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Jacobs Journal of Nanomedicine and Nanotechnology

Editorial

Biological Effects of Functionalized Multi-Walled Carbon Nanotubes on Human Cancer and Normal Cell Lines

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Received: 07-24-2014

Accepted: 07-28-2014

Published: 09-04-2014

ABSTRACT

Multi-walled carbon nanotubes (MWCNTs) have a great biomedical potential, even though their toxicology in vitro remain still controversial.

Since it is well known that nanotube aggregates are more toxic than monodispersed nanotubes, the aim of our study was, firstly, to provide a new method that makes the nanotubes themselves monodispersed.

Then, we determined the cytotoxicity of pristine, as well as functionalized MWCNTs with hydroxyl (MWCNTs-OH) and carboxyl (MWCNTs-COOH) groups on the human cancer cell lines MCF-7, Caco-2, and HL-60 and normal human dermal fibroblasts (HFs).

All three types of nanotubes, at the highest concentrations, led to a delay of cell cycle and a slight increase of apoptosis in MCF-7 cells, while only MWCNTs-OH led to a slowing down of cell cycle in Caco-2 cell line. In HL-60 and HFs no effect was shown. Moreover, we observed that not only MCF-7 cells but also Caco-2 cells and HFs were able to internalize all types of nanotubes.

The absence of apoptosis in Caco-2, HL-60 and HFs, and the low degree of apoptosis in MCF-7, led us to assume that MWCNTs, MWCNTs-COOH, and MWCNTs-OH are not cytotoxic in our in vitro systems. This could be due to the high degree of solubilization of the nanotubes; so that, methods that increase nanotubes dispersion are desirable for future biomedical applications.

Keywords: Multi-Walled Carbon Nanotubes; Functionalization; MCF-7, Caco-2; HL-60; HFs; Apoptosis; Cell cycle

Introduction

The large interest gained in the field of nanotechnology by carbon nanotubes (CNTs) [21], within the class of carbon-based nanomaterials, is due to their unique properties and applications [2,18]. Among them, also applications for medical purposes have been widely advocated [1,3,23,26]. Complexes based on CNTs can be considered for use in biosensors and as markers in biomedical diagnostics. Moreover, the use of such nanoparticle composites can also be extended for achieving drug or radiation delivery. Indeed, CNTs based composites can be engineered for potential applications in cardiovascular surgery, whereas CNTs themselves show remarkable properties as intracellular protein transporters. Taking into account also the many possibilities yielded by CNTs for industry, which would eventually give rise to innumerable commercial products, it is of crucial importance to assess the toxicity of CNTs [9,16,17,28].

CNTs functionalization and chemical modification can play an important role for applications, hence it must be considered carefully from the toxicity viewpoint. Multi-walled CNTs (MWCNTs) consist of several concentric graphene tubes and diameters of up to a few hundred nm. Pristine MWCNTs are chemically inert and insoluble in aqueous media, what can severely hinder their use in biological or medical applications. They are hydrophobic and tend to aggregate, something that explains the damage they produce for living cells in culture [14,27]. For many applications, CNTs are functionalized endowing them with hydroxyl and carboxyl groups [24], at their defective sites. These oxidized CNTs are much more readily dispersed in aqueous solutions and have been coupled to biomolecules or other nanomaterials [8].

In a previous paper [7] data on CNT hazards were reviewed, with particular emphasis on toxic effect on lung and in cell culture of lung origin, investigating the relation between toxicity and particle characteristics, involving size, surface area and surface chemistry. Moreover, buckypaper, an innovative material for pharmacological and prosthetic employment, was shown to decrease proliferation of human colorectal, breast and leukemic cancer cell lines in vitro and to be nontoxic in vivo [3].

Based on a previous investigation on human cell lines [12], which singled out the effect of MWCNTs on human breast adenocarcinoma MCF-7 cells and on human colorectal cancer Caco-2 cells, we studied the effects of COOH or OH functionalized MWCNTs on MCF-7 and Caco-2 in order to assess the possible increase of toxicity compared to pristine (not functionalized) nanotubes. All three types of nanotubes were administered to the cells after an innovative treatment that made them monodisperse. In addition, we studied the functionalized MWCNT biological effects even on human dermal fibroblast (HFs), and on a human promyelocytic leukaemia cell line HL-60. Moreover, to correlate the biological effects on human cancer and normal cells of the functionalized and not functionalized MWCNTs to their internalization into the cells, we observed their cellular localization by transmission electron microscopy.

Material and methods

Carbon nanotubes

The employed MWCNTs were synthesized by chemical vapor deposition, having a diameter of 20–40 nm, inner diameter 5–15 nm and length 0.5–200 μm (specifications given by the manufacturer, i.e. HeJi Inc.). The MWCNTs purity was up to 93.37%, and impurities present in the sample were Cl 0.20%, Fe 0.55%, Ni 1.86% and S 0.02%. The COOH (OH) functionalized nanotubes had a concentration COOH (OH)> 5wt%. The SSA 40-600 m2/g, bulk density 0.05 g/m3, true density 2.1 g/cm3 complete the physico-chemical characterization of the commercial nanotube samples. MWCNTs, and functionalized MWCNTs-COOH and MWCNTs-OH were placed in bidistilled water at concentration of 5 mg/ml and dispersed by sonication for 90 minutes to get a homogeneous suspension. After centrifugation at 14000 rpm, to remove the biggest agglomerates, the resulting suspension was used immediately for the in vitro studies. To determine the real nanotubes concentration in the solution, the pellets resulting from centrifugation were weighed and their values were subtracted from the initial concentration.

Cell cultures

The human breast adenocarcinoma cell line MCF-7, the human colorectal cancer cell line Caco-2, the human promyelocytic leukaemia cell line HL-60 were obtained from European Collection of Cell Cultures (ECACC). Primary HFs were isolated from healthy dermis by a collagenase type II digestion. Caco-2, MCF-7 cells, and HFs were seeded into 25-cm2 flasks (Falcon; Becton Dickinson Labware; Franklin Lakes NJ, USA) in Dulbecco modified Eagle medium (DMEM; Euroclone Ltd, Cramlington, UK) supplemented with 10% Fetal Calf Serum (FCS; Euroclone Ltd) and antibiotics (Penicillin 100 IU/ml, Streptomycin 100 mg/ml, Gentamycin 200 mg/ml; all from Euroclone Ltd). HL-60 leukemic cells were seeded into 25-cm2 flasks (Falcon; Becton Dickinson Labware) in RPMI 1640 medium (Euroclone Ltd) supplemented with 10% Fetal Calf Serum (FCS) and antibiotics (Penicillin 100 IU/ml, Streptomycin 100 mg/ml, Gentamycin 200 mg/ml). The cultures were kept at 37°C in an atmosphere of 5% CO2 in air. In Caco-2 cells, MCF-7 cells, and HFs the medium was changed every third day, and, at confluence, the cells were subcultured after removal with 0.05% trypsin-0.01% EDTA (Euroclone Ltd). HL-60 cells were maintained at low density (below 1 x 106 cells/ml) and used for ex-

periments from passages 5 to 15 to avoid differentiation. Cell viability was assessed with the Trypan Blue (Sigma Chemical Co., St. Louis MO, USA) dye exclusion method.

Cell proliferation assay

MCF-7, Caco-2, HL-60 cells, and HFs were seeded in 6-well culture plates (Falcon, Becton Dickinson Labware) at a concentration of 1 x 105 cells/well in a standard medium. The following day, the cells were refed with standard medium containing MWCNTs, MWCNTs-COOH, or MWCNTs-OH at concentrations ranging from 1 μg/ml to 100 μg/ml. The plates were incubated for 24, and 72 hours at 37°C in an atmosphere of 5% CO2 in air. The cells were then detached from wells by trypsinization (except for HL-60 cells) and centrifuged, and cell pellets were resuspended in PBS. Cell count was performed by a particle count and size analyzer (Beckman Coulter, Inc., Fullerton, CA, USA), and by a Thoma hemocytometer, after staining with the vital stain Trypan Blue. In all cell types no difference has been observed between the cell counts performed by the Beckman Coulter cell counter and by the Thoma hemocytometer, after staining with the vital stain Trypan Blue. Two replicate wells were used for each data point, and every experiment was performed six times.

Cell Cycle Analysis

MCF-7, Caco-2, HL-60 cells, and HFs were seeded into 25-cm2 flasks (Falcon, Becton Dickinson Labware) in a standard medium. The following day, the cells were refed with standard medium containing MWCNTs, MWCNTs-COOH, or MWCNTs-OH at a concentration of 100 μg/ml. The cultures were incubated for 24 and 72 hours at 37°C in an atmosphere of 5% CO2 in air. The cells were then harvested, washed twice with PBS, fixed with 70% ethanol at 4°C for 24 hours and stained with aqueous staining solution containing 0.75 mM (0.5 mg/mL) Propidium Iodide (PI), 4 mM sodium citrate, 1% Triton-X100 and 1% bovine serum albumin (all from Sigma Chemical Co.) at 4°C overnight. PI-stained cells were measured by flow cytometry. Cell cycle analysis was performed three times.

Annexin V/7-AAD Staining

MCF-7, Caco-2, HL-60 cells, and HFs were cultured at confluence into 25-cm2 flasks (Falcon, Becton Dickinson Labware) in a standard medium. The following day, the cells were refed with standard medium containing MWCNTs, MWCNTs-COOH, or MWCNTs-OH at a concentration of 100 μg/ml. The cultures were incubated for 24 and 72 hours at 37°C in an atmosphere of 5% CO2 in air. After the incubations, for each sample, the cells and the supernatants were collected, centrifuged at 1.500 rpm for 10 minutes, and washed twice with PBS. The cells were stained with fluorescein isothiocyanate labelled Annexin V/7-AAD (7-aminoactinomycine-D) according to the instructions of the manufacturer (Instrumental Pro3 Laboratory, Cavenago, MI, Italy). Briefly, a washed cell pellet was resuspended in 1.0 ml binding buffer at a concentration of 106 cells/ml; 10 L of Annexin V together with 20 l 7-AAD were added to 400 l cell suspension. The cells were incubated for 15 min on ice in the dark. The samples were analyzed by flow cytometry. Fluorescence intensity for annexinV – fluorescein isothiocyanate is plotted on the x-axis and 7-AAD is plotted on the y-axis. The lower left quadrant cells (Annexin V-/7-AAD-) were defined as viable cells, the lower right quadrant cells (Annexin V+/7-AAD-) as apoptotic cells, and the upper right quadrant cells (Annexin V+/7-AAD+) as necrotic and late apoptotic cells. Fluorescence compensation between FITC (green) and 7-AAD (red) was automatically calculated by the algorithm inside the EXPO 32 Software, which manages the EPICS Coulter XL cytometer (Beckman-Coulter Inc. Fullerton, CA, USA). Apoptosis assay was performed three times.

Flow Cytometry

The samples for both cell cycle and apoptosis assays were analyzed by flow cytometry performed using an EPICS Coulter XL (Beckman-Coulter). The fluorescence of 20,000 events was measured. An excitation wavelength of 488 nm was used in combination with standard filters to discriminate between the FL1 and FL3 channels, forward scatter and side scatter. Data were analyzed by Modfit LT Software (Veruty software Inc. USA).

Transmission electron microscopy analysis

MCF-7, Caco-2, HL-60 cells, and HFs were cultured at confluence into 25-cm2 flasks (Falcon, Becton Dickinson Labware) in a standard medium. The following day, the cells were refed with standard medium containing MWCNTs, MWCNTs-COOH, or MWCNTs-OH at a concentration of 100 μg/ml. The cultures were incubated for 24 and 72 hours at 37°C in an atmosphere of 5% CO2 in air. The cells were then harvested and washed twice with PBS. Cultured cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), postfixed in 1% OsO4 in 0.1 M cacodylate buffer, de-hydrated in ethanol, and embedded in epoxy resin. Ultrathin sections were contrasted in lead-hydroxide, studied and photographed by a Hitachi 7000 Transmission Electron Microscope (Hitachi, Tokyo, Japan). Also, nanotube morphological appearance was studied applying similar procedures to centrifuged MWCNTs, MWCNTs-COOH, or MWCNTs-OH. Transmission electron microscopy analyses were performed in three independent experiments. At least ten ultrathin sections were analyzed for each sample in each experiment.

Statistical analysis

Data were expressed as mean standard deviation (SD). Data

were statistically analyzed with the analysis of variance (ANOVA) followed by Bonferroni post-test. Differences were considered significant at the level of $p<0.05$. Statistical analysis was performed by using GraphPad Instat software (GraphPad Software, Inc.; San Diego, CA, USA).

Results

Cell proliferation assay

In human breast adenocarcinoma cell line, MCF-7, treatment with MWCNTs, MWCNTs-COOH, and MWCNTs-OH, added to culture medium for 24 and 72 hours, determined a reduction of cell number as compared with the control at the highest nanotube concentration (100 μg/ml), but this decrease was statistically significant only after 72 hours of culture (Figure 1A).

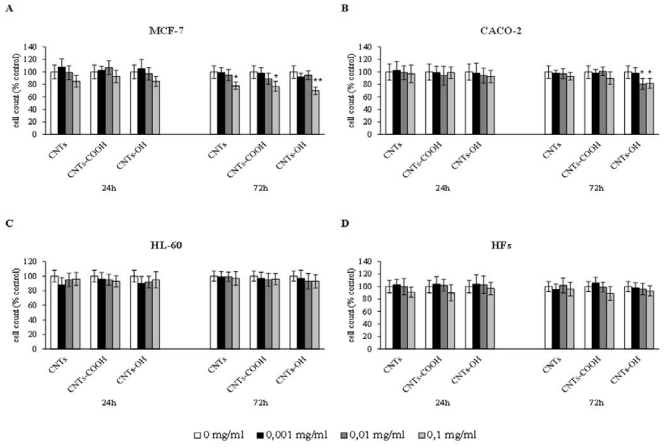

Figure 1. Effects of MWCNTs, MWCNTs-COOH, and MWCNTs-OH on the viability of the human cell lines MCF-7 (A), Caco-2 (B), HL-60 (C), and HFs (D). Control samples is considered 100%. Each column represents the mean value ± SD of six separate experiments performed in duplicate. *p<0.05, **p<0.01 versus control by ANOVA followed by Bonferroni post-test.

In human colorectal cancer cell line, CaCO-2, treatment with MWCNTs, MWCNTs-COOH and MWCNTs-OH, added to culture medium for 24 hours, did not induce any modification in cell growth as compared with not treated cells used as control (Figure.1B). After 72 hours of culture only the treatment with MWCNTs-OH at the concentrations of 10 and 100 μg/ml determined a statistically significant reduction of cell number, whereas the treatment with MWCNTs and MWCNTs-COOH did not evidence any difference in cell viability as compared with the control cells (Figure 1B).

In human promyelocytic leukaemia cell line HL-60 and in normal dermal HFs, treatment with MWCNTs, MWCNTs-COOH, and MWCNTs-OH, added to culture medium at concentrations from 1 to 100 μg/ml for 24 and 72 hours, did not induce any relevant decrease of cell viability as compared with the respective controls (Figure 1C, D).

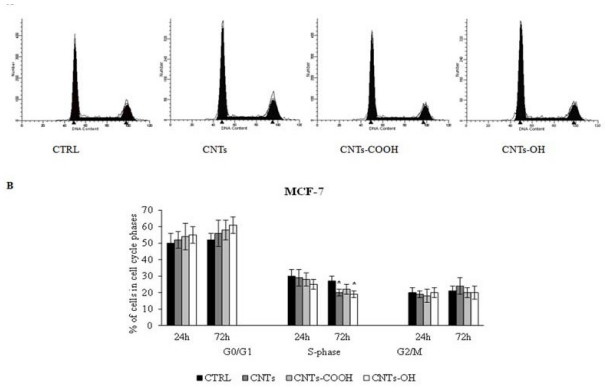

Figure 2. Effects of MWCNTs, MWCNTs-COOH, and MWCNTs-OH on cell cycle distribution in MCF-7 cells. (A) Hystograms showing the percentage of cells in various phases of the cell cycle. Each column represents the mean value ± SD of three separate experiments. *p<0.05 versus control by ANOVA followed by Bonferroni post-test. (B) Representative flow cytometric profiles of cell cycle of MCF-7 cells treated with all types of MWCNTs for 72 hours.

Cell Cycle Analysis

In human breast adenocarcinoma cell line, MCF-7, treatment with 100 μg/ml MWCNTs, MWCNTs-COOH, and MWCNTs-OH for 24 hours, did not determine any statistically significant change in cell cycle distribution as compared with the control (Figure 2). After 72 hours of culture, the treatment with all MWCNT types induced a reduction of the percentage of MCF-7 cells in the S-phase of the cell cycle, when compared with the control. However, the decrease of S-phase was statistically significant only in MCF-7 cells treated with MWCNTs and MWCNTs-OH (Figure 2A, 2B). The reduction in the percentage of cells in S-phase corresponded to an increase, even if not significant, of cells in G0/G1 phase.

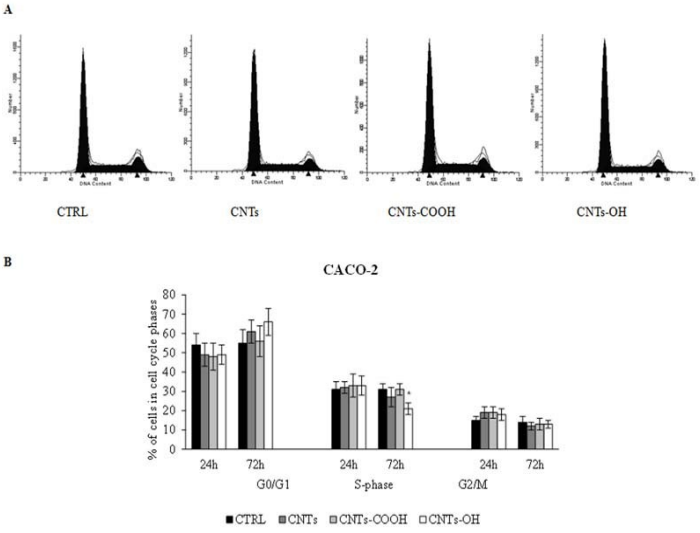

Figure **3.** Effects of MWCNTs, MWCNTs-COOH, and MWCNTs-OH on cell cycle distribution in Caco-2 cells. (A) Hystograms showing the percentage of cells in various phases of the cell cycle. Each column represents the mean value ± SD

of three separate experiments. *p<0.05 versus control by ANOVA followed by Bonferroni post-test. (B) Representative flow cytometric profiles of cell cycle of Caco-2 cells treated with all types of MWCNTs for 72 hours.

In human colorectal cancer cell line, Caco-2, treatment with 100 μg/ml MWCNTs, MWCNTs-COOH and MWCNTs-OH for 24 hours, did not induce any modification of cell cycle distribution as compared with the not treated cells (Figure 3). After 72 hours of culture, only the treatment with MWCNTs-OH induced a statistically significant reduction of the percentage of Caco-2 cells in S-phase, and a moderate increase of cells in G0/G1 phase of cell cycle (Figure 3A, 3B). No modulation in cell cycle distribution was observed in Caco-2 cells treated with MWCNTs, and MWCNTs-COOH for 72 hours.

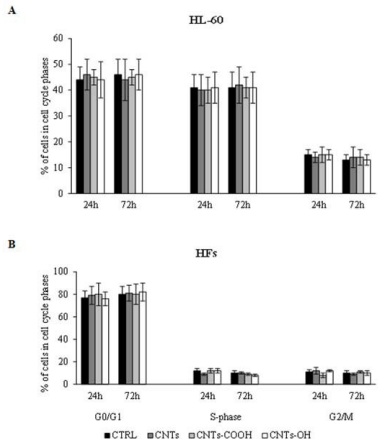

Figure **4.** Effects of MWCNTs, MWCNTs-COOH, and MWCNTs-OH on cell cycle distribution in HL-60 (A) and HFs (B). Hystograms show the percentage of cells in various phases of the cell cycle. Each column represents the mean value ± SD of three separate experiments.

In human promyelocytic leukaemia cell line HL-60 and in normal dermal HFs, treatment with 100 μg/ml CNTs, CNTs-COOH, and CNTs-OH for 24 and 72 hours, did not induce any alteration of cell cycle distribution as compared with the respective controls (Figure 4A, 4B).

Apoptosis assay

Data obtained by cytofluorimetric assays showed a statistically significant increase in apoptotic cells only in MCF-7 cell line treated with 100 μg/ml CNTs, CNTs-COOH, and CNTs-OH (Figure 5). Particularly, CNTs and CNTs-OH induced in MCF-7 an increase of apoptosis with respect to control after 24 hours of treatment, whereas no increase of apoptosis was found in MCF-7 cells cultured in presence of CNTs-COOH (Figure 5A, 5B). At 72 hours all three types of carbon nanotubes induced a statistically significant increase in the percentage of apoptotic cells (Figure 5A, 5C).

No increase of apoptosis was found in Caco-2, HL-60, and HFs treated with 100 μg/ml CNTs, CNTs-COOH, and CNTs-OH for 24 and 72 hours as compared with their respective controls. The treatment of the three cell types with the apoptotic inducer Camptothecin (CPT) at a concentration of 10μg/ml (positive control) triggered a statistically significant increase in apoptosis (Figure 6A, 6B, 6C).

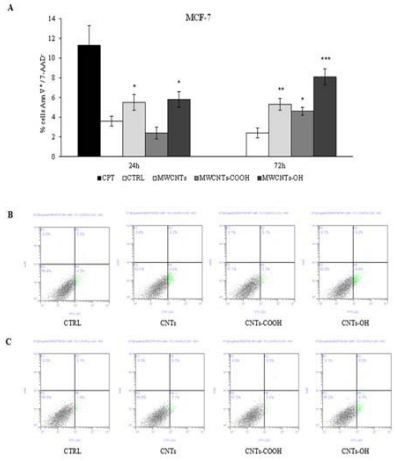

Figure 5. Effects of MWCNTs, MWCNTs-COOH, and MWCNTs-OH on apoptosis of MCF-7 cells determined by cytofluorimetric assays after Annexin V/7-AAD staining. (A) Histogram showing the percentage of apoptotic cells after treatment with all types of MWCNTs. Each column represents the mean value ± SD of four independent experiments. *p<0.05, **p<0.01, ***p<0.001 versus control (CTRL) by ANOVA followed by Bonferroni post-test. CPT, camptothecin. (B) Representative dual-parameter flow cytometric density dot plots for MCF-7 treated with all types of MWCNTs at 24 hours (B) and 72 hours (C).

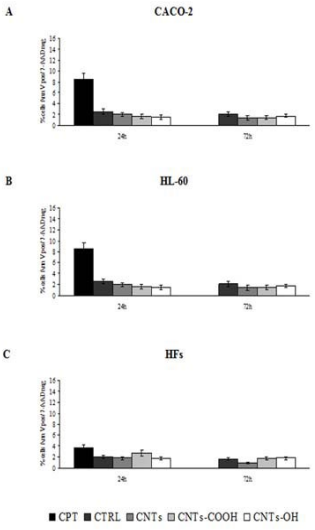

Figure 6. Effects of MWCNTs, MWCNTs-COOH, and MWCNTs-OH on apoptosis of Caco-2 (A), HL-60 (B) and HFs (C) determined by cytofluorimetric assays after Annexin V/7-AAD staining. Histograms show the percentage of apoptotic cells after treatment with all types of MWCNTs. Each column represents the mean value ± SD of four independent experiments.

Transmission electron microscopy analysis

In Figure 7 the ultrastructural characterization of MWCNTs

(Figure 7A), MWCNTs-COOH (Figure 7B), and MWCNTs-OH (Figure 7C) by transmission electron microscopy analysis of functionalized and not functionalized MWCNTs pellet is reported. In MCF-7, Caco-2, and HFs, after 24 hours of treatment, all types of MWCNTs showed a low efficiency of cellular internalisation. After 72 hours of treatment, the efficiency of cellular internalization of all types of MWCNTs was really increased. MWCNTs, MWCNTs-COOH, and MWCNTs-OH were clearly observable into the cytosol of all three cell lines, both as free nanotubes as well as in vesicles (Figure 8, 9, 10). In HL-60, nanotubes within the cells were not observed (data not shown).

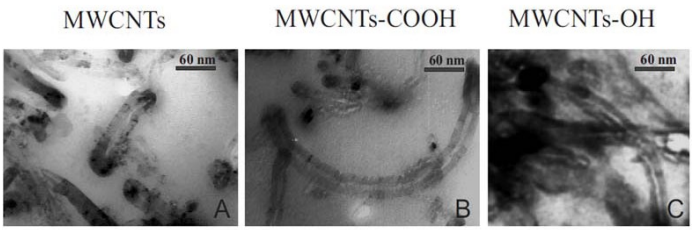

Figure 7. Transmission electron microscopy analysis of functionalized and not-functionalized MWCNTs pellet. A) MWCNTs, B) MWCNTs-COOH, C) MWCNTs-OH.

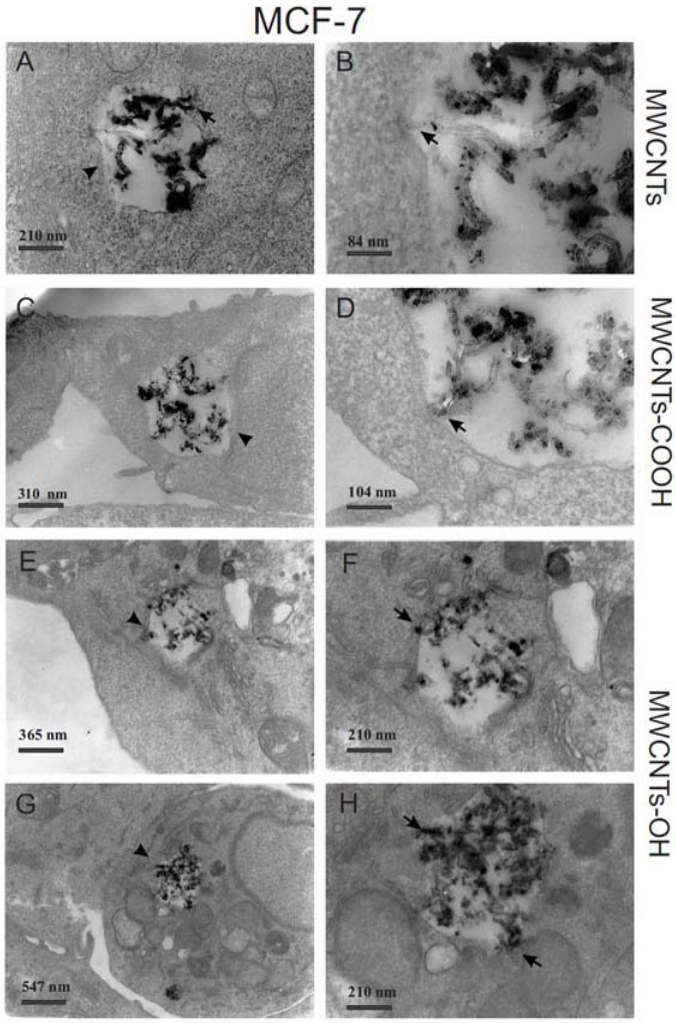

Figure 8. Transmission electron microscopy analysis of MCF7 cells cultured for 72h in medium additioned of functionalized and not-functionalized MWCNTs. A) MCF7 internalization of not-functionalized MWCNTs. In B) higher magnification of the same field is shown. C) MCF7 internalization of MWCNTs-COOH. In D) higher magnification of the same field is shown. E-G) MCF7 internalization of MWCNTs-OH. In F) and H) higher magnification of the same fields are shown. Arrowheads indicate cytoplasmic vesicles filled of nanotubes whereas arrows indicate nanotubes that are emerging from the vesicle to the cytoplasm.

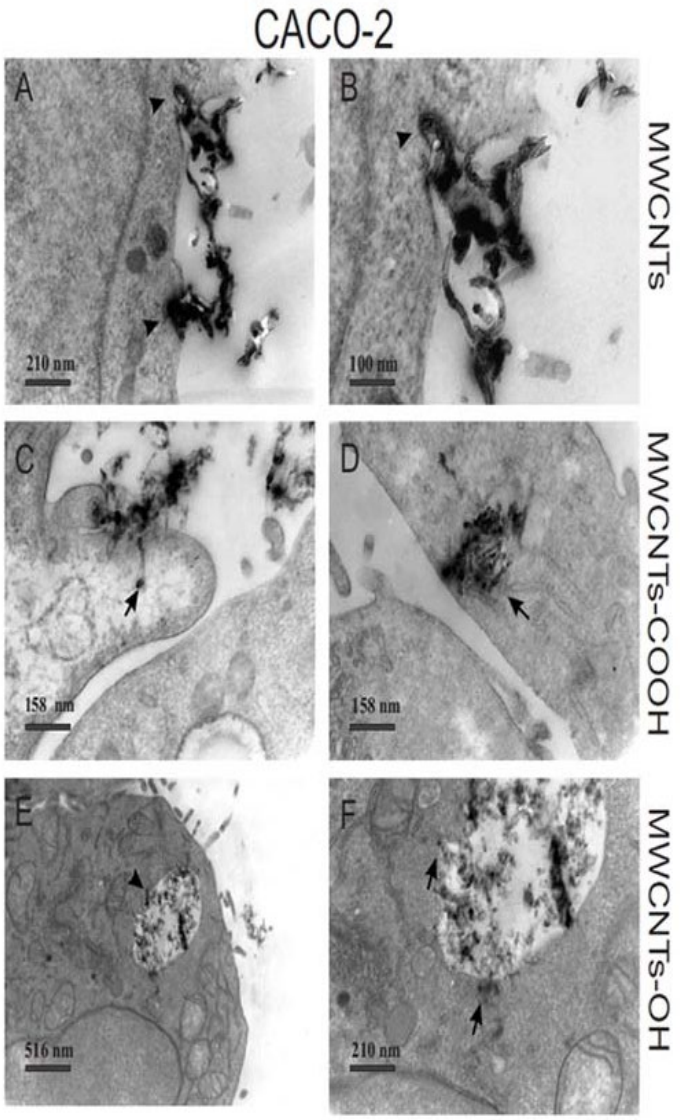

Figure 9. Transmission electron microscopy analysis of Caco-2 cells cultured for 72h in medium additioned of functionalized and not-functionalized MWCNTs. A) Picture describing Caco-2 plasma membrane invaginations with engulfed not-functionalized MWCNTs. In B) higher magnification is shown. Arrowheads indicate plasma membrane invaginations caused by nanotubes interaction. C) Direct penetration of MWCNTs-COOH trough Caco-2 plasma membrane. Arrow indicate nanotubes embedded in the cytoplasm. D) Picture showing MWCNTs-COOH free in the cytoplasm of Caco2 cell (arrow). E) Caco-2 internalization of MWCNTs-OH in cytoplasmic vesicle. In F) higher magnification of the same field is shown. Arrowhead indicates the vesicle containig nanotubes whereas arrows indicate nanotubes emerging from the vesicles in the cell cytoplasm.

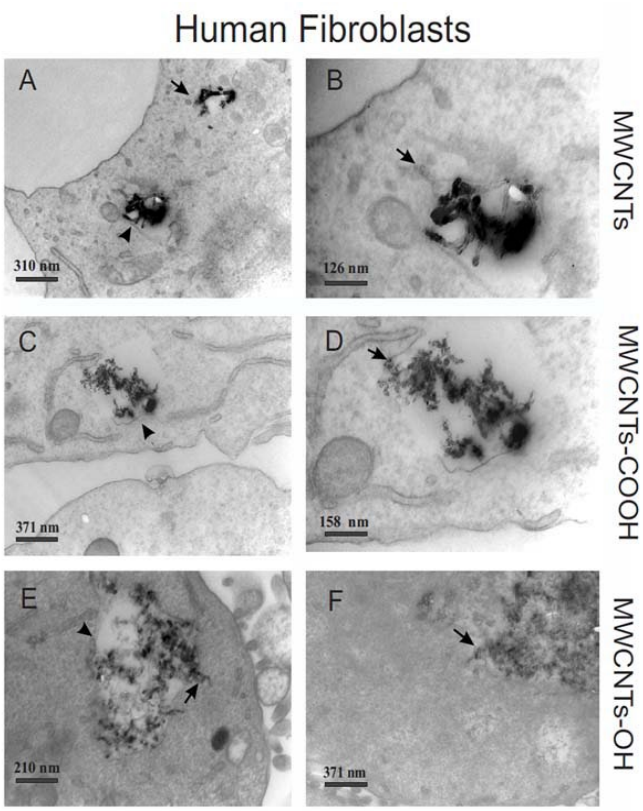

Figure 10. Transmission electron microscopy analysis of HFs cultured for 72h in medium additioned of functionalized and not-functionalized MWCNTs. A) Not-functionalized MWCNTs internalized in the cytoplasm of human fibroblast. Arrow indicates nanotubes free in the cytoplasm whereas arrowhead indicates a vesicle filled of nanotubes. In B) higher magnification of the same field is shown. Arrow indictes nanotube emerging from the vesicle to the cytoplasm. C) HF internalization of MWCNTs-COOH. Nanotubes appear engulfed in a vesicle (arrowhead) that is open in the cell cytoplasm. In D) higher magnification of the same field is shown. Arrow indicates nanotubes emerging from the vesicles in the cell cytoplasm. E) Microphotograph showing HF vesicle containing MWCNTs-OH (arrowhead). Arrow indicates nanotubes emerging from the vesicles in the cell cytoplasm. F) MWCNTs-OH internalized in HFs free in the cytoplasm (arrow).

Discussion

Scientific data on toxicological effects of CNTs in vitro are currently controversial, depending on the CNTs type, their functionalization and solubilization methods [Cui et al., 2010]. Because of the great potential for the biomedical use of CNTs, several in vitro studies have been performed in order to determine CNT cytotoxicity in a large number of cell types. The results in the literature have been contradictory: some studies demonstrated CNT cytotoxic effect, whereas others showed their biocompatibility [Cui et al., 2010]. It is well known that nanotube aggregates are more toxic than the monodispersed ones [Belyanskaya et al., 2009] and numerous studies have been performed to make them more soluble in cell culture medium [Coccini et al., 2010]. In the present study, we provided a new method, constituted by a long time sonication, a subsequent centrifugation for the removal of aggregates, and the immediate administration of all considered types of MWCNTs to the cell cultures. This new methodological approach makes the nanotubes themselves monodispersed and, probably, less toxic. To assess this hypothesis, four human cell lines, the breast adenocarcinoma MCF-7, the colorectal cancer Caco-2, the promyelocytic leukaemia HL-60, and the normal dermal HFs were treated with MWCNTs, MWCNTs-COOH, or MWCNTs-OH. These four cell lines were chosen because they derived from different tissues, so that they may provide an overview of the effects of nanotubes in biological systems. To determine the possible toxic effects of nanotubes, we combined three types of experiments: cell proliferation assay, cell cycle analysis and apoptosis.

MCF-7 cells showed a significant decrease of cell growth only after 72 hours of incubation with the highest concentration of all types of MWCNTs. These data are in agreement with those obtained in our previous study, in which pristine (not functionalized) MWCNTs induced a decrease of MCF-7 cell proliferation [12]. In both studies, we analyzed cell growth in the different cell lines through cell count assays. Indeed, it has been shown that the most common colorimetric methods, such as MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H- tetrazolium bromide), WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium), Alamar BlueTM, and Neutral Red, do interact with CNTs providing unreliable results [5, 10,30]. In MCF-7 cell count data were confirmed by cell cycle analysis. In fact, treatment of MCF-7 cells with 100 µg/ml MWCNTs, MWCNTs-COOH, or MWCNTs-OH for 72 hours triggered a significant reduction of the percentage of cells in S-phase and an increase in G0/G1 phase, results compatible with a delay in cell cycle progression. This result is in agreement with some studies in which SWCNTs treatment slowed down cell proliferation through cell cycle arrest (G1 block) in the FE1-MutaTMMouse lung epithelial cell line [22] and in human embryo kidney HEK293 cells [14].

Furthermore, other studies confirmed that MWCNTs induced a cell cycle arrest at the G1/S transition in the HEK293 cells, human neuroblastoma NB1691cells, and mouse mesenchymal stem cells C2C12 [32]. To investigate whether nanotubes are cytotoxic on MCF-7 cells we performed an apoptosis assay. Data obtained by flow cytofluorimetry demonstrated a slight increase of the apoptotic rate in MCF-7 cells treated with 100 µg/ml MWCNTs, MWCNTs-COOH, or MWCNTs-OH. Anyway, though the apoptotic rate showed by all types of nanotubes has been statistically significantly higher, the numerical values were not so relevant as to consider these nanomaterials cytotoxic for breast cancer MCF-7 cells.

In colon cancer Caco-2 cells only the treatment with MWCNTs-OH produced a decrease in cell number that was associated with a slight slowing of the cell cycle progression. In this cell

line, treatment with all three types of MWCNTs did not induce any increase of apoptosis and neither cytotoxic effects. In the latter case, we observed differences in the biological effects of the three types of nanotubes. The MWCNTs-OH, although not toxic, have slowed cell proliferation, while the MWCNTs and MWCNTs-COOH did not have any effects on colon cancer cells. Several studies have demonstrated that the functionalization of nanotubes induces different biological effects on a large variety of cell lines. In some cases the addition of functional groups, such as hydroxyl, or carboxyl groups to nanotubes seems to induce higher cytotoxicity compared to pristine CNTs [20,25], meanwhile in other cases the functionalization seems to make the nanotubes more biocompatible [11,29]. In particular, it has been reported that the functionalization of MWCNTs with hydroxyl groups induced a higher apoptosis in human lung epithelial A549 cells, with respect to not functionalized MWCNTs, although the latter produced membrane damage at lower concentrations compared to MWCNTs-OH [31]. Indeed, in Caco-2 cells, only MWCNTs-OH showed a moderate biological effect; meanwhile, in MCF-7 cell line both pristine and functionalized nanotubes presented the same cytotoxicity, even though in a very limited manner. In HL-60 cell line and HFs, the treatment with MWCNTs, MWCNTs-COOH and MWCNTs-OH did not show relevant biological effects on proliferation, cell cycle progression and apoptosis. These results confirmed that the nanotubes effects on cell cultures are highly dependent on cell type, as has been widely reported in the literature [15, 19]. We wondered if the absence of biological effects on some cell types was due to the lack of internalization of nanotubes into these cells. So, we observed the cellular localization of MWCNTs functionalized and not by transmission electron microscopy. In MCF-7, Caco-2, and HFs all types of nanotubes accumulated in cells both as isolated particles as well as in endocytotic vesicles. The transmission electron microscopy images showed that nanotubes penetrated into the cells either directly or through endocytosis. Furthermore, nanotubes present in vesicles often penetrated into the cytoplasm both directly, through the vesicle plasma membrane, and because of its breaking. No significant differences in the amount and distribution of the different types of nanotubes were observed in the same cell type and among the different cell lines. Conversely, in HL-60 nanotubes within the cells were not observed.

These results lead us to suppose that the reported biological effects of nanotubes do not merely depend on their ability to be internalized into the cells. In fact, while HL-60 cells did not show nanotubes inside them and neither changes in proliferation, cell cycle distribution and apoptosis, following treatment with all types of MWCNTs; on the other hand HFs showed the presence in their cytoplasm of nanotubes that still did not exert any biological effect. Based on the absence of apoptosis in Caco-2 and on the low degree of apoptosis in MCF-7, we can assume that MWCNTs, MWCNTs-COOH, and MWCNTs-OH are not cytotoxic in our in vitro systems, independently whether they are functionalized or not. Thus, it could be due to the high solubilization of the nanotubes themselves into the culture medium and, consequently, to the poor formation of agglomerates of carbon nanoparticles. So, using methods that increase dispersion and solubilization of nanotubes and choosing the appropriated biological target, functionalized and not functionalized MWCNTs might be considered useful in therapeutic nanomedicine for drug and biomolecule delivery.

Conflict of Interest

All authors have no personal or financial conflicts of interest and they have not entered into any agreement that could interfere with our access to the data on the research or on our ability to analyse the data independently, to prepare manuscripts and to publish them.

Acknowledgments

We are grateful to Mrs. Stefania De Grossi for Transmission Electron Microscopy technical assistance (DAHFMO facility).

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