least 70 cells were included in each set of experiments and total 300-350 cells were examined). CTL, control group; MWNT, multi-walled carbon nanotubes.

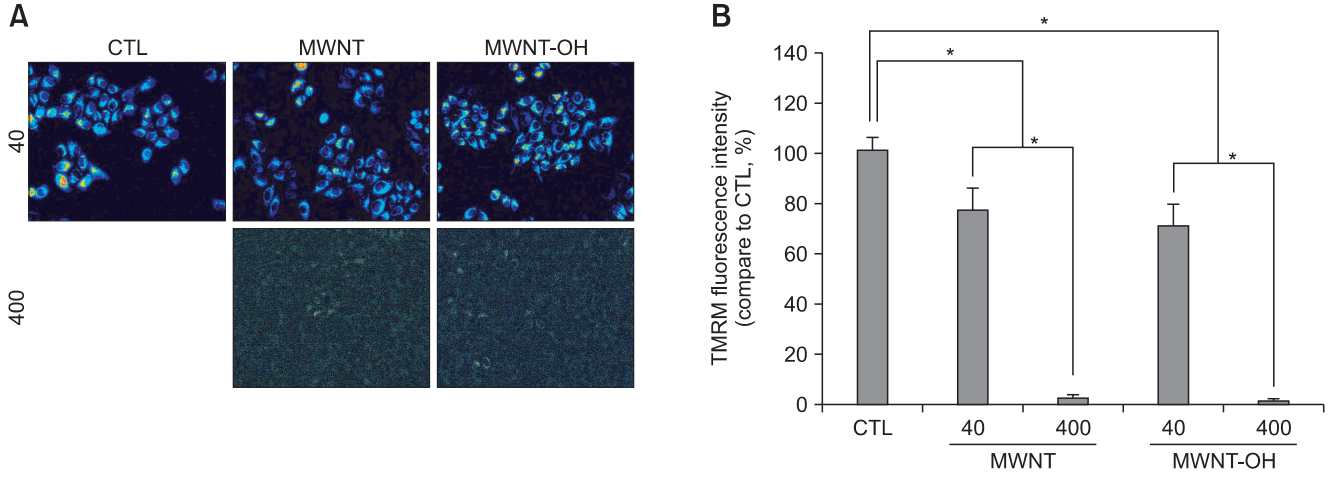

Fig. 4. Mitochondrial membrane potential in carbon nanotubes treated condition. (A) Representative images of tetramethyl rhodamine methyl ester (TMRM) fluorescence in each condition. Scale bar=40 μm. (B) Quantification of TMRM fluorescence intensities. *p<0.05, n>20 in each group. CTL, control group; MWNT, multi-walled carbon nanotubes.

known that slight stress, such as starvation, can increase the mitochondrial length, while strong damage conversely promotes mitochondrial fragmentation and subsequent apoptosis (Youle & van der Bliek, 2012). In this respect, it suggests that CNT exposure is mild stress to the cells and promote mitochondrial elongation without noticeable cell death.

In addition to the morphology, we also found that mitochondrial

membrane potential was decreased in a dose-dependent manner. Mitochondria membrane potential has been considered as a measure of mitochondria's functional integrity (Chaturvedi & Flint Beal, 2013). Loss of mitochondrial membrane potential depolarization is closely associated with the impairment of ATP synthesis from the mitochondria. Therefore, these results suggest that the changes in the mitochondrial morphology are also accompanied by the functional impairment. Although high concentration of CNTs greatly impaired mitochondrial membrane potential, this condition did not promote cell death. It is important to note that many cancer cells including HeLa cells generate ATP via cytosolic glycolysis, and the contribution of mitochondrial ATP synthesis is only marginal (Rossignol et al., 2004; Benard et al., 2007; Aguer et al., 2011). In this respect, loss of mitochondrial potential and failure of ATP synthesis from the mitochondria did not appear to promote cell death. Therefore, while we did not directly explore in this study, it is plausible that non-tumor cells with high demand of mitochondrial ATP generation might be more sensitive to the CNT-derived cellular toxicity.

It is also yet unclear how CNT treatments affect mitochondrial morphology/function. MWNT can be internalized into the cells, and there is a report demonstrating that nano materials can interact with mitochondria (Karatas et al., 2009). Therefore, physical association of CNT-mitochondria might trigger mitochondrial impairment, although other indirect influences are not entirely rule out.

CONCLUSIONS

CNTs appear to promote mild stress to the cells and evoke morphological and functional changes of mitochondria, although it does not affect the viability of the cells. Based on our observation, we propose that CNT exposure is potentially harmful to the cell, and assessment of mitochondrial function is useful and sensitive means to address the effect of CNTs on cells.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

REFERENCES

Aguer C, Gambarotta D, Mailloux R J, Moffat C, Dent R, McPherson R, and Harper M E (2011) Galactose enhances oxidative metabolism and reveals mitochondrial dysfunction in human primary muscle cells. *PLOS ONE* **6**, e28536.

Al Faraj A, Bessaad A, Cieslar K, Lacroix G, Canet-Soulas E, and Cremillieux Y (2010) Long-term follow-up of lung biodistribution and effect of instilled SWCNTs using multiscale imaging techniques. *Nanotechnology* **21**, 175103.

Bachtold A, Hadley P, Nakanishi T, and Dekker C (2001) Logic circuits with carbon nanotube transistors. *Science* **294**, 1317-1320.

Bates K and Kostarelos K (2013) Carbon nanotubes as vectors for gene therapy: past achievements, present challenges and future goals. *Adv. Drug Deliver Rev.* **65**, 2023-2033.

Benard G, Bellance N, James D, Parrone P, Fernandez H, Letellier T, and Rossignol R (2007) Mitochondrial bioenergetics and structural network organization. *J. Cell Sci.* **120**, 838-848.

Bhatnagar I, Venkatesan J, and Kiml S K (2014) Polymer functionalized single walled carbon nanotubes mediated drug delivery of gliotoxin in cancer cells. *J. Biomed Nanotechnol.* **10**, 120-130.

Bottini M, Bruckner S, Nika K, Bottini N, Bellucci S, Magrini A, Bergamaschi A, and Mustelin T (2006) Multi-walled carbon nanotubes induce T lymphocyte apoptosis. *Toxicol. Lett.* **160**, 121-126.

Chaturvedi R K and Flint Beal M (2013) Mitochondrial diseases of the brain. *Free Radical Biol. Med.* **63**, 1-29.

Che Abdullah C A, Lewis Azad C, Ovalle-Robles R, Fang S, Lima M D, Lepro X, Collins S, Baughman R H, Dalton A B, Plant N J, and Sear R P (2014) Primary liver cells cultured on carbon nanotube substrates for liver tissue engineering and drug discovery applications. *ACS Appl. Mater. Interfaces* [Epub ahead of print].

Chen H and Chan D C (2004) Mitochondrial dynamics in mammals. *Curr. Top. Dev. Biol.* **59**, 119-144.

Deheer W A C A and Ugarte D (1995) A carbon nanotube field-emission electron source. *Science* **270**, 1179-1180.

Firme C P 3rd and Bandaru P R (2010) Toxicity issues in the application of carbon nanotubes to biological systems. *Nanomed-Nanotechnol.* **6**, 245-256.

Galloway C A, Lee H, and Yoon Y (2012) Mitochondrial morphology-emerging role in bioenergetics. *Free Radical Biol. Med.* **53**, 2218-2228.

Gerlier D and Thomasset N (1986) Use of MTT colorimetric assay to measure cell activation. *J. Immunol. Methods* **94**, 57-63.

Hoppins S (2014) The regulation of mitochondrial dynamics. *Curr. Opin. Cell Biol.* **29C**, 46-52.

Jia G, Wang H, Yan L, Wang X, Pei R, Yan T, Zhao Y, and Guo X (2005) Cytotoxicity of carbon nanomaterials: single-wall nanotube, multi-wall nanotube, and fullerene. *Environ. Sci. Technol.* **39**, 1378-1383.

Kane L A and Youle R J (2010) Mitochondrial fission and fusion and their roles in the heart. *J. Mol. Med.* **88**, 971-979.

Karatas O F, Sezgin E, Aydin O, and Culha M (2009) Interaction of gold nanoparticles with mitochondria. *Colloids Surf. B Biointerfaces* **71**, 315-318.

Kim H W, Park S N, Moon Y, Oh S H, and Rhyu I J (2013) Morphological diversity of mitochondria in cultured astrocytes, Hela, COS7 cells under high voltage electron microscopy. *Appl. Microsc.* **43**, 117-121

Knott A B, Perkins G, Schwarzenbacher R, and Bossy-Wetzel E (2008) Mitochondrial fragmentation in neurodegeneration. *Nat. Rev. Neurosci.* **9**, 505-518.

Kostarelos K (2008) The long and short of carbon nanotube toxicity. *Nat. Biotechnol.* **26**, 774-776.

Liesa M and Shirihai O S (2013) Mitochondrial dynamics in the regulation of nutrient utilization and energy expenditure. *Cell Metab.* **17**, 491-506.

Liu R, Jin P, LiqunYu, Wang Y, Han L, Shi T, and Li X (2014) Impaired mitochondrial dynamics and bioenergetics in diabetic skeletal muscle. *PLOS ONE* **9**, e92810.

Meng L, Zhang X, Lu Q, Fei Z, and Dyson P J (2012) Single walled carbon nanotubes as drug delivery vehicles: targeting doxorubicin to tumors. *Biomaterials* **33**, 1689-1698.

Monteiro-Riviere N A, Nemanich R J, Inman A O, Wang Y Y, and Riviere J E (2005) Multi-walled carbon nanotube interactions with human epidermal keratinocytes. *Toxicol. Lett.* **155**, 377-384.

Muller J, Huaux F, Moreau N, Misson P, Heilier J F, Delos M, Arras M, Fonseca A, Nagy J B, and Lison D (2005) Respiratory toxicity of multi-wall carbon nanotubes. *Toxicol. Appl. Pharm.* **207**, 221-231.

Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington S J, and Capaldi R A (2004) Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. *Cancer Res.* **64**, 985-993.

Youle R J and van der Bliek A M (2012) Mitochondrial fission, fusion, and stress. *Science* **337**, 1062-1065.