The response effect of pheochromocytoma (PC12) cell lines to oxidized multi-walled carbon nanotubes (*o*-MWCMTs)

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Abstract

Background: The applications of oxidized carbon nanotubes (o-CNTs) have shown potentials in novel drug delivery including the brain which is usually a challenge. This underscores the importance to study its potential toxic effect in animals. Despite being a promising tool for biomedical applications little is known about the safety of drugs in treating brain diseases. The toxicity of oxidized multi-walled carbon nanotubes (*o*-MWCNTs) are of utmost concern and in most *in-vitro* studies conducted so far are on dendritic cell (DC) lines with limited data on PC12 cell lines.

Objectives: We focused on the effect of *o*-MWCNTs in PC12 cells *in vitro:* a common model cell for neurotoxicity.

Methods: The pristine multi-walled carbon nanotubes (*p*-MWCNTs) were produced by the swirled floating catalytic chemical vapour deposition method (SFCCVD). The *p*-MWCNTs were then oxidized using purified H_2SO_4/HNO_3 (3:1v/v) and 30% HNO_3 acids to produce *o*-MWCNTs. The Brunauer-Emmett-Teller (BET), transmission electron microscopy (TEM), Scanning electron microscopy (SEM), thermogravimetric analyser (TGA) and Raman spectroscopy techniques were used to characterize the MWCNTs. The PC12 cells were cultured in RPMI medium containing concentrations of *o*-MWCNTs ranging from 50 to 200 µg/ml.

Results: The *o*-MWCNTs demonstrated slight cytotoxicity at short time period to PC12 neuronal cells whilst at longer time period, no significant ($p > 0.05$) toxicity was observed due to cell recovery.

Conclusion: In conclusion, the *o*-MWCNTs did not affect the growth rate and viability of the PC12 cells due to lack of considerable toxicity in the cells during the observed time period but further investigations are required to determine cell recovery mechanism.

Keywords: Oxidized carbon nanotubes, PC12, cellular response, toxicity

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Introduction

Carbon nanotubes (CNTs) are molecular-scale tubes of graphitic carbon that possess excellent mechanical, magnetic and electrical properties due to their unique topology and structure.[1,2]. The versatility has attracted intense research in biomedical applications such as drug delivery[3,4], medical imaging[5], cancer treatment[6,7] and the treatment of brain diseases[8,9]. However, toxicological information on functionalized carbon-containing particles is limited and published data on the toxic effects of CNTs are often contradictory[10,11,12]. The toxicity studies of both *p*-MWCNTs (un-functionalized carbon nanotubes) and *o*-MWCNTs (functionalized carbon nanotubes versatile for drug and other bio-conjugates attachment) conducted so far on pheochromocytoma (PC12) cells are limited. Most of the studies are done in dendritic cells with very limited studies on PC12 cells. The PC12 cells are derived from rat adrenal medullary tumor and are widely used to study responses of differentiated neuronal cells[13]. The aim of this study is to study the effect on *o*-MWCNTs in PC12 cells *in vitro:* a common model cell for neurotoxicity. Reports by Xu et al[14] have showed that carboxyl-terminated MWCNTs can suppress potassium channel activities in PC12 cells in a time dependent and irreversible manner. Other reports by Wang et al[15] found a

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decrease in cell viability of PC12 cells due to single walled CNTs (SWCNTs) expressing oxidative stress. Using other cell lines, Zhang et al[16] showed that acid treated MWCNTs and SWCNTs have an increased cytotoxic effect in human cervical carcinoma HeLa cells when compared to the untreated CNTs (pristine CNTs). Also Bottini and colleagues[17] compared the toxicity of pristine MWCNTs to *o*-MWCNTs on human T lymphocyte cells. They found that *o*-MWCNTs were more toxic than *p*-MWCNTs, with the formal inducing loss of cell viability through apoptosis at dosses of 400 μg/ml.

Methods

Chemicals and reagents

The ferrocene, sulphuric and nitric acid, methanol, diethyl ether and ethanol were obtained from Merck Chemicals (Pty) Ltd, South Africa. The argon and acetylene gases were obtained from Afrox Ltd South Africa. The RPMI medium, fetal bovine serum, and horse serum (Sigma-Aldrich, St Louis, MO, USA) and other reagents used were all of analytical grade.

Production of carbon nanotubes

The modification of the chemical vapour deposition (CVD), termed the swirled floating catalyst (SFCCVD)[18] was used to produce the *p*-MWCNTs. It consists of a vertical silica plug flow reactor that is immersed in a furnace connected to a temperature regulator with valves, rotameter and a pressure controller. The CNTs were produced by the catalytic decomposition of acetylene in argon gas flow using ferrocene as the catalyst. The production was carried out at a reactor temperature range of 700 – 900 ºC and ferrocene heated at 150ÚC for 1 h reaction time using acetylene with flow rate of 100 ml/min. The transmission electron microscopy(TEM, JEOL JEM-100S), scanning electron microscopy (SEM), Brunauer-Emmett-Teller (BET, Micromeritics Tristar), Raman spectroscopy, and Thermogravimetric analysis (TGA, Perkin Elmer TGA 4000 Thermogravimetric Analyser) were used to characterise the synthesized CNTs.

Functionalization of carbon nanotubes.

The pristine MWCNTs (p-MWCNTs) were oxidised using $H2SO_4$/HNO3 in a 3:1 (v/v) and 30% HNO_3 acids to create the carboxylic groups (-COOH) by sonication. The solution was then filtered and washed several times with distilled water to remove residual acids (pH neutal) and then dried at 50ºC in an oven. The oxidation procedure modifies the *p*-MWCNTs surfaces with carboxylic groups resulting into MWCNT-COOH (figure. 1), which are required for further coupling[4]. The *o*-MWCNTs were then sterilely (filtration) and serially (10-fold dilution) diluted in RPMI medium in a biosafety cabinet and sonicated as stock solution.

PC12 neuronal cell culture

PC12 cells (obtained from the Department of Pharmacy and Pharmacology, University of the Witwatersrand, South Africa) were maintained in RPM1 medium supplemented with 10% v/v donor horse serum, 5% v/v fetal bovine serum and 1% v/v PenStrap (penicillin and streptomycin). All cells were cultured in plastic culture flasks at 37ÚC in a 5% CO_2 humidified incubator (HERA Cell, Thermo Electron Corporation).

Cell-viability

A CytoTox-Glo assay was used to measure cell viability. All samples were sterilized for 24 h under UV irradiation. The PC12 cells were seeded in each well containing 0, 50, 100 and 200 μg/ml of *o*-MWCNTs. They were then incubated at 0, 2, 4, 22 and 24 hr.

Cytotoxicity of carbon nanotubes towards PC-12 neuronal cells

To determine the cytotoxicity of CNTs in PC-12 neuronal cells, the CytoTox-Glo™ Promega assay (Madison, USA) was used according to the manufacturer's instructions (Niles et al 2007).

In a 96-well plate, cells were prepared by adding 12μl of PC12 cell culture to each well in triplicates. Twelve microliter of freshly prepared sterile media containing CNTs (containing 2, 50, 100 and 200 μg/ml of *o*-MWCNTs) were then added to the 96-well plates containing cells. The cells were then added and cultured for not more than 24 hr, as recommended by the manufacturer. After 2, 4, 22 and 24hr of incubation 12.5μl of CytoTox-Glo Cytotoxicity Reagent was added to all wells. The 96-well plate was then taken to the Victor X3 and mixed by orbital shaking for about 30 seconds and incubated at room temperature for 15 min. The luminescence was then measured onVictor X3 (PerkinElmer) and recorded (representing signal obtained from dead cells). 12.5μl of lysis reagent (Assay kit) was then added to each and mixed again by orbital shaking for 30 seconds and incubated at room temperature for 15 minutes. The Luminescence

was recorded again on Victor X3 (Signal obtained from total cell population). This procedure was repeated at 4, 22 and 24 hr incubation, to obtain the luminescent contribution to viable cells.

Morphometric analysis of PC12 cells

Cells were placed onto a glass slide using a micropipette and covered with a cover slip before viewing. The morphology of PC12 cells after treatment was studied under a bright field microscope (Olympus Optical CO. Ltd, Tokyo, Japan).

Statistical Analyses

Statistical analyses were performed using the analysis of variance (One-Way ANOVA) to compare between the triplicates and Student T-test to compared between p-MWCNTs and *o*-MWCNTs using the Origin 6.0 Professional software and was considered statistically significant $p < 0.05$. All the experiments were done in triplicates and data shown as mean ± SD.

Ethics Clearance

The proposed study was submitted for ethical consideration and approval by the University of the Witwatersrand Research Ethics Committee.

Results

Figure 1 shows the creation of the *o*-MWCNTs from *p*-MWCNTs and how carboxyl (-COOH) groups are aligned on the *o*-MWCNTs. The o-MWCNTs in this case is known as functionalized MWCNT with carboxylic ends (MWCNT-COOH).

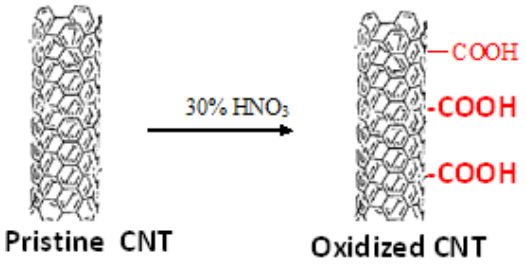

Figure 1: Oxidation of pristine MWCNT to create MWCNT-COOH

The *o*-MWCNTs and *p*-MWCNTs morphologies were viewed using the TEM and SEM. The TEM micrograph structures of *p*-MWCNTs are shown in figure. 2a, while those of the *o*-MWCNTs (1:3 HNO_3:H_2SO_4 and 30% HNO_3 acids) are shown in figures 2b and 2c. Figure 3a shows the SEM micrograph of *p*- MWCNTs while Figure 3b 30% HNO_3 oxidized and figure 3c 1:3 HNO_3:H_2SO_4 oxidized MWCNTs. Both the *p*-MWCNTs and o-MWCNTs sizes ranged from 53 ± 12 nm in diameter and length of 2.5 ± 0.5 μm.

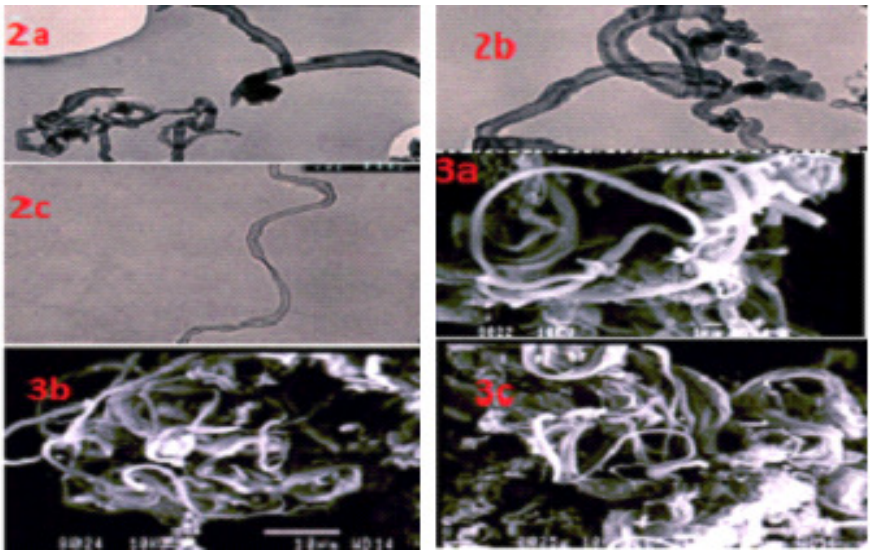

Figure 2a: TEM micrograph of pristine MWCNTs, 2b: Oxidized MWCNTs after1:3 HNO_3:H_2SO_4 acid treatment and 2c: 30% HNO_3 oxidized MWCNTs.
Figures 3a: SEM Micrograph of *p*- MWCNTs, 3b: 30% HNO_3 oxidized and 3c: 1:3 HNO_3:H_2SO_4 oxidized MWCNTs.

The Brunauer-Emmett-Teller (BET) analysis of the various MWCNT are shown in table 1. The surface areas (SA) ranged from 29.5-36.0m^2/g, average pore volume (0.1-0.16 cm^2/g) and average pore diameter of pristine (13.4-20.6nm).

The Raman spectroscopy analysis of the pristine, 30% HNO_3 and 1:3 HNO_3:H_2SO_4 treated MWCNTs are shown in table 2. The band positions were found to range from 1321 cm^{-1}- 1327 cm^{-1} with ID/IG ration of 30% HNO_3 treated MWCNTs at 1.01 while that of pristine 1.03 and 1:3 HNO_3:H_2SO_4 at 1.39.

The TGA analysis was employed to determine the quality[19] and chemical stability of MWCNTs and to estimate the amount of residual catalyst present in the sample before and after oxidation/purification[20]. The TGA curves of *p*-MWCNTs, 30% HNO_3 and 1:3 HNO_3:H_2SO_4 acid treated MWCNTs are represented in Figure. 4. The *p*-MWCNTs were found to decompose easily and reported the highest value of metal catalyst (13.20%). The 30% HNO_3 oxidation showed 9.70% while 1:3 HNO_3:H_2SO_4 reported catalyst impurities of 11.67%. Thus the 30% HNO_3 treatment removed more metal catalysts than the 1:3 HNO_3:H_2SO_4. Thermal stability of MWCNTs depend on the side wall "defects" and the amount of metallic impurities.

Table 1: BET results of MWCNTs

Sample	SA (m^2/g)	Average pore volume (cm^3/g)	Average pore diameter (nm)
Pristine MWCNTs	31.6	0.16	20.6
30% HNO_3 treated MWCNTs	36.0	0.16	17.3
1:3 HNO_3:H_2SO_4	29.5	0.10	13.4

Table: 2 Raman spectra results of pristine, 30% HNO_3 and 1:3 HNO_3:H_2SO_4 MWCNTs treated

Sample intensity	Band position Intensity ratio		Band position Band width		Band intensity Band width		B a n d
	D1	G	ID	IG	ID/IG	D1	G
Pristine MWCNTs	1324	1587	37	36	1.03	194	75
30% HNO_3 acid treated MWCNTs	1321	1584	72	71	1.01	182	74
1:3 HNO_3:H_2SO_4 acid treated MWCNTs	1327	1582	36	26	1.39	159	97

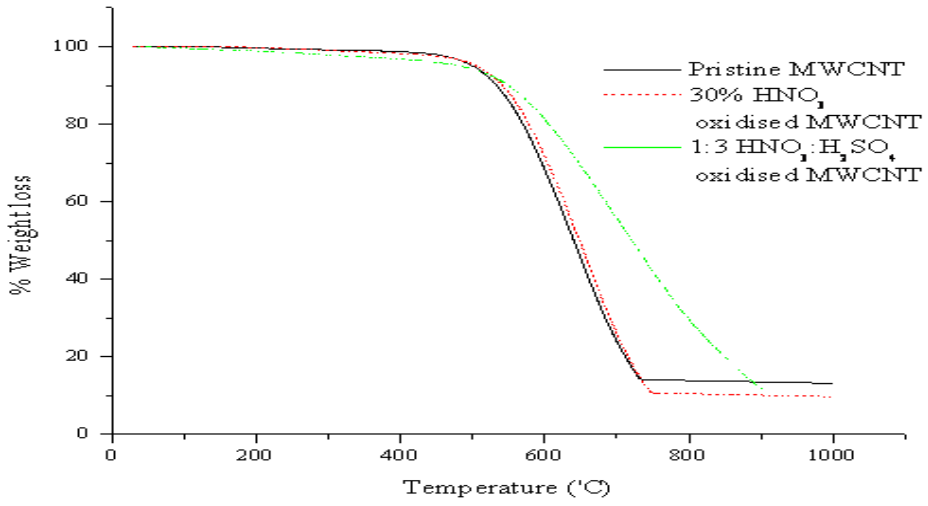

Figure 4: Thermal stability of pristine, 30% HNO_3 and 1:3 HNO_3:H_2SO_4 oxidised MWCNTs

The undifferentiated PC12 cells were used to evaluate the cell viability of 30% HNO_3 as shown in Figure 5. The 30% HNO_3 *o*-MWCNTs was used because of the purity when compared to *p*-MWCNTs and 1:3 HNO_3:H_2SO_4 acid treated. There was a no significant difference between the *p*-MWCNTs and *o*-MWCNTs ($p<0.05$).

In this study the Promega assay was utilized to determine the effect of various concentrations of o-MWCNTs on the viability of cultured PC12 cells as shown in figure 5. After 2 hours of incubation, the 50 µg/ml *o*-MWCNTs resulted in no loss in cell viability whilst after 4hr of incubation the percentage of viable cells was reduced to 79% as compared to the control group (incubated with no MWCNTs).The 100 µg/ml and 200 µg/ml *o*-MWCNT showed a reduction in cell viability from 84 and 61% respectively in 2 hours when compared to the control group. After 4 hours the reduction in viability was observed in all the 3 concentrations (50, 100 and 200 µg/ml). The cells were found to recover at longer period times of incubation. At 22 hours, 87 % of the cells in 50 to 200 µg/ml o-MWCNT concentrations recovered when compared to the control groups. The recovery was found to increase up to 24 hours of incubation with no loss in cell viability. The effect of high concentrations of *o*-MWCNTs on the viability of PC12 cells was found suppressive only at earlier periods of incubation and this suppressive effect was concentration dependent. This indicates that at shorter (2 and 4hours) incubation in *o*-MWCNTs affect PC12 cells while at longer period, regeneration can occurs due to adaptation in the *o*-MWCNTs.

To examine the morphology of PC12 cells, a bright field microscope was used, figure 6 (a)-(b). Figure 6a shows the cells cultured without MWCNTs while Figure 6b shows a typical image of PC12 cells cultured with *o*-MWCNTs .The microscopic observation showed that the PC12 cells grew and spread with the *o*-MWCNTs by adhering to the *o*-MWCNTs (figure 6b). The untreated cells exhibited normal growth with organized cellular structures (figure 6a).

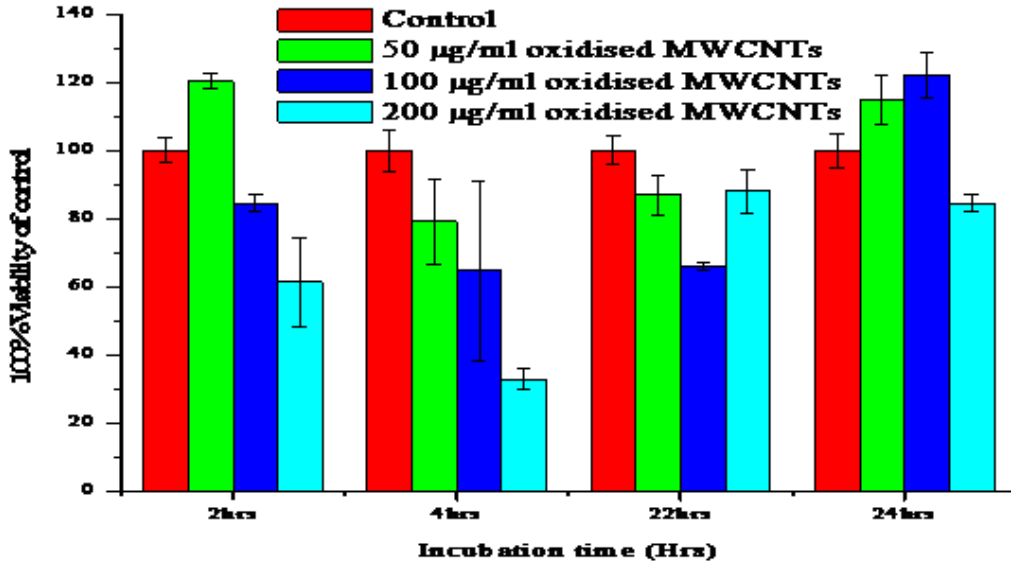

Figure 5: The response effect of PC12 cells to oxidised MWCNTs. Error bars represent standard deviation (SD) of one set of triplicate data

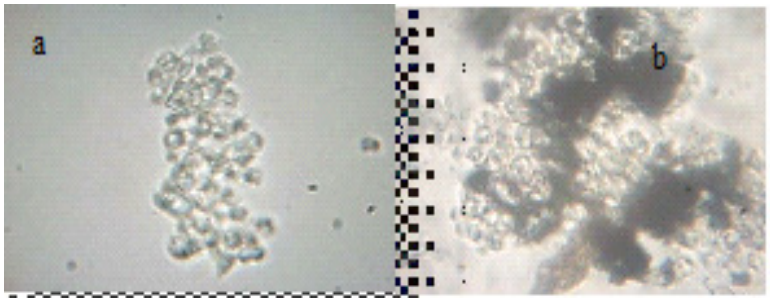

Figure 6: Optical phase contrast microscope photographs of PC12 cells (6a) incubated without oxidized MWCNTs (6b) incubated with oxidized MWCNTs

Discussion

The synthesis of *p*-MWCNTs are widely reported in literature with varied diameters and lengths[1,18] as well as *o*-MWCNTS[2]**.** The results obtained were similar to those earlier reported by Abdulkareem et al[21] and Yah et al[22] with similar diameters. The SEM images of the *p*-MWCNTs and *o*-MWCNTS are shown in Figures.3a-c with length distribution of 1± 0.5 μm. The *o*-MWCNTs showed different degrees of oxidation with shorter lengths as compared to the *p*-MWCNTs. These results were similar to those reported by Cheng et al[20] who showed the oxidation MWCNTs with 1:3 v/v HNO_3:H_2SO_4 acid. However, their lengths and sizes (500nm to 5 μm and 940- 974nm) were by far greater than those reported in this study (1± 0.5 μm and 53 ± 12 nm) which may have resulted from the sonication process. The sonication process may lead to destruction of the conjugate bonds on the *o*-MWCNTS as well as preventing aggregation and agglomeration[4].

There was no significant difference between the surface area of the *p*-MWCNTS and *o*-MWCNTS. Though the surface area was not as large as those reported by Bacsa et al[23], it was sufficient enough for analysis.

The Raman spectra differed from those reported by Bacsa et al[23] and Yah et al[18] with higher proportion of tubular carbon. However there was a no significant difference between the *p*-MWCNTs and *o*-MWCNTs ($p<0.05$) because the p-MWCNTS and *o*-MWCNTS ID/IG ratio were closer to 1. The determination of the purity of the *o*-MWCNTS showed 30% HNO_3 acid oxidation the most applicable method similar to that reported by Phillips et al [2], Dettlaff-Weglikowska et al[24] and Kim et al[25].

Reports on functionalized MWCNTs toxicity pro-file on neuronal cells are limited. In this study we reported the effect *o*-MWCNTs on PC12 neuronal cells. The findings demonstrate that high (100μg/ml and 200μg/ml) concentrations of *o*-MWCNTs can inhibit PC12 cells proliferation when compared to lower (50 μg/ml) concentrations. These were similar to those reported by Bang et al[26] that at sufficiently high concentrations, MWCNTs can inhibit reduced PC12 viability. According to reports by Meng et al[27] short exposure of MWCNTs to neuronal cells can lead to up-regulation and expression of neurotrophin pathways with unknown underlying mechanism. Earlier reports by Xu et al [14] showed that carboxyl-terminated MWCNTs can antagonize the 3 types of potassium channels on undifferentiated PC12 cells. However the potassium channels cell alteration had no significant effect in the generation of reactive oxygen species (ROS)[14]. The ROS may not have been involved in the potassium suppression but the elemental suppression can give a huge impact on neurone electrical impulse transmission and excitation processes[14].

Since the inhibition of the PC12 cells was found to be dose dependent and at longer incubation period could recover has been observed in similar earlier reports[11,14]. This phenomenon has been reported by Cheng et al[11] that shorter exposure time of 0.01mg/ml of *o*-MWCNTs on endothelial cells can cause a slight reduction in cells viability and at longer period a huge cells recovery. This was supported by Xu et al [14] that MWCNT-COOH are time-dependent on PC12 cells but can be irreversible as well. Furthermore Wang et al[15] reported that long and short pristine SWCNTs can cause decrease in PC12 cells viability which are time and dose dependent arising from oxidative stress to nervous system due to neuronal cells injuries. The bright field morphological structures of *o*-MWCNTs on PC12 cells (Figures 6a & 6b) showed that of *o*-MWCNTs has no effect on PC12 cells. They were found to be internalized in the PC12 cells. The bright field micrographs results were similar to those reported by Raffa et al[28] where *o*-MWCNTs was found to internalize in PC12 cells. Although this can be influence by the size and length of the *o*-MWCNTs[28]. However, the mechanism of *o*-MWCNT penetration into PC12 cells is yet to be elucidated and the more information needed to augment *o*-MWCNTs PC12 cells toxicity. The induction of PC12 cell viability according to the findings by Meng et al[27] Iron (Fe) impurities embedded in the *o*-MWCNTs during the production process can be responsible for the PC12 induction. Therefore, the dynamics of *o*-MWCNTs cellular involvement in PC12 toxicity and safe applications need further studies.

Conclusion

The quality of purified MWCNTs resulting from the 30% HNO_3 acid treatment was higher than that of 1:3 HNO_3:H_2SO_4 acid treated MWCNTs. The response of *o*-MWCNTs in the PC12 cells was both concentration and time dependent. PC12 cells inhibition was observed at shorter incubation period (2-4 hours). However, at long exposure periods (22-24 hours) there was massive cells recovery in the present of *o*-MWCNT doses. Generally the *o*-MWCNTs *in-vitro* toxicity studied in PC12 cells

showed very minimal adverse effect but their use in biomedical applications need further safety investigation as well as mechanism of cell recovery at longer periods of exposure.

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