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Full Length Research Paper

In vitro cytotoxicity and induction of apoptosis by multiwalled carbon nanotubes in human peripheral lymphocytes: Correlation with physicochemical properties

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Multiwalled carbon nanotubes (MWCNTs) consist of more than 80% of the current nanomaterials' applications worldwide. Despite their wide application, little information is known concerning their impact on human health. The current study aims to identify the *in vitro* effects of exposure of the human peripheral blood lymphocytes (PBL) to MWCNTs and the possible associations with their physiochemical properties. Two doses (50 and 500 µg/ml) of four different commercially available MWCNTs (obtained from Frascati Laboratory, Sigma Co., Sun Nanotech Co. and Shenzhen Co.) were used in this study. Cell viability and apoptotic activity were evaluated by trypan blue exclusion test and Annexin-V/PI staining in peripheral blood leukocytes (PBL). Physicochemical properties of the different MWCNTs were determined and correlated with the cytotoxicity results after exposure for 12 and 48 h. Results indicate that PBL exposed to MWCNTs showed decreased cell viability and increased apoptosis in a dose- and time-dependent manner. The Pearson's correlation test showed a lack of relationship between diameter of nanoparticles and their pro-apoptotic activity (r^2= 0.282), whereas significant correlations were found between pro-apoptotic activity and the presence of some metal contaminants such as nickel, cobalt and gold (r^2= 0.919, 0.698 and 0.520, respectively). Our results therefore suggest that carbon nanotubes at high concentrations lowered cell viability *in vitro* and induced apoptosis in human cells *in vitro*. In addition, the results indicate that metal contaminants in carbon nanotubes may be causative of some of the adverse outcomes observed. Finally, our finding provides important information on the biohazard potential of some carbon nanotubes in humans.

Key words: Multiwalled, carbon, nanotubes, cytotoxicity, apoptosis, flow cytometry, viability, lymphocytes.

INTRODUCTION

Carbon nanotubes (CNTs) have unique chemical, physical, optical, and magnetic properties that make them potentially useful for multiple industrial products in the field of nanotechnology, including in nanomedicine. However, previous reports have suggested that due to their fibrous shapes CNTs could be as toxic as asbestos (Ryman-Rasmussen et al., 2009). Injecting carbon nanotubes into mice shows they can trigger similar toxic responses to asbestos fibers, causing a strong immune response and possibly cancer in the abdominal cavity (Poland et al., 2008), warning further research and careful attentions on their potential toxicities.

While previous work has shown that an enzyme present in human immune cells can break down nanotubes (Kagan et al., 2010), yet other studies have

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reported that CNTs suppress systemic immune functions (Mitchell et al., 2009) and induce massive loss of cell viability in human T-lymphocytes through programmed cell death at doses of 400 µg/ml, which corresponds to approximately 10 million carbon nanotubes per cell (Bottini et al., 2006). Discrepancies observed with respect to the toxicity of CNTs in such reports, can be attributed to the differences in physicochemical properties of the batches of CNTs and cellular models used in these studies (Kunzmann et al., 2011; Liu et al., 2008). A majority of published reports lack information on the CNTs production method, purity and functionalization treatments that can influence bioavailability and therefore toxicity (Firme et al., 2010). It is therefore critical to conduct toxicity studies on CNTs batches that have been well characterized with respect to their physicochemical properties. Therefore, in the present study, we investigated physiochemical properties of four different commercially available multiwalled carbon nanotubes (MWCNTs) and correlated it with their cytotoxicity potential.

MATERIALS AND METHODS

Nanotubes sources

Multiwalled carbon nanotubes were obtained from different sources: MWCNTs were obtained from Frascati National Laboratory, Nanotechnology Laboratory, Italy (MWCNTs Frascati); Multiwalled carbon nanotubes, 110 to 170 nm diameters, were purchased from Sigma-Aldrich (MWCNTs Sigma); MWCNTs were purchased from Sun Nanotech, China (MWCNTs Sunnano); short multiwalled carbon nanotubes (SMWCNTs), 60 - 100 nm diameters, were obtained from Shenzhen Nano-Technologies, China (MWCNTs Shenzhen). All carbon nanotubes were used as received.

Physiochemical analyses

Transmission electron microscopy

The morphology of the carbon nanotubes and the composite was characterized by transmission electron microscope (type JEOL JEM-1230 operating at 120 kV attached to a CCD camera).

Inductively coupled plasma spectroscopy (ICP-OES)

The concentration of metal ions was determined by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Optima 4100 DV, PerkinElmer) after metal extraction by concentrated nitric acid as previously described (Pumera, 2007).

Surface area analysis

Surface area was determined at 77K from nitrogen adsorption/desorption isotherms measurements using a model NOVA 3200e automated gas sorption system (Quantachrome, USA). Prior to measurement, each sample was degassed for 6 h at 100°C. The specific surface area was calculated by applying the Brunauer-Emmett-Teller (BET) equation as previously described (Shim et al., 2006).

In vitro analyses

Preparation of carbon particle suspension

MWCNT suspensions were prepared as previously described (Jia et al., 2005). Briefly, nanoparticles were freshly suspended in culture medium (RPMI 1640 medium supplemented with 10% FBS) and sonicated for 20 min with a short break every 2 min for vortexing on ice. A stable suspension of carbon particles was obtained in this way and used immediately.

Isolation of peripheral blood lymphocytes (PBL)

Blood samples from of healthy female donors with age range of 23 to 30 years, nonsmokers, who had not been exposed to known environmental mutagen agents were collected into heparinized vacutainers tubes. PBL were further isolated by histopaque 1077 density gradient centrifugation at 400 g for 30 min at 25°C (Sigma-Aldrich, St. Louis MO, USA), and then washed twice with FBS-free medium (RPMI-1640) at 250 g for 10 min at 25°C and finally adjusted to 5×10^3 cells/well for analysis.

Viability assay

PBL (5×10^3 cells/well) were plated on 96 flat-bottom well plates, and incubated 48 h at 37°C in 5% CO_2 atmosphere. After incubation, culture medium was removed, and nanotubes diluted in the same medium were added at concentrations ranging from 10 to 500 ug/ml. The plates were then incubated for 5 h at 37°C and 5% CO_2 atmosphere. The supernatant was removed and cells were washed twice with serum-free media. Cell viability was determined by the trypan blue exclusion method, and results were given as the mean ± SD of three independent experiments.

Analysis of cellular apoptosis by flow cytometry

To determine cellular apoptosis, 2×10^5 cells/well were seeded into in 6 well plates (Nunc) with the total working volume of 2 ml for each well and incubated overnight. Cells were treated in duplicate with two different particle suspension (50 and 500 ug/ml) of MWCNTs for 48 h. After treatment, the cells were harvested by trypsinization. The pellets were resuspended and incubated in the dark for 15 min in 100 µl Annexin labeling solution consisting of 2% Annexin-V FITC and 0.1 µg/ml PI in HEPES buffer (10 mM HEPES, 140 mM NaCl, 2mM $CaCl_2$, 5 mM KCL and 1 mM $MgCl_2$, pH 7.4). Stained cells were measured on FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and CellQuest analysis software. A minimum of 15,000 events were analyzed per sample after cell debris were excluded from the analysis based on scatter signals. The percentage of Annexin-V^+ cells correlates with the extent of apoptosis in the samples (Darzynkiewicz and Bedner 2000). Every experiment was repeated at least three times. The results represent the average ± SD in triplicate samples.

Statistical analyses

Results were calculated as the mean ± SD of the triplicate experiments. The significance of the results was statistically analyzed by a one-way analysis of variance (ANOVA) with Tukey's multiple comparison for pair-wise comparison. A Pearson's correlation analysis was used to establish associations of physiochemical properties of MWCNTs and the cellular changes studied. Statistical significance was set at $P<0.05$.

RESULTS

Characterization of MWCNT

Morphological structure

Transmittance electron microscope (TEM) was used to analyze the morphological structure of the MWCNTs included in this study and the images are presented in Figure 1. It is clear from the TEM images that all types of carbon nanotubes are tangled around each other and they were in the nano-size scale.

MWCNT diameter

The average diameter was between 23 to 40 nm for Sigma MWCNTs, 26 to 47 nm for Shenzhen MWCNTs, 37 to 100 nm for Sunnano MWCNTs, and 12 to 40 nm for Frascati MWCNTs. The length of the carbon nanotubes was not clear as the tubes are very tangled around each other.

MWCNT specific surface area

The specific surface area, which indicates the dispersion of the nano particles within the reaction matrix, was determined at 77K from nitrogen adsorption/desorption isotherms, and the results are presented in Figure 2. The BET specific surface areas were found to be 12.9 $m^2 g^{-1}$, 6.66 $m^2 g^{-1}$, 170 $m^2 g^{-1}$ and 79.2 $m^2 g^{-1}$ for the MWCNTs Frascati, MWCNTs Sigma, MWCNTs Sunnano, and MWCNTs Shenzhen, respectively (Figure 3).

Metal ions content of MWCNT

The total metals analysis for the different carbon nanotubes under investigation was measured and the results are tabulated in Table 1. It is clear from the analysis that MWCNTs Sigma had the least metals concentrations, followed by MWCNTs Frascati. For the other two MWCNTs, the metal concentration was very high, especially Fe, Si, Al and Ni for the MWCNTs Sunnano, and Fe, Cr, Ni, and Au for the MWCNTs Shenzhen (Table 1).

In vitro cell response to MWCNT

Viability of PBL

As observed in Figure 4, nanotube suspensions induced dose-dependent cytotoxic effect on PBL. The median cytotoxic concentration (CC50) for the studied nanotube suspensions was 500 µg/ml, at which PBL showed lowest viability after 48 h of addition of Shenzhen and Sunnano nanotubes whereas, at 500 ug/ml of Frascati and Sigma nanotubes, PBL showed higher viability than the other two nanotubes.

Pro-apoptotic activity of MWCNT

The effect of carbon nanotubes on PBL apoptosis was investigated using two different doses; 500 and 50 µg/ml at two different time intervals; 12 and 48 h and the results are presented in Figure 5. It is clear that MWCNTs Shenzhen and MWCNTs Sunnano had the highest pro-apoptotic potential compared with MWCNTs Frascati and MWCNTs Sigma; 7.79, 6.05, 4.26 and 5.33%, respectively, when 50 µg/ml dose was used and after 12 h of exposure. This effect was significantly enhanced after 48 h of exposure as the percent of apoptosis increased to 25.79, 21.05, 14.26 and 14.32% for MWCNTs Shenzhen, MWCNTs Sunnano, MWCNTs Sigma and MWCNTs Frascati, respectively.

It is clear that at this dose and exposure time, both Shenzhen and Sunnano MWCNTs had almost 150% more pro-apoptotic potential compared with Frascati and Sigma MWCNTs. Moreover, the effect of 500 µg/ml MWCNTs on the apoptosis of the PBL was studied and the results showed that after 12 h of exposure, the MWCNTs Shenzhen, and MWCNTs Sunnano had higher effect on the PBL cells compared with the MWCNTs Sigma and MWCNTs Frascati; 11.84, 9.32, 6.44 and 7.74%, respectively. Increasing the exposure time to 48 h had increased the percentage of apoptotic PBL in comparison to that at 12 h exposure (Figure 5).

Table 2 presents the correlation between and physiochemical properties of carbon and the apoptosis occurred to the PBL. In this table, the low correlation between carbon nanotubes specific surface area and apoptosis at both low and high doses are clearly observed. The correlation between the metal ions contaminants and PBL apoptosis after 48 h revealed a strong correlation between nickel and the apoptosis (r^2=0.9833 and 0.9198, respectively). In addition, there was also a considerable correlation between cobalt and PBL apoptosis (r^2=0.5893 and 0.6989, respectively).

Moreover, there was a considerable correlation between gold concentration and the apoptosis occurred to the PBL cells at low and high dose (r^2= 0.5476 and 0.5893, respectively). On the other hand, iron showed no correlation with apoptosis occurred to the PBL cells either at low or high dose; (r^2=0.0149 and 0.0697, respectively).

DISCUSSION

The present study investigated the physiochemical properties of four different commercially available MWCNTs and correlated it with their cytotoxicity

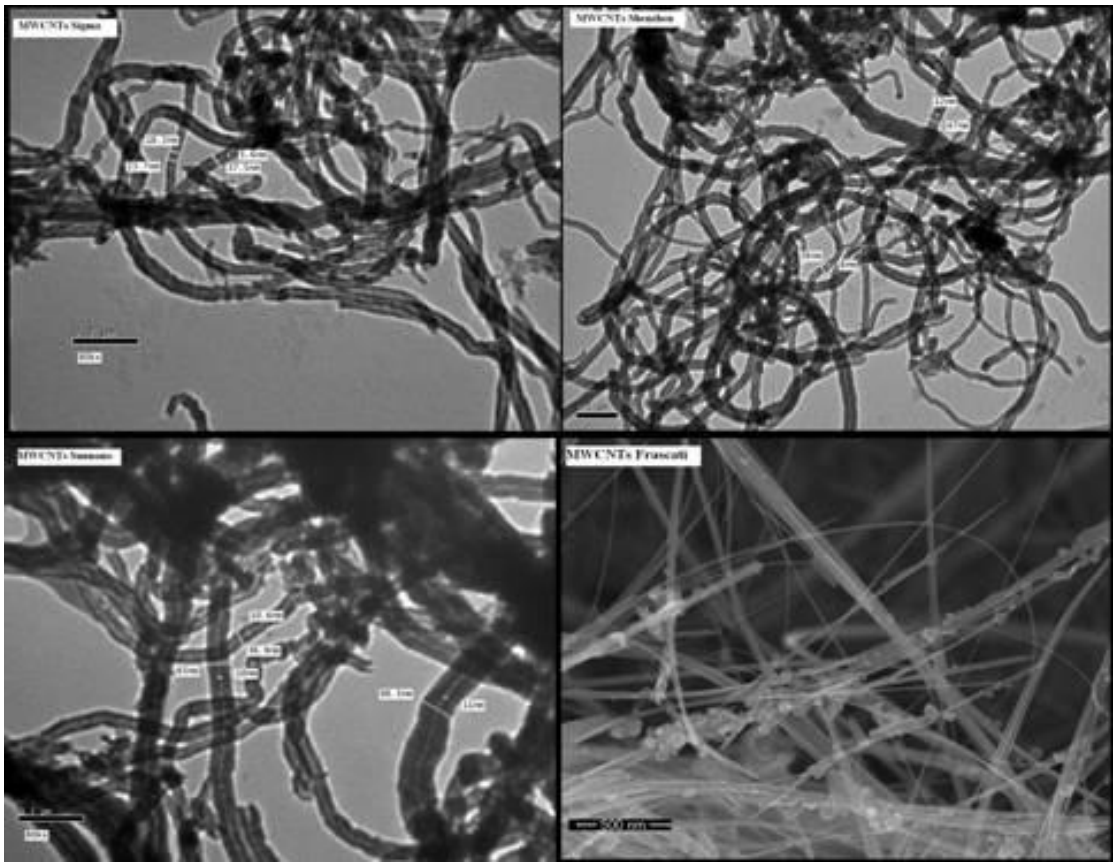

Figure 1. TEM of different multi-walled carbon nanotubes.

Table 1. Total metal analysis for the different MWCNTs.

Metal	MWCNTs Frascati	MWCNTs Sigma	MWCNTs Sunnano	MWCNTs Shenzhen
	Concentration (ppb or ng metal/g CNTs)			
Ag	0	0	0	5.625
Au	1.625	2.125	2.5	2.375
Co	3.75	0.625	0	25.375
Cr	33.75	16.125	24.375	20
Mn	0	5.125	33.25	7.75
Ru	0	2.375	3.625	0.625
Sr	0.125	35.25	179.875	0
Ti	0	16.875	0	0
V	0	6.125	1.75	0
Fe	319.375	114.25	7957.125	244.375
Si	0	0	2724.5	0
Ni	18.625	0	3253.375	4487.625
Al	0	0	1191.25	0

potentials. Doses of 50 and 500 µg/ml were selected because according to a previous study (Bottini et al., 2006), a safe concentration of carbon nanotubes was about 40 µg/ml. Also, in one of the medical application, carbon nanotubes in dose ranging from 5 to 500 µg/ml were applied for the thermal destruction of cancer cells (Gannon et al., 2007). Our investigation showed that the viabilities of the MWCNT-treated cells decreased above

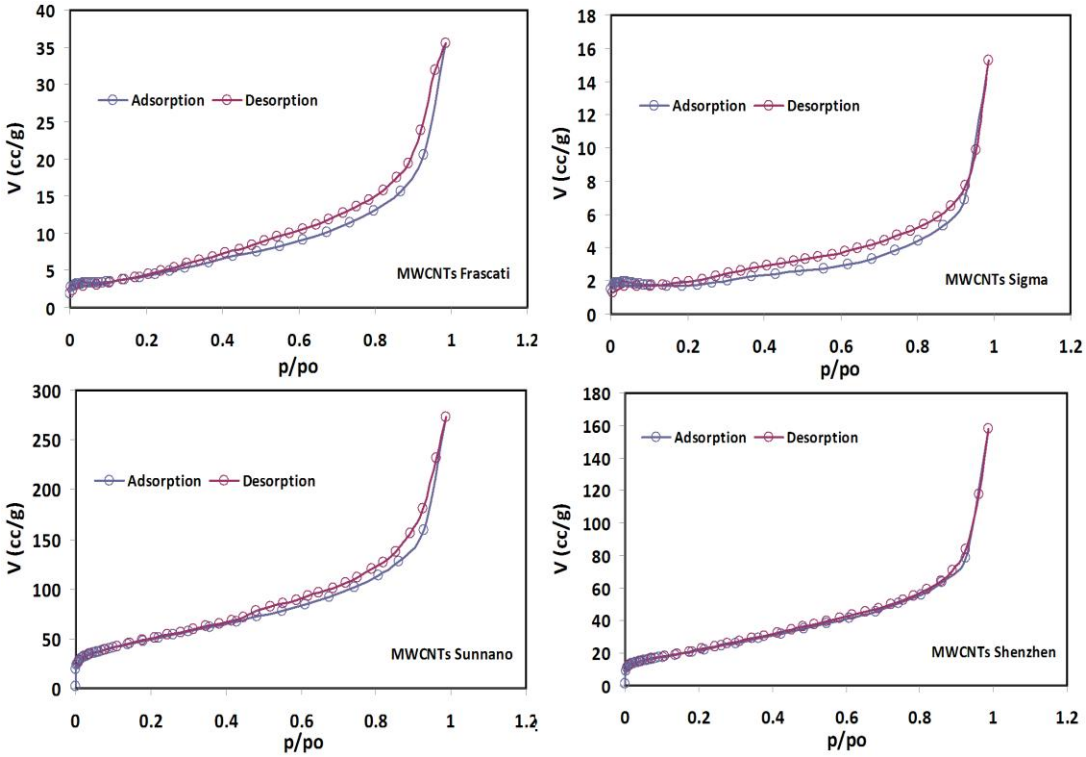

Figure 2. Adsorption/desorption curves of different multi-walled carbon nanotubes.

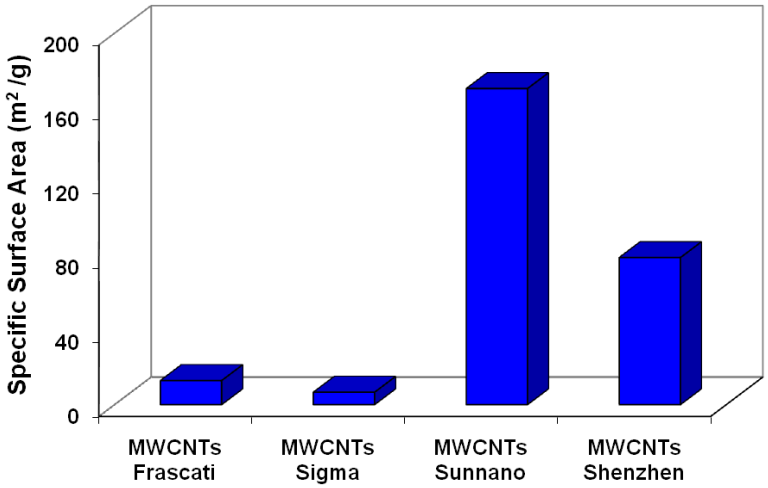

Figure 3. Specific surface area of different multi-walled carbon nanotubes.

concentration 50 ug/ml. The viability of PBL decreased significantly at 10-fold concentrations (500 ug/ml) of MWCNTs. The mechanism for causing cell death in this study was attributed to apoptosis as manifested by increasing Annexin-V expression post treatment.

At a dose of 500 µg/ml and an exposure time 12 h, both Shenzhen and Sunnano MWCNTs had almost 150% more effect compared with Frascati and Sigma MWCNTs on the apoptosis of the PBL. This effect was significantly enhanced after 48 h of exposure as the percentage of apoptotic cells increased. Although MWCNTs were found less toxic at 10-fold lower concentration, however, increasing the exposure time leads to great effect on the PBL as it enhanced the percentage of apoptosis significantly, regardless of the carbon nanotubes type. This may indicate that the cytotoxicity of MWCNTs is time and dose-dependent behavior in human blood lymphocytes. These data are in agreement with previous

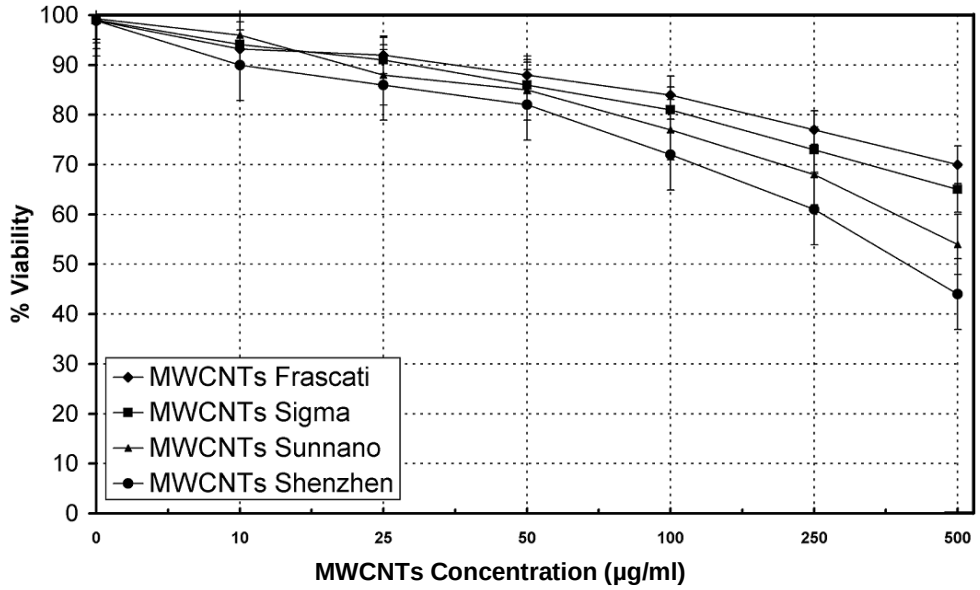

Figure 4. Cellular viability at different multi-walled carbon nanotubes concentrations.

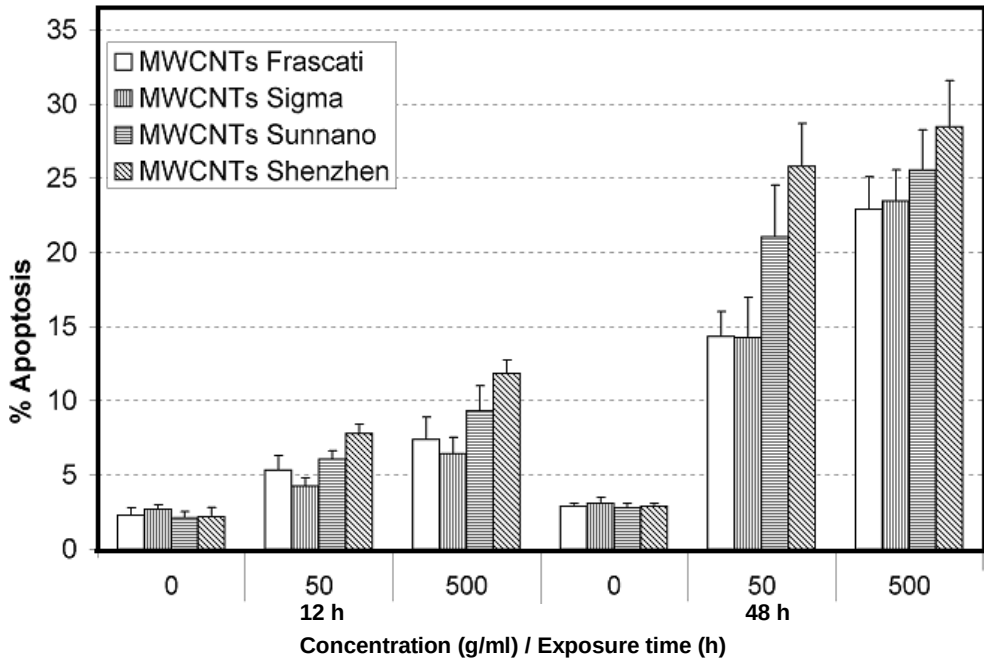

Figure 5. Percent of apoptotic cell death at different multi-walled carbon nanotubes concentrations.

studies that reported a decreased metabolic activity post treatment with MWCNTs, which might affect long-term viability of blood lymphocytes (Thurnherr et al., 2011). However, these studies did not evaluate particle-related characteristics that may have caused cell death.

It is clear from the TEM images that all types of carbon nanotubes are tangled around each other and they were in the man-size scale. However, the length of the carbon nanotubes was not clear as the tubes were extremely tangled around each other. So, in general, most of the carbon nanotubes under investigation had the same diameter range, except the Sunnano carbon nanotubes which had a higher diameter ranges. Sunnano MWCNTs; with average diameter between 37 to 100 nm, reduced cellular viability and induced apoptosis more than relatively lower diameter Sigma MWCNTs (average

Table 2. Correlation between metals' concentration and the apoptosis caused by different carbon nanotubes at different doses (48 h of exposure).

Contaminant metal	Correlation coefficient (r^2)	
	50 (µg/ml)	500 (µg/ml)
Nickel	*0.9833	*0.9198
Cobalt	*0.5893	*0.6989
Iron	0.0149	0.0697
Gold	*0.5476	*0.5205
Specific surface area	0.4238	0.2826

*P-value <0.05.

diameter between 23 to 40 nm) and Frascati MWCNTs (average diameter between 12 to 40 nm). Despite this, the MWCNTs Shenzhen with medium average diameter (between 26 to 47 nm) showed increased cytotoxicity and pro-apoptotic activity than Sunnano MWCNTs, suggesting that other factors could be contributing to this behavior. The specific surface area, which indicates the dispersion of the nanoparticles within the reaction matrix have been suggested as a contributing factor in CNTs toxicity (Hsieh et al., 2012). Our results showed poor correlation between carbon nanotubes specific surface area and apoptosis occurred in blood lymphocytes.

One of the key factors which affect the reactivity and toxicity of nanoparticles is their content of metal impurities (Haniu et al., 2010). Generally, most the carbon nanotubes produced using metallic nano particles as catalysts. These metals encapsulated within the walls of the carbon nanotubes and may affect the carbon nanotubes toxicity. Most of the carbon nanotubes production facilities used iron, cobalt and nickel basically as catalysts beside many other metals (Cao et al., 2006).

So, it is important to correlate between the metals concentrations and the apoptosis occurred to the PBL cells. The total metals analysis for the different carbon nanotubes under investigation was measured using the ICP-OES. It is clear from the metal analysis that MWCNTs Sigma had the least metals concentrations, followed by MWCNTs Frascati. For the other two MWCNTs, the metal concentration was very high, especially Fe, Si, Al and Ni for the MWCNTs Sunnano, and Fe, Cr, Ni, and Au for the MWCNTs Shenzhen.

Correlation analyses between the metal contaminants and PBL apoptosis after 48 h of exposure showed a very high correlation between nickel concentration and PBL apoptosis, (r^2=0.9833 and 0.9198, respectively). This result is in agreement with previous study by Joshi et al. (2005) who observed an increased generation of hydroxyl radicals that was associated with a significant decrease in viability and decline in glutathione levels in cells. In addition, a considerable correlation between cobalt concentration and the apoptosis occurred to the PBL both at low and high dose (r^2= 0.5893 and 0.6989, respectively). The potential toxic effect of the cobalt on the T-cells has been attributed to its clastogenic potential that cause DNA breaks in chromosomes (Beyersmann and Hartwig 1992).

Moreover, the concentration of gold in the carbon nanotubes was correlated to blood lymphocytes; (r^2= 0.5476 and 0.5893, respectively). Similar effect has been reported previously (Li and GU 2010; Qiu et al., 2010), and it was concluded that the gold in nanoparticles induces selective optical stimulation inside cells leading to induction of apoptosis. In good agreement with other studies (Pulskamp et al., 2007; Worle-Knirsch et al., 2006), iron content of nanoparticles showed no correlation with the apoptosis of the PBL neither at low- nor high dose; (r^2= 0.0149 and 0.0697, respectively).

Conclusion

Our results suggest that carbon nanotubes at high concentrations lowered cell viability *in vitro* and induced apoptosis in human cells. In addition, the results also indicate that metal contaminants are a relevant factor in modulating cytotoxicity of CNTs. Finally, our findings recommend conducting further toxicity studies, particularly in conjunction with nanomedical applications of carbon nanotubes.

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