Original Article

Endoplasmic reticulum stress mediated apoptosis via JNK in MWCNT-exposed *in vitro* systems: size, surface functionalization and cell type specificity

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ABSTRACT — The aim of the present study was to evaluate the underlying mechanism of multi-walled carbon nanotubes (MWCNT) induced cellular response and their potential cross-talk, specifically, between endoplasmic reticulum (ER) stress, MAPK activation and apoptosis and how these nano-bio interactions depend on the physico-chemical properties of MWCNT. For this purpose, human bronchial epithelial (Beas2B) and human hepatoma (HepG2) cell lines, were exposed to five kinds of MWCNTs which differ in functionalization and aspect ratios. Tissue-specific sensitivity was evident for calcium homeostasis, ER-stress response, MAPK activation and apoptosis, which further depended on surface functionalization as well as aspect ratios of MWCNT. By applying specific pharmaceutical inhibitors, relevant biomarkers gene and proteins expressions, we found that possibly MWCNT induce activation of IRE1 α -XPB1 pathway-mediated ER-stress response, which in turn trigger apoptosis through JNK activation in both type of cells but with variable intensity. The information presented here would have relevance in better understanding of MWCNT toxicity and their safer applications.

Key words: MWCNTs, *In vitro* cell type specificity, Calcium homeostasis, Endoplasmic reticulum stress, MAPK activation, Apoptosis

INTRODUCTION

The unique physical, thermal and electrical properties of multi-walled carbon nanotubes (MWCNT), concentrically stacked cylindrical tubes of graphitic carbon, have led to wide range of applications not only in the fields of electronics, emission devices, and energy storage devices but also as drug delivery carriers, biosensor, therapeutics, and diagnostic materials in the biomedical and nanomedicine fields (Castranova *et al.*, 2013; Heister *et al.*, 2013; Lanone *et al.*, 2013). Surface functionalization of MWCNT by introducing hydrophilic chemical groups (such as, hydroxyl -OH or carboxyl groups-COOH) is the principle way to overcome low solubility and to improve the efficacy of MWCNTs' applications, specifically in the biomedical field (Prato *et al.*, 2008; Vardharajula *et al.*, 2012).

Considerable health concerning environmental, occupational as well as in consumer domain has immensely increased with the wide production and applications of MWCNT and their functionalized derivatives (Castranova *et al.*, 2013; Li *et al.*, 2013; Albini *et al.*,

2015). The foregoing original research as well as recent reviews highlighted that length, diameter, surface functionalization, aspect ratio as well as the target cell types are the principle determinants of the nano-bio interactions of MWCNT (Sweeney *et al.*, 2014; Hamilton *et al.*, 2013; Han *et al.*, 2012; Kim *et al.*, 2011; Liu *et al.*, 2012; Nagai *et al.*, 2011; Bussy *et al.*, 2012). A noted apprehension is that the modification of any one or a combination of these properties could modulate the mode of nano-bio interactions of MWCNT (Lanone *et al.*, 2013). The well-known mechanisms of MWCNT-induced toxicity include oxidative stress, inflammatory responses, lung granulomas formation, interstitial fibrosis, DNA damage, autophagy, apoptosis, malignant transformations, DNA methylation etc. (Liu *et al.*, 2013; Wang *et al.*, 2011a; Ghosh *et al.*, 2020; Toyokuni, 2013; Sargent *et al.*, 2014; Chatterjee *et al.*, 2017; Shvedova *et al.*, 2012; Poulsen *et al.*, 2015; Wang *et al.*, 2011b; Hamilton *et al.*, 2013). However, only a few studies reported on endoplasmic reticulum (ER) stress (Chang *et al.*, 2018; Long *et al.*, 2019; Sun *et al.*, 2019) and MAPK activation (Lee *et al.*, 2012; Sweeney

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et al., 2014) in mammalian cell model due to MWCNT exposure.

The endoplasmic reticulum (ER) is an essential organelle which plays critical role in cell survival and functions, such as, calcium homeostasis, lipid biosynthesis, etc. The ER maintains a surveillance mechanism for protein folding and works as a sensor for misfolded protein to guide them in the degradatory pathway (Malhotra and Kaufman, 2007). The misfolded or unfolded proteins caused ER stress, also known as unfolded protein response (UPR), which is an evolutionarily conserved cell signaling pathway (Harding *et al.*, 2002; Garg *et al.*, 2012; Shen *et al.*, 2004). The activation of transmembrane stress-sensing proteins in the ER, including inositol-requiring protein 1 (IRE1), PKR-like endoplasmic reticulum kinase (PERK), and activating transcription factor-6 (ATF-6), either gives rise to signals for cellular self-recovery or, alternatively, promotes cell death (Garg *et al.*, 2012; Harding *et al.*, 2002; Tabas and Ron, 2011). Calcium homeostasis also plays crucial role in ER/mitochondria cross-talk and hence induces various modes of cell death, autophagy, apoptosis, necrosis (Pinton *et al.*, 2008; Romagnoli *et al.*, 2007; Kaufman and Malhotra, 2014; Malhotra and Kaufman, 2011).

The detailed mechanisms of ER-stress induction by MWCNT and how it is related to cell death remains to be elucidated. Here, we addressed the potentiality of MWCNT to induce ER stress response-mediated apoptosis in *in vitro* systems and how it differs with the cell types as well as MWCNTs' physicochemical properties. To this end, we used five types of MWCNT which differ in length, diameter as well as surface functionalization and two different cultured cell systems, human bronchial epithelial cells (Beas2B cell) and human hepatoma cell line (HepG2 cell). The mechanisms were evaluated with/without relevant inhibitor, gene and protein expressions of ER-calcium depletion and mitochondrial calcium loading, ER-stress response, MAPK activation and apoptosis as endpoints.

MATERIALS AND METHODS

Characterization of MWCNTs

The commercial MWCNTs [pristine, functionalized derivatives – carboxylated (-COOH), hydroxylated (-OH) and hydroxylated with different tube length and diameter] were purchased from CheapTubes.com (Cambridgeport, VT, USA) and were additionally characterized, further, with X-ray photoelectron spectroscopy (XPS), transmission electron microscopy (TEM) and a dynamic light scattering spectrometer (DLS).

XPS (Sigma Probe, ThermoVG, UK) was used to examine the surface chemical modification of the MWCNTs. The data were obtained by a hemispherical analyzer equipped by a monochromatic Al X-ray source (15 kV, 100W, 400 micrometer) operating at a vacuum (2×10^{-9} mb). The XPS peaks were analyzed by using Gaussian components after a Shirley background subtraction. The O/C atomic ratios of the samples were obtained by using peak area ratios of the XPS core levels and the sensitivity factor (SF) of each element in XPS.

Structure and shape were investigated by TEM (Carl Zeiss LIBRA 120). The samples for TEM were prepared by drop casting a diluted suspension (50 mg/L) onto a carbon film with 300 square mesh copper grids and dried at room temperature for 24 hr.

The size distribution and ζ -potential of the MWCNTs (30 mg/L in DMEM culture media) were evaluated by using a photal dynamic light scattering spectrometer (DLS) (ELSZ-1000, Otsuka Electronics Co., Inc., Osaka, Japan).

Cell culture and treatment with MWCNTs and inhibitors

Beas2B cells (human bronchial epithelial cells) were cultured in DMEM/F12 (GIBCO, Grand Island, NY, USA) and HepG2 (human liver carcinoma cells) were cultured in MEM (GIBCO) and both were supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotics, at 37°C in a 5% CO₂ atmosphere.

The MWCNTs were freshly prepared in cell culture medium (DMEM/F12 or MEM) at the desired concentrations (respective EC₂₀ of each MWCNT for each cell lines) with appropriate amount from the stock (1000 mg/L in distilled water) and were sonicated for 15 min before biological exposure.

Approximately, 5×10^3 cells/ well were seeded in 96-well plates 24 hr prior to treatment. Then the cells were pre-incubated with 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) (5 μ M), 4-Phenylbutyrate (4-PBA) (5 mM) and solubralin (25 μ M) pretreatment for 1 hr followed by addition of MWCNTs (respective EC₅₀ doses), and after incubation for 24 hr, cytotoxicity was measured with the EZ-Cytox method as described previously (Chatterjee *et al.*, 2017). The dose of each inhibitor was selected by testing less than 10% cytotoxicity with the inhibitors exposure alone (data not shown).

Total RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNA from MWCNT-treated samples (respective

EC₂₀ for 24 hr) were extracted by using RNA extraction kit (NucleoSpin, MachereyNagel, Düren, Germany) and the quantity and quality of RNAs were detected in spectrophotometer as well as with agarose gel separation.

Synthesis of cDNAs was performed by a reverse transcriptase (RT) reaction and the PCR amplification was carried out with a thermal cycler (Bio-Rad, Hercules, CA, USA). Real-time RT-PCR analysis was accomplished with CFX manager (Bio-Rad) using the IQ™ SYBR Green SuperMix (Bio-Rad). The primers were constructed (by Primer3plus) based on sequences available in NCBI and the qRT-PCR conditions were optimized (efficiency and sensitivity tests) for each primer prior to the experiment (Supplementary Table S2). Three biological replicates each in triplicate were used for each qRT-PCR analysis. Analysis of negative control reactions (without RT and all reagents except template) confirmed no DNA contamination. The gene expressions were normalized by using GAPDH and ACTB as a housekeeping genes.

Western blotting

Cell samples were harvested from each treatment and cell extracts were prepared in NP40 protein extraction buffer and the protein concentration was measured by Bradford method. Equal amounts (50 µg) of proteins were separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 3% BSA in TBST at room temperature. The primary antibody was used at 1:1000 dilutions and the secondary antibody was used at 1:2000 dilutions. Blots were developed using enhanced chemiluminescence western blotting detection kit (Amersham, Little Chalfont, UK). The tested proteins were Bax, Bcl-2, γ-H2AX and β-Actin (antibodies purchased from Cell Signaling, Beverly, MA, USA).

Image analysis

The cells were plated on poly-L-lysine-coated cover glass (at 1 × 10⁵ cells/mL) and exposed to MWCNTs respective EC₂₀ doses for 24 hr. After incubation the respective fluorescence dyes were added to the cells, and the cells were further incubated for 30 min under the same growth conditions and were then washed with PBS after removing the loading dye. Microscopic images were collected using the fluorescence microscope (Leica DM IL) equipped with Leica DCF 420C camera. The endoplasmic reticulum (ER) stress was detected with ER-Tracker Blue-White DPX (1 µM, Molecular Probes, Life Technologies), mitochondrial Ca²⁺ level was determined with Rhod2 AM (Molecular Probes, Life Technologies) and the apoptotic bodies or condensed nuclei were detected after staining with Hoechst 33342 (10 µg/mL, Sigma-

Aldrich, St. Louis, MO, USA).

Statistical analysis

Significance of differences among/between treatments was determined using one-way analysis of variance (ANOVA) and followed by a post-hoc test (Tukey, $p < 0.05$) in SPSS 12.0KO (SPSS Inc., Chicago, IL, USA) and graphs were prepared in Sigma Plot (Version 12.0).

RESULTS AND DISCUSSION

Characterization of MWCNTs

The characterizations of all MWCNTs (including in-house analysis as well as from manufacturer) were previously published (Chatterjee *et al.*, 2016, 2017) and summarized in Table 1. In total, five kinds of MWCNTs were used in this study and divided in two groups, one group differing in surface functionalization [pristine (Prist-SS), hydroxylation (OH-SS) and carboxylation (COOH-SS)] but possessing similar length and diameter (length: 0.5-2.0 µm, diameter: 8-15 nm) and the other group with same surface functionalization (hydroxylation) but differing in length and diameter (OH-SS with length: 0.5-2.0 µm, diameter: 8-15 nm, aspect ratio: 33.33-250; OH-LS with length: 10-30 µm, diameter: 8-15 nm, aspect ratio: 666.67-3750; OH-LB with length: 10-30 µm, diameter 20-30 nm, aspect ratio: 333.33-1500) (Table 1). The order of carbon content was exhibited as Prist-SS > OH-LB > COOH-SS > OH-LS > OH-SS in XPS measurement and oxygen content was in a reverse order (Table 1). The aggregation behavior and colloidal stability of MWCNTs under different time points (0 and 24 hr) were evaluated with simple visual evidence of settling in cell culture media as well as with DLS for ζ-potential (Table 1). The order of visual dispersion stability in culture media after 24 hr was OH-SS > COOH-SS > OH-LS > Prist-SS > OH-LB, which was also in agreement with measured ζ-potential (Table 1).

The EC₂₀ of 24-hr doses (Table S1) of each MWCNT to each type of cells (Chatterjee *et al.*, 2016, 2017), were chosen for further mechanistic endpoint, ER stress, MAPK-activation, DNA damage and apoptosis. For viability rescue due to inhibitor exposures, the respective EC₅₀ of 24-hr doses (Table S1) were selected.

Alterations in Calcium Homeostasis and Endoplasmic Reticulum Stress

The treatment with MWCNTs at 24 hr caused depletion of ER calcium stores and significant increase in mitochondrial Ca²⁺ levels in both HepG2 (Fig. 1A) and Beas2B (Fig. 1B) cell lines. Almost all types of MWC-

Table 1. Physicochemical characteristics of MWCNTs.

The MWCNTs Name, [abbreviation], (Catalogue No.)		Pristine: short-short [Prist-SS] (sku-030402)	Carboxylation: short-short [COOH-SS] (sku-03040302)	Hydroxylation: short-short [OH-SS] (sku-03040202)	Hydroxylation: long-short [OH-LS] (sku-030202)	Hydroxylation: long-broad [OH-LB] (sku-030204)
Dimensions and aspect Ratio	Outer Diameter*	8-15 nm	8-15 nm	8-15 nm	8-15 nm	20-30 nm
	Inside Diameter *	3-5 nm	3-5 nm	3-5 nm	3-5 nm	3-5 nm
	Length*	0.5-2.0 μm	0.5-2.0 μm	0.5-2.0 μm	10-30 μm	10-30 μm
	Aspect Ratio (length /outer diameter)	33.33 – 250	33.33 – 250	33.33 – 250	666.67 – 3750	333.33 - 1500
Functionalization measured with XPS	Atomic % of Carbon (C)	99.15	96.72	95.86	96.53	98.07
	Atomic % of Oxygen (O)	0.84	3.28	4.13	3.47	1.94
Zeta Potential (mV) measured with DLS		-19.51 $\pm$ 0.66	-25.40 $\pm$ 1.21	-29.76 $\pm$ 0.45	-21.78 $\pm$ 0.7	-17.59 $\pm$ 0.05
TEM Measurements
Dispersions in cell culture media	0 hr
	24 hr

*Source Material: CCVD MWCNTs; *Process: Split plasma; *Impurity: < 1.5%; *True density: $\sim$ 2.1 g/cm³

* - Data supplied by manufacturer

NTs displayed clear mitochondrial Ca^{2+} level overloading except the OH-LB one, and the order in HepG2 cells was OH-SS > OH-LS $\geq$ Prist-SS > COOH-SS > OH-LB (Fig. 1A) while the order in Beas2B cells was COOH-SS $\geq$ Prist-SS > OH-SS $\geq$ OH-LS > OH-LB (Fig. 1B). Moreover, marked time-dependent increases in mitochondrial Ca^{2+} levels from 4 hr to 24 hr were evident in both types of cell lines (Fig. S1A and S1B). Likewise, depletion of ER Ca^{2+} stores and mitochondrial Ca^{2+} overloading, all types of MWCNTs displayed sharp endoplasmic reticulum (ER) stress. The order of ER stress in HepG2 cells was OH-SS > Prist-SS > OH-LS > COOH-SS > OH-LB (Fig. 1C), whereas, the order of COOH-SS > Prist-SS $\geq$ OH-SS > OH-LS > OH-LB was noticed in Beas2B Cells (Fig. 1D). In addition, the time-dependent increases in ER stress were evident between 4-hr to

24-hr exposures to MWCNTs (Fig. S1C and S1D). The pre-treatment with known calcium chelator, BAPTA, showed rescue in viability, mainly in OH-SS, in both type of cells and COOH-SS in Beas2B (Fig. 1E) which was actually in agreement with mitochondrial Ca^{2+} level overloading (Fig. 1A and Fig. 1B). It is a well-known fact that calcium homeostasis is tightly linked with endoplasmic reticulum function with mitochondrial bioenergetics (Kaufman and Malhotra, 2014). In addition, the ER stress inhibitors, 4 BPA, exhibited viability rescue in almost all MWCNT treatments except OH-LB (Fig. 1E) which further support that the MWCNTs caused ER stress in both types of cells (Fig. 1C and Fig. 1D). To shed light on the effects of MWCNT-mediated Ca^{2+} depletion from ER, we have chosen gene expressions of the three luminal ER Ca^{2+} -regulating proteins (Clapham, 2007; Prins and

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Fig. 1. Determination of calcium homeostasis and endoplasmic reticulum (ER) stress in HepG2 and BEAS-2B cells due to MWCNTs exposures for 24 hr at their respective EC_{20} doses. A. Measurement of mitochondrial Ca^{2+} levels by Rhod2 AM (1 μ M) probing in HepG2 cells. B. Measurement of mitochondrial Ca^{2+} levels by Rhod2 AM (1 μ M) probing in BEAS-2B cells. C. Evaluation of ER staining with ER tracker blue-white DPX probing in HepG2 cells. D. Evaluation of ER staining with ER tracker blue-white DPX probing in BEAS-2B cells. E. Determination of mechanism of mitochondrial Ca^{2+} levels and ER-stress with BAPTA, 4-PBA and solubrilal pretreatment and the rescue of viability due to MWCNTs exposure at their respective EC_{50} doses. F. Alteration of calcium channel-related genes (CAL, IP3R1 and PPP3CA) expressions. G. Variation of ER-stress-related genes (GPR94, GPR78, CHOP, ATF4, ATF6, PERK, eIF2 α , IRE1 α , XBP1) expressions. Data presented as mean $\pm$ SEM, * p < 0.05, ** p < 0.001, *** p < 0.0001.

Fig. 1. (Continued).

Michalak, 2011). The IP3R1 receptor-mediated calcium release from ER were evident in MWCNT-treated HepG2 cells but not in Beas2B cells, while calcineurin (PPP3-CA) up-regulations were observed in both types of cells, except OH-LB-treated Beas2B cells (Fig. 1F). The deregulation of calreticulin (CALR), the Ca^{2+} binding pro-

tein in ER lumen, was observed both in HepG2 cells and Beas2B cells, specifically in the OH-LS- and COOH-SS-exposed ones (Fig. 1F). Furthermore, the expressions of ER stress sensor genes, GRP94, GRP78, PERK, ATF4, eIF2a, CHOP, IRE1a, ATF6, and spliced XBP1 were measured to elucidate the underlying mechanism of

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E **F** **G**

Fig. 1. (Continued).

Fig. 2. Effects of MWCNTs on MAPK (ERK, p-ERK, JNK, p-JNK, p38 and p-p38) protein expressions due to MWCNTs exposure for 24 hr at their respective EC_{20} doses.

Fig. 3. Double-stranded DNA damage measured with γ -H2AX protein expression after 24 hr of exposure of MWCNTs at their respective EC_{20} doses.

MWCNT-mediated ER stress response. The highly significant increases in IRE1a gene expressions were evident in both types of MWCNT-exposed cells, particularly in COOH-SS-treated cells (Fig. 1G). Besides, GRP78 and XBP1 (spliced) gene expressions were also found as significant, specifically in COOH-SS-exposed cells. Conversely, PERK and eIF2a genes did not exhibit significant alterations in their expression, except in OH-SS-exposed cells (Fig. 1G). Thus, MWCNTs treatment, in general, causes ER stress through IRE1a-XBP1 pathway but does not evoke the PERK-eIF2a pathway, except OH-SS, which might possibly elicit both pathways in both cells (Fig. 1G). This hypothesis was further supported by the pretreatments with solubralin, the ER stress inhibitor specific for eIF2a inhibition. The solubralin pretreatment did not show significant rescue in viability of MWCNT-exposed cells, except both OH-SS-treated cells (Fig. 1E). In summary, tissue-specific ER-stress response varies among

surface functionalization as well as length and diameter. Our results showed that in respect of ER-stress and mitochondrial Ca^{2+} overloading, COOH-SS is the most sensitive in Beas2B cells whereas OH-SS is the most sensitive in HepG2 cells and shorter diameter and shorter length possess higher response than their longer counterparts. In agreement with our study, a previous study reported that significant modulation of autophagy-ER stress mediated cytotoxicity in shorter diameter MWCNT in comparison to longer ones (Zhao *et al.*, 2019). Unlike another previous report, we found shorter length MWCNT caused more profound ER-stress response (Long *et al.*, 2017). The reason of differential response might lie in the choice of cell model (Sweeney *et al.*, 2014).

Effect of MWCNTs on MAPK expressions

Very interesting tissue-specific and functionalization-specific effects were evident in MAPKs activa-

Fig. 4. Evaluation of MWCNTs induced apoptosis in HepG2 cells and BEAS-2B cells at their respective EC_{20} doses for 24 hr. A. Measurement of DNA condensation and fragmentation measured by Hoechst 33342 staining in HepG2 cells. B. Measurement of DNA condensation and fragmentation measured by Hoechst 33342 staining in BEAS-2B cells. C. Changes in Bcl-2 (antiapoptotic) and Bax (pro-apoptotic) proteins expression. D. Alteration of FAS and caspases genes (CASP9, CASP8, CASP3) expressions. Data presented as mean $\pm$ SEM, * $p < 0.05$, ** $p < 0.001$.

tions (Fig. 2). The phospho ERK1/2 (p-ERK1/2) were only differentially expressed in Beas2B cells but not in HepG2 cells. The suppression of p-ERK1/2 was evi-

dent due to MWCNTs exposure, specifically in Prist-SS-exposed Beas2B cells. By the same token, phospho p38 (p-p38) was only activated in Beas2B cells and showed

Fig. 4. (Continued).

no changes in HepG2 cells. In particular, OH-LB- and OH-SS-treated Beas2B cells showed significant higher activation of p-p38 than control (Fig. 2). By the way of contrast, phospho JNK (p-JNK) was commonly activated in both types of cells, and the order of activation in Beas2B cells was Prist-SS > COOH-SS > OH-SS ≥ OH-LS > OH-LB while the order of activation in HepG2 cells was OH-LS > OH-SS > Prist-SS > OH-LB > COOH-SS (Fig. 2). In agreement with our study, Sweeney *et al.* (2014) reported markedly altered MAPK (ERK, JNK and p38) signaling proteins responses that vary according to the target cell type, as well as the aspect ratio of the MWCNT. Similar MAPK activation, i.e., activation

of JNK but not ERK1/2, mode was evidenced in MWCNT-exposed IL-1 cell model (Arnoldussen *et al.*, 2015). Besides, MAPK (JNK, 38MAPK, ERK) activation were reported in COOH-MWCNT-treated macrophages (Zhang *et al.*, 2015), conversely, no significant MAPK (ERK, JNK and p38) activation was reported in MWCNT-treated mouse macrophages (Hirano *et al.*, 2008).

DNA damage

The order of double strand break potency, the expressions of γ -H2AX, in MWCNT-exposed HepG2 cells was OH-SS > COOH-SS ≥ Prist-SS > OH-LS ≥ OH-LB, conversely, the order in MWCNT-treated Beas2B cells was

COOH-SS > OH-LS > OH-SS > OH-LB $\geq$ Prist-SS (Fig. 3). Previous studies also support that MWCNT cause DNA strand break and higher γ -H2AX protein expressions (Guo *et al.*, 2011; Cveticanin *et al.*, 2010). The genotoxicity, specifically the DNA double strand break, shed light on the fact of carcinogenic potentiality of MWCNTs (Wang *et al.*, 2011a; Sargent *et al.*, 2014; Nagai *et al.*, 2011; Lindberg *et al.*, 2013; Toyokuni, 2013).

MWCNT-mediated Apoptosis

The MWCNT-induced apoptosis was measured in reference to nuclear morphological criteria by employing Hoechst 33342 staining. The MWCNT-treated cells showed DNA condensation and fragmentation at 24 hr in HepG2 cells (Fig. 4A) as well as Beas2B cells (Fig. 4B) at their respective EC₂₀ doses. In general, Prist-SS showed the highest fluorescence intensity in both types of cells. But the time dependency of apoptosis induction was not clear from 4 hr to 24 hr with Hoechst 33342 staining (Fig. S2A and S2B). The prominent apoptotic features, an increase in BAX and a corresponding decrease in Bcl-2 expressions, were evident in MWCNT-treated cells at respective EC₂₀ doses (Fig. 4C). The imbalance of BAX/BCL2 ratio was most significant in COOH-SS-followed by Prist-SS-treated Beas2B cells; conversely, it was highest in Prist-SS-exposed HepG2 cells followed by OH-SS ones; but the highest BAX, the pro-apoptotic marker, expression was found in OH-SS-exposed HepG2 cells (Fig. 4C). To find out the ER-stress mediated apoptosis, Bax/Bcl-2 expressions were also measured in 4-PBA $\pm$ MWCNT-treated conditions. The alterations in Bax (increased) and Bcl-2 (decreased) expressions due to MWCNT exposures were rescued in 4-PBA pre-treatment (Fig. 4C). In general, MWCNT activates apoptosis pathway through ER-stress, however, the intensity of this cellular response depends on aspect ratio, surface functionalization (Sun *et al.*, 2019). In addition, other executioners of apoptosis, the caspases (CASP9, CASP8, CASP3) and FAS gene expressions displayed further tissue type as well as MWCNTs functionalization specificities (Fig. 4D). The FAS gene was noticeably expressed only in OH-LB and Prist-SS exposed HepG2 cells, whereas it was significantly expressed in all MWCNT-treated Beas2B cells, except OH-SS ones. CASP9 was the most significant gene expressed in HepG2 cells and the order of gene expressions was OH-SS > COOH-SS > Prist-SS > OH-LS > OH-LB. However, CASP8 was the most ascertainably expressed gene in Beas2B cells and the order of expressions was COOH-SS > OH-SS > Prist-SS > OH-LS > OH-LB (Fig. 4D). Therefore, the MWCNT-exposed HepG2 cells might use mitochondrial apoptotic pathway

and Beas2B cells use extrinsic apoptotic pathway. The extrinsic apoptotic pathway was most common in Prist-SS and OH-LB MWCNTs treatment, irrespective of cell types. In agreement with our study, it was demonstrated that MWCNT induced apoptosis via mitochondrial pathway in macrophage cells (Wang *et al.*, 2012), in rat lung epithelial cells (Ravichandran *et al.*, 2009). In contrast to our study, it is reported that hydroxylation (Liu *et al.*, 2014b) and carboxylation (Liu *et al.*, 2014a) of MWCNT decreased the mitochondrial apoptotic pathway and thereby functionalization of MWCNT reduced cytotoxicity. This might be because of variations in cell types as well as dimension and aspect ratio of MWCNT.

Taken together, we postulate that MWCNT induce activation of IRE1 α -XPB1 pathway mediated ER-stress response, which triggers apoptosis through JNK (Tabas and Ron, 2011; Mekahli *et al.*, 2011).

In summary, we found that cellular responses vary according to the target cell type, aspect ratio as well as the surface functionalization of the MWCNT. In general, HepG2 cells were more sensitive to hydroxylated MWCNT (short diameter, short length) while Beas2B cells were more sensitive to carboxylated (short diameter, short length) MWCNT. Significant increase in ER Ca²⁺ depletion and mitochondrial Ca²⁺ overloading, ER-stress, DNA double strand damage, MAPK (JNK) activation and increased apoptosis in MWCNT exposed cells. Most probably, the IRE1 α -XPB1 pathway mediated ER-stress response trigger apoptosis through JNK in MWCNT-treated cells. The results of the present study could be useful for regulatory consideration as well as safe-by-design concept of MWCNT applications, in particular, for biomedical applications.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

- Albini, A., Pagani, A., Pulze, L., Bruno, A., Principi, E., Congiu, T., Gini, E., Grimaldi, A., Bassani, B., De Flora, S., de Eguileor, M. and Noonan, D.M. (2015): Environmental impact of multi-wall carbon nanotubes in a novel model of exposure: systemic distribution, macrophage accumulation, and amyloid deposition. *Int. J. Nanomedicine*, **10**, 6133-6145.
- Arnoldussen, Y.J., Skogstad, A., Skaug, V., Kasem, M., Haugen, A., Benker, N., Weinbruch, S., Apte, R.N. and Zienolddiny, S.

- (2015): Involvement of IL-1 genes in the cellular responses to carbon nanotube exposure. *Cytokine*, **73**, 128-137.
- 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.
- Castranova, V., Schulte, P.A. and Zumwalde, R.D. (2013): Occupational nanosafety considerations for carbon nanotubes and carbon nanofibers. *Acc. Chem. Res.*, **46**, 642-649.
- Chang, S., Zhao, X., Li, S., Liao, T., Long, J., Yu, Z. and Cao, Y. (2018): Cytotoxicity, cytokine release and ER stress-autophagy gene expression in endothelial cells and alveolar-endothelial coculture exposed to pristine and carboxylated multi-walled carbon nanotubes. *Ecotoxicol. Environ. Saf.*, **161**, 569-577.
- Chatterjee, N., Yang, J., Kim, S., Joo, S.W. and Choi, J. (2016): Diameter size and aspect ratio as critical determinants of uptake, stress response, global metabolomics and epigenetic alterations in multi-wall carbon nanotubes. *Carbon*, **108**, 529-540.
- Chatterjee, N., Yang, J., Yoon, D., Kim, S., Joo, S.W. and Choi, J. (2017): Differential crosstalk between global DNA methylation and metabolomics associated with cell type specific stress response by pristine and functionalized MWCNT. *Biomaterials*, **115**, 167-180.
- Clapham, D.E. (2007): Calcium signaling. *Cell*, **131**, 1047-1058.
- Cveticanin, J., Joksic, G., Leskovic, A., Petrovic, S., Sobot, A.V. and Neskovic, O. (2010): Using carbon nanotubes to induce micronuclei and double strand breaks of the DNA in human cells. *Nanotechnology*, **21**, 015102.
- Garg, A.D., Kaczmarek, A., Krysko, O., Vandenabeele, P., Krysko, D.V. and Agostinis, P. (2012): ER stress-induced inflammation: does it aid or impede disease progression? *Trends Mol. Med.*, **18**, 589-598.
- Ghosh, M., Murugadoss, S., Janssen, L., Cokic, S., Mathysen, C., Van Landuyt, K., Janssens, W., Carpentier, S., Godderis, L. and Hoet, P. (2020): Distinct autophagy-apoptosis related pathways activated by Multi-walled (NM 400) and Single-walled carbon nanotubes (NIST-SRM2483) in human bronchial epithelial (16HBE14o-) cells. *J. Hazard. Mater.*, **387**, 121691.
- Guo, Y.Y., Zhang, J., Zheng, Y.F., Yang, J. and Zhu, X.Q. (2011): Cytotoxic and genotoxic effects of multi-wall carbon nanotubes on human umbilical vein endothelial cells *in vitro*. *Mutat. Res.*, **721**, 184-191.
- Hamilton, R.F. Jr., Wu, Z., Mitra, S., Shaw, P.K. and Holian, A. (2013): Effect of MWCNT size, carboxylation, and purification on *in vitro* and *in vivo* toxicity, inflammation and lung pathology. *Part. Fibre Toxicol.*, **10**, 57.
- Han, Y.G., Xu, J., Li, Z.G., Ren, G.G. and Yang, Z. (2012): In vitro toxicity of multi-walled carbon nanotubes in C6 rat glioma cells. *Neurotoxicology*, **33**, 1128-1134.
- Harding, H.P., Calfon, M., Urano, F., Novoa, I. and Ron, D. (2002): Transcriptional and translational control in the Mammalian unfolded protein response. *Annu. Rev. Cell Dev. Biol.*, **18**, 575-599.
- Heister, E., Brunner, E.W., Dieckmann, G.R., Jurewicz, I. and Dalton, A.B. (2013): Are carbon nanotubes a natural solution? Applications in biology and medicine. *ACS Appl. Mater. Interfaces*, **5**, 1870-1891.
- Hirano, S., Kanno, S. and Furuyama, A. (2008): Multi-walled carbon nanotubes injure the plasma membrane of macrophages. *Toxicol. Appl. Pharmacol.*, **232**, 244-251.
- Kaufman, R.J. and Malhotra, J.D. (2014): Calcium trafficking integrates endoplasmic reticulum function with mitochondrial bioenergetics. *Biochim. Biophys. Acta*, **1843**, 2233-2239.
- Kim, J.S., Lee, K., Lee, Y.H., Cho, H.S., Kim, K.H., Choi, K.H., Lee, S.H., Song, K.S., Kang, C.S. and Yu, I.J. (2011): Aspect ratio has no effect on genotoxicity of multi-wall carbon nanotubes. *Arch. Toxicol.*, **85**, 775-786.
- Lanone, S., Andujar, P., Kermanizadeh, A. and Boczkowski, J. (2013): Determinants of carbon nanotube toxicity. *Adv. Drug Deliv. Rev.*, **65**, 2063-2069.
- Lee, J.K., Sayers, B.C., Chun, K.S., Lao, H.C., Shipley-Phillips, J.K., Bonner, J.C. and Langenbach, R. (2012): Multi-walled carbon nanotubes induce COX-2 and iNOS expression via MAP kinase-dependent and -independent mechanisms in mouse RAW264.7 macrophages. *Part. Fibre Toxicol.*, **9**, 14.
- 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 multiwall carbon nanotubes determine pulmonary toxicity. *ACS Nano*, **7**, 2352-2368.
- Lindberg, H.K., Falck, G.C., Singh, R., Suhonen, S., Järventaus, H., Vanhala, E., Catalán, J., Farmer, P.B., Savolainen, K.M. and Norppa, H. (2013): Genotoxicity of short single-wall and multi-wall carbon nanotubes in human bronchial epithelial and mesothelial cells *in vitro*. *Toxicology*, **313**, 24-37.
- Liu, D., Wang, L., Wang, Z. and Cuschieri, A. (2012): Different cellular response mechanisms contribute to the length-dependent cytotoxicity of multi-walled carbon nanotubes. *Nanoscale Res. Lett.*, **7**, 361.
- 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. (2014a): 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.
- Liu, Z., Liu, Y. and Peng, D. (2014b): Hydroxylation of multi-walled carbon nanotubes reduces their cytotoxicity by limiting the activation of mitochondrial mediated apoptotic pathway. *J. Mater. Sci. Mater. Med.*, **25**, 1033-1044.
- Long, J., Ma, W., Yu, Z., Liu, H. and Cao, Y. (2019): Multi-walled carbon nanotubes (MWCNTs) promoted lipid accumulation in THP-1 macrophages through modulation of endoplasmic reticulum (ER) stress. *Nanotoxicology*, **13**, 938-951.
- Long, J., Xiao, Y., Liu, L. and Cao, Y. (2017): The adverse vascular effects of multi-walled carbon nanotubes (MWCNTs) to human vein endothelial cells (HUVECs) *in vitro*: role of length of MWCNTs. *J. Nanobiotechnology*, **15**, 80.
- Malhotra, J.D. and Kaufman, R.J. (2007): Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid. Redox Signal.*, **9**, 2277-2293.
- Malhotra, J.D. and Kaufman, R.J. (2011): ER stress and its functional link to mitochondria: role in cell survival and death. *Cold Spring Harb. Perspect. Biol.*, **3**, a004424.
- Mekahli, D., Bultynck, G., Parys, J.B., De Smedt, H. and Missiaen, L. (2011): Endoplasmic-reticulum calcium depletion and disease. *Cold Spring Harb. Perspect. Biol.*, **3**, a004317.
- Nagai, H., Okazaki, Y., Chew, S.H., Misawa, N., Yamashita, Y., Akatsuka, S., Ishihara, T., Yamashita, K., Yoshikawa, Y., Yasui, H., Jiang, L., Ohara, H., Takahashi, T., Ichihara, G., Kostarelos, K., Miyata, Y., Shinohara, H. and Toyokuni, S. (2011): Diameter and rigidity of multiwalled carbon nanotubes are critical factors

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- in mesothelial injury and carcinogenesis. *Proc. Natl. Acad. Sci. USA*, **108**, E1330-E1338.
- Pinton, P., Giorgi, C., Siviero, R., Zecchini, E. and Rizzuto, R. (2008): Calcium and apoptosis: ER-mitochondria Ca^{2+} transfer in the control of apoptosis. *Oncogene*, **27**, 6407-6418.
- Poulsen, S.S., Saber, A.T., Williams, A., Andersen, O., Købler, C., Atluri, R., Pozzebón, M.E., Mucelli, S.P., Simion, M., Rickerby, D., Mortensen, A., Jackson, P., Kyjovska, Z.O., Mølhave, K., Jacobsen, N.R., Jensen, K.A., Yauk, C.L., Wallin, H., Halappanavar, S. and Vogel, U. (2015): MWCNTs of different physicochemical properties cause similar inflammatory responses, but differences in transcriptional and histological markers of fibrosis in mouse lungs. *Toxicol. Appl. Pharmacol.*, **284**, 16-32.
- Prato, M., Kostarelos, K. and Bianco, A. (2008): Functionalized carbon nanotubes in drug design and discovery. *Acc. Chem. Res.*, **41**, 60-68.
- Prins, D. and Michalak, M. (2011): Organellar calcium buffers. *Cold Spring Harb. Perspect. Biol.*, **3**, a004069.
- Ravichandran, P., Periyakaruppan, A., Sadanandan, B., Ramesh, V., Hall, J.C., Jejelowo, O. and Ramesh, G.T. (2009): Induction of apoptosis in rat lung epithelial cells by multiwalled carbon nanotubes. *J. Biochem. Mol. Toxicol.*, **23**, 333-344.
- Romagnoli, A., Aguiari, P., De Stefani, D., Leo, S., Marchi, S., Rimessi, A., Zecchini, E., Pinton, P. and Rizzuto, R. (2007): Endoplasmic reticulum/mitochondria calcium cross-talk. *Novartis Found. Symp.*, **287**, 122-131, discussion, 131-139.
- Sargent, L.M., Porter, D.W., Staska, L.M., Hubbs, A.F., Lowry, D.T., Battelli, L., Siegrist, K.J., Kashon, M.L., Mercer, R.R., Bauer, A.K., Chen, B.T., Salisbury, J.L., Frazer, D., McKinney, W., Andrew, M., Tsuruoka, S., Endo, M., Fluharty, K.L., Castranova, V. and Reynolds, S.H. (2014): Promotion of lung adenocarcinoma following inhalation exposure to multi-walled carbon nanotubes. *Part. Fibre Toxicol.*, **11**, 3.
- Shen, X., Zhang, K. and Kaufman, R.J. (2004): The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum. *J. Chem. Neuroanat.*, **28**, 79-92.
- Shvedova, A.A., Pietroiusti, A., Fadeel, B. and Kagan, V.E. (2012): Mechanisms of carbon nanotube-induced toxicity: focus on oxidative stress. *Toxicol. Appl. Pharmacol.*, **261**, 121-133.
- Sun, Y., Gong, J. and Cao, Y. (2019): Multi-Walled Carbon Nanotubes (MWCNTs) Activate Apoptotic Pathway Through ER Stress: Does Surface Chemistry Matter? *Int. J. Nanomedicine*, **14**, 9285-9294.
- Sweeney, S., Berhanu, D., Misra, S.K., Thorley, A.J., Valsami-Jones, E. and Tetley, T.D. (2014): Multi-walled carbon nanotube length as a critical determinant of bioreactivity with primary human pulmonary alveolar cells. *Carbon N Y*, **78**, 26-37.
- Tabas, I. and Ron, D. (2011): Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. *Nat. Cell Biol.*, **13**, 184-190.
- Toyokuni, S. (2013): Genotoxicity and carcinogenicity risk of carbon nanotubes. *Adv. Drug Deliv. Rev.*, **65**, 2098-2110.
- Vardharajula, S., Ali, S.Z., Tiwari, P.M., Eroglu, E., Vig, K., Dennis, V.A. and Singh, S.R. (2012): Functionalized carbon nanotubes: biomedical applications. *Int. J. Nanomedicine*, **7**, 5361-5374.
- Wang, L., Luanpitpong, S., Castranova, V., Tse, W., Lu, Y., Pongrakhananon, V. and Rojanasakul, Y. (2011a): Carbon nanotubes induce malignant transformation and tumorigenesis of human lung epithelial cells. *Nano Lett.*, **11**, 2796-2803.
- Wang, X., Guo, J., Chen, T., Nie, H., Wang, H., Zang, J., Cui, X. and Jia, G. (2012): Multi-walled carbon nanotubes induce apoptosis via mitochondrial pathway and scavenger receptor. *Toxicol. In Vitro*, **26**, 799-806.
- 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. (2011b): 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.
- Zhang, T., Tang, M., Kong, L., Li, H., Zhang, T., Xue, Y. and Pu, Y. (2015): Surface modification of multiwall carbon nanotubes determines the pro-inflammatory outcome in macrophage. *J. Hazard. Mater.*, **284**, 73-82.
- Zhao, X., Chang, S., Long, J., Li, J., Li, X. and Cao, Y. (2019): The toxicity of multi-walled carbon nanotubes (MWCNTs) to human endothelial cells: The influence of diameters of MWCNTs. *Food Chem. Toxicol.*, **126**, 169-177.