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Cytotoxicity of functionalized carbon nanotubes in J774A macrophages

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Abstract

Cytotoxicity of carbon nanotubes (CNTs) is a prime concern for its use as antigen carriers. Here we evaluated the cytotoxic effect of unpurified (UP-CNTs), purified (P-CNTs), fluorescein isothiocyanate–functionalized (FITC-CNTs), and *Entamoeba histolytica* 220-kDa lectin–functionalized CNTs (L220-CNTs) in J774A macrophage (MOs) cell line. Cell viability and apoptosis were analyzed by MTT and TUNEL assays, respectively. Cyclooxygenase-2 (COX-2) was analyzed by reverse transcription–polymerase chain reaction. Cytotoxicity at 6.0 mg/L was higher with UP-CNTs > P-CNTs > FITC-CNTs, showing a decrease in cell viability and an increase in apoptosis. In contrast, MOs interacted with L220-CNTs showed an increase in cell viability without signs of apoptosis. Although UP-CNTs and P-CNTs exhibited COX-2 induction with 6.0 mg/L, functionalized CNTs were able to induce COX-2 at concentrations as low as 0.06 mg/L. These results suggest that functionalization decreases toxicity, and that L220-CNTs may be an excellent candidate for the production of a nanovaccine against amebiasis.

From the Clinical Editor: Cytotoxicity of carbon nanotubes may significantly limit their use as delivery systems. This study demonstrates that functionalization of these nanoparticles actually greatly reduces toxicity in J774A macrophages, and that lectin-functionalization may be a viable approach for the production of anti-amebiasis nanovaccines utilizing carbon nanotubes.

Key words: Carbon nanotubes; Purification; Cyclooxygenase; *Entamoeba histolytica*; Macrophages; Nanovaccines

Carbon nanotubes (CNTs) have many chemical properties that allow them to be used in biotechnological applications.[1] Because of their ability to penetrate plasma membranes,[2] CNTs can be used as carriers of substances in biological systems[3,4] and as biosensors that detect cellular tumors[5]; they can even be used for nanovaccine fabrication.[6-8] Nevertheless, these applications are limited because CNTs are nanomaterials that are almost insoluble and can accumulate in cells, organs, and tissues, causing toxic effects.[9] Several studies have demonstrated that CNTs provoke cellular apoptosis and a decrease in viability in lung tumor cells,[10] human fibroblasts,[11] and human T lymphocytes.[12] It has also been observed that CNTs induce the release of interleukin 8 as well as the production of reactive oxygen species in human epidermal keratinocytes (HEK).[13] The exact mechanism by which CNTs cause these effects is not completely known at this date. Nevertheless, some authors attribute these effects to the hydrophobic nature of the CNTs, fabrication residues, and high surface area or size.[14] Additionally, studies carried out in cell cultures have shown that functionalized CNTs (f-CNTs) do not exhibit cytotoxicity.[6,7,15] On the other hand, the distribution and excretion of f-CNTs in murine models has already been described by several groups.[2,16,17]

A potential use for f-CNTs is nanovaccine fabrication[8]; however, information about their potential cytotoxicity and the immunological response that these particles induce is contradictory. In particular, it would be of interest to make a nanovaccine against *Entamoeba histolytica*, a parasite that is responsible for intestinal amebiasis, an infection that affects more than 4 million individuals annually and that causes over 10,000 deaths each year.[18] Among other virulence factors already reported for this parasite, the 220-kDa lectin (L220) has been described and studied as an immune-modulatory molecule.[19,20] L220 is a highly immunogenic protein that has been shown to have peptides with the ability to develop a type 1 T-helper cell

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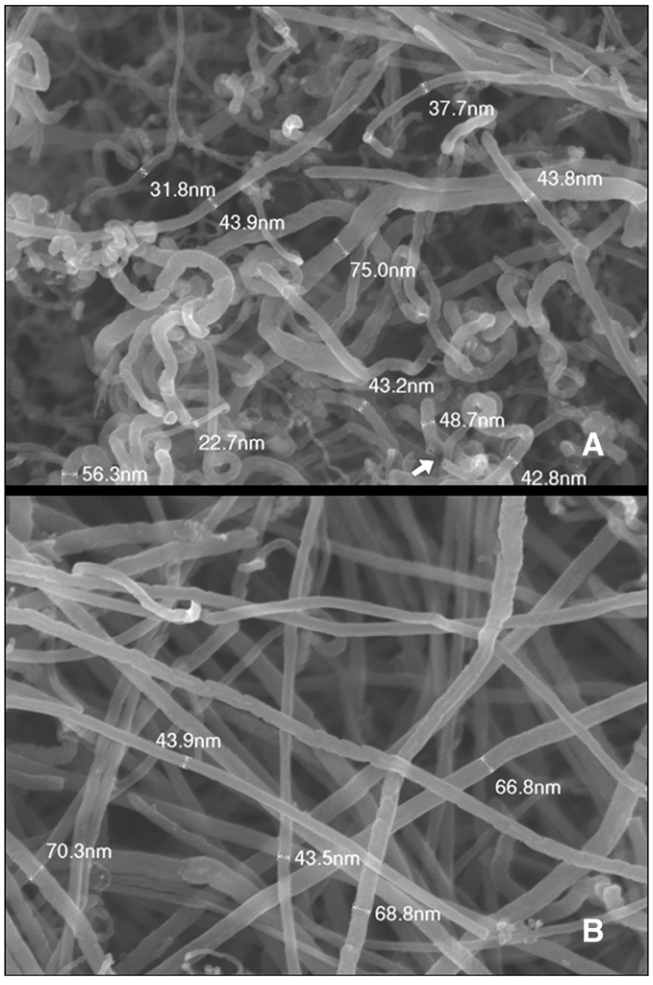

Figure 1. Photomicrographs obtained by SEM of UP-CNTs and P-CNTs. **(A)** UP-CNTs measuring 23–75 nm in diameter. The UP-CNTs are hollow structures that tend to fold over themselves like a plate of spaghetti. **(B)** P-CNTs are more dispersed. Several of the CNTs are observed with open ends.

response.[20] The objective of this study was to evaluate the cytotoxicity of unpurified, purified, fluorescein isothiocyanate (FITC)–functionalized, and L220 (from *E. histolytica*)-functionalized CNTs in macrophages (MOs) from the J774 cell line.

Methods

Synthesis and purification of CNTs

CNTs were synthesized by spray pyrolysis, using toluene and ferrocene as the carbon source and the catalyst, respectively.[21] Purification was carried out with 0.1 g of unpurified CNTs (UP-CNTs) and 150 mL of 3M HNO_3. The mixture was dispersed by sonication for 90 minutes and was put in reflux for 24 hours. Purified CNTs (P-CNTs) were washed with distilled water and dried at 90°C for 8 hours. The UP-CNTs and P-CNTs were characterized by scanning electron microscopy (SEM) (Figure 1, *A*). The dimensions obtained for CNTs varied from 29 to 100 nm in diameter and 123 μm in length. P-CNTs showed that higher particle dispersion and open-end formation were favored. Semiquantitative analysis of elemental composition (Table 1) showed a 2.77% decrease in Fe content among samples, as well a slight increase of O_2 (3%).

Table 1
Analysis of elements found in unpurified (UP) or purified (P) samples of CNTs

UP-CNTs		P-CNTs	
Element	% weight*	Element	% weight*
C	94.83	C	93.23
O	1.26	O	4.26
Si	0.02	Si	1.39
Fe	3.89	Fe	1.12
Total	100	Total	100

* Element content was determined by semiquantitative analysis of elemental composition.

CNT functionalization

Ten milligrams of P-CNTs were functionalized with 1 g of N-Boc-2,2-dioxyethylene diethylamine linker (Boc) (Sigma-Aldrich, St. Louis, Missouri) by a thermal reaction at 120°C for 6 days[22] and dispersed in 100 mL of absolute ethanol by stirring for 2 hours. The CNT-Bocs were washed with dimethylformamide using a filter of 0.45-μm polytetrafluoroethylene and dried at room temperature (25°). The CNT-Bocs obtained had a weight increase of 61%, which is equivalent to 9.82% of COOH groups on the P-CNT surface.[23] Functionalization with FITC was done as described elsewhere.[24] To confirm the FITC functionalization, FITC-CNTs were visualized by means of epifluorescence microscopy (data not shown).

To carry out L220 conjugation, 0.0852 mg of previously purified L220 was conjugated in accordance with previously described methodology[7]. L220-CNTs were lyophilized and reconstituted in sterile high-glucose Dulbecco's minimal essential medium (DMEM-HG), supplemented with 2 mM L-glutamine and HEPES. Binding efficiency was 5.28%.[22]

Viability tests in J774 MOs cell line

Cell viability was determined by MTT assays (Sigma-Aldrich) in 96-well plates.[25] For this assay, 10^5 cells were cultivated in DMEM-HG supplemented with 10% heat-inactivated bovine fetal serum, 100 IU/mL of penicillin, 100 μm/mL streptomycin, and 2 mM L-glutamine; cells were interacted with UP-CNTs, P-CNTs, FITC-CNTs, and L220-CNTs at 0.06, 0.6, and 6 mg/L concentrations. Cultures were incubated for 24 hours at 37°C in a humid atmosphere at 5% CO_2. MOs without stimulus were used as control. At 20 hours cultivation time, 0.1 mg of MTT dissolved in sterile phosphate buffered saline (PBS) was added to each well, followed by incubation for 4 more hours. Cells were lyzed with acidified isopropanol, and absorbances at 590 nm were quantified using a Bio-Rad ELISA microreader (Bio-Rad Laboratories, Hercules, California).

Apoptosis assays

Cultures in the Lab Tek Chamber slide system (Nalge Nunc International, Rochester, New York) with 1×10^6 cells/mL per well were interacted with the UP-CNTs, P-CNTs, FITC-CNTs, and L220-CNTs at a concentration of 0.06, 0.6, and 6 mg/L, and

incubated for 24 and 48 hours. After the incubation time, culture supernatants were washed out and the cells were fixed by adding 200 μL of 4% w/v buffered paraformaldehyde. and permeabilized with 0.02% v/v PBS–Triton X-100 solution for 15 minutes at room temperature. Cells were washed several times with PBS 1×, and apoptosis determination was carried out using a TUNEL-based assay with the Apo-BrUd Kit (Biovision, Mountain View, California) according to the manufacturer's instructions. Previous to microscopic observation, the multichamber wall was taken out, a drop of Vectashield (Vector Laboratories, Burlingame, California) was applied, and then a coverglass was placed and sealed. Photomicrographs were obtained by using an Olympus BX41 microscope (Olympus, Miami, Florida) equipped with a Pixera charge-coupled device camera (Pixera, Miami, Florida) and analyzed with IMAGE Pro-Plus 4.1 software (Media Cybernetics, Silver Spring, Maryland).

GAPDH and COX-2 amplification

Macrophages (3×10^6) were interacted with the UP-CNTs, P-CNTs, FITC-CNTs, and L220-CNTs at 0.06, 0.6, and 6 mg/L, and incubated for 24 hours; total RNA was extracted by using Trizol (Invitrogen, Carlsbad California). Complementary DNA was obtained by retrotranscription from 5 μg of total RNA using the Super Script reverse transcriptase II kit (Invitrogen). An aliquot of 1 μL of complementary DNA from each of the cultures was used to amplify the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and COX-2 genes by the polymerase chain reaction (PCR). The following primers were used: for COX-2 gene, sense primer 5′-CTG TAT CCC GCC CTG CTG GTG-3′ and antisense primer 3′-ACT TGC GTT GAT GGT GGC TGT CTT-5′; for GAPDH, sense primer 5′-CCT TCA TTG ACC TCA ACT AC-3′ and antisense primer 3′-GGA AGG CCA TGC CAG TGA GC-5′.[26] These primers yielded amplified products of 279 base pairs (bp) for COX-2 and 574 base pairs for GAPDH; the cycling program and reaction conditions are described elsewhere.[27] PCR products were analyzed by electrophoresis in a 1% w/v agarose gel containing ethidium bromide; photographs were taken using a laboratory imaging GDS-8000 Chemi System (UVP, Inc., Upland, California) and analyzed with LabWorks 3.0 software (UVP, Upland, California). Controls were run to rule out DNA contamination in samples.

Statistical analysis

Statistical analysis was carried out by the Minitab software (State College, Pennsylvania) and using a one-way analysis of variance machine to determine the difference between the MOs' interactions with different CNTs at all the concentrations used. Cellular apoptosis data from MOs' interactions with different CNTs at previously mentioned concentrations, for 24 and 48 hours were compared and analyzed by the STATA 9.0 program for Windows (Stata Statistical Software, Release 9.0., Stata, College Station, Texas).

Results

Cell viability of J774 MQ cell line

As shown in Figure 2, MOs interacted with UP-CNTs showed a significant decrease to 63% of cell viability that was independent of the dose of UP-CNTs tested. In the case of the P-CNTs and FITC-CNTs, a dose-dependent toxic effect was observed, because at 0.06 mg/L no significant difference in cell viability was found, whereas cells interacted with 0.6 and 6 mg/L had a significant decrease to 60% and 40% in cellular viability, respectively, when compared to control. On the other hand, MOs interacted with L220-CNTs showed a significant increase in the number of viable cells at any of the tested concentrations compared with control without stimulus, or with cultures interacted with other CNTs. This result suggests that protein bound on the CNTs' surface may not only decrease the cytotoxicity but also most probably elicit cell proliferation.

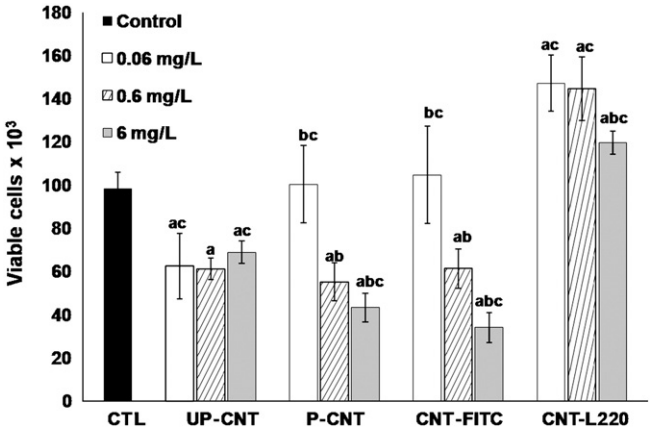

Figure 2. Viability of MOs interacted with CNTs at different concentrations at 24 hours. Each bar represents mean ± SD of two experiments done in triplicates ($n = 6$). a, $P < 0.01$ denotes significant differences between mean values measured in the indicated group as compared to control without stimulus (CTL). b, $P < 0.01$ denotes significant differences between mean values for CNTs at different concentrations. c, $P < 0.01$ denotes significant differences between mean values for a particular concentration among different CNTs.

Apoptosis assays

After 24 hours of interaction with the different types of CNTs, low levels of cellular apoptosis were observed, except for P-CNTs at 0.06 mg/L, where a 17% of cellular apoptosis was detected. At 48 hours, an increase in apoptotic cell number was observed in almost all interaction conditions, except for P-CNTs at 0.06 mg/L, where apoptosis decreased. At 48 hours the highest apoptosis level was observed with UP-CNTs, and no difference was found between concentrations. In the case of P-CNTs at 48 hours cells showed an increase in percentages of apoptosis in a dosage-dependent manner (Figure 3, Table 2); apoptosis percentage with 6 mg/L was significantly higher when compared with apoptosis observed at lower concentrations. However, apoptosis with 0.06 mg/L at 24 hours was significantly higher than percentages detected for the same concentration at 48 hours. With regard to MOs interacted with FITC-CNTs and L220-CNTs, apoptosis was significantly lower in relation to that observed for UP-CNTs and P-CNTs (Table 2). Particularly, the condition of MOs interacted with L220-CNTs showed a lower percentage of apoptosis compared with other particles. Additionally, when cells were interacted with FITC-CNTs and L220-CNTs, an increase in cell density was evident (Figure 3) compared with cultures interacted with UP-CNTs or P-CNTs.

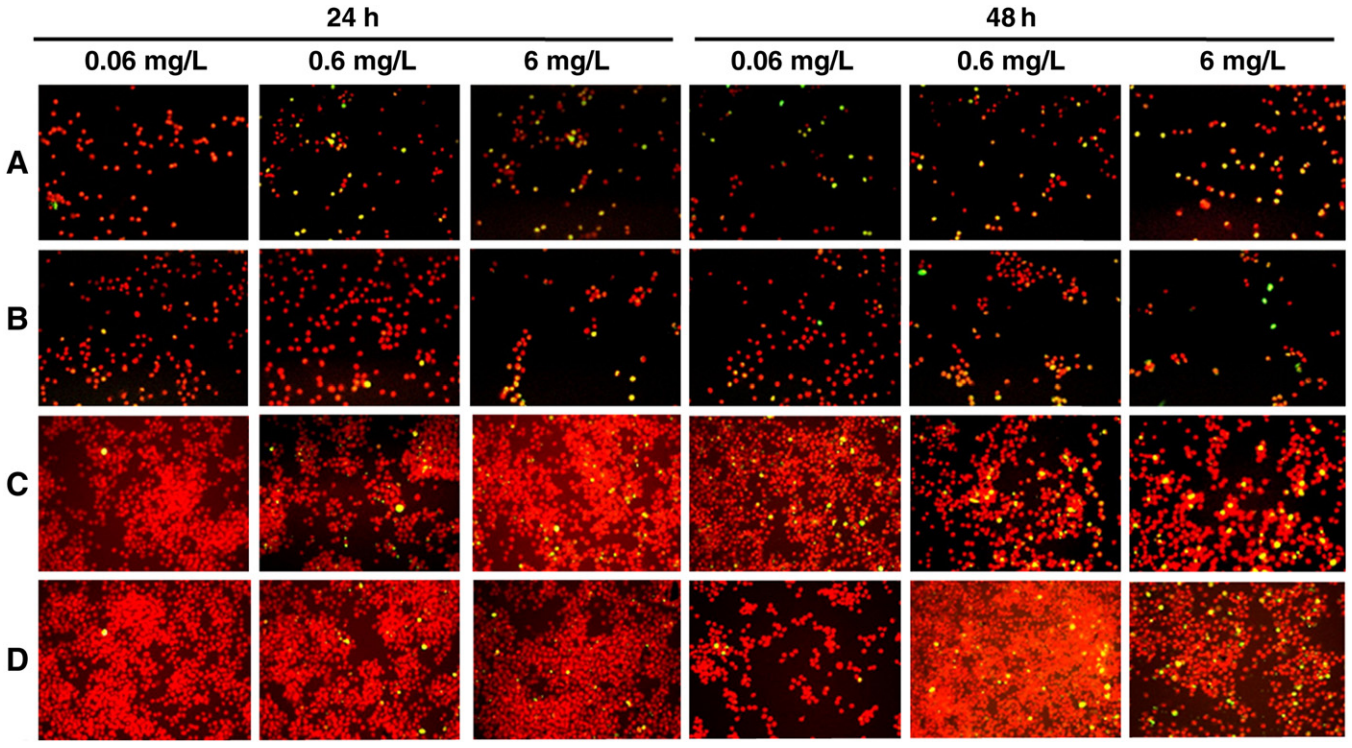

Figure 3. Apoptosis induction in MOs interacted with different CNTs at 24 and 48 hours. Concentration tested appears in upper line; apoptotic cells are distinguished by the green nucleus, and cells with intact DNA show a red nucleus. Photomicrographs show MOs interacted with **(A)** UP-CNTs, **(B)** P-CNTs, **(C)** FITC-CNTs, and **(D)** L220-CNTs.

Table 2
Percentage of cell apoptosis in MOs interacted with different CNTs

CNTs	(mg/L)	Apoptosis (%)*	
		24 hours	48 hours
UP	0.06	4.95 ± 1.18	29.01 ± 9.01‡,§
	0.6	5.510 ± 1.36‡	22.08 ± 6.28‡
	6	3.29 ± 0.73	28.85 ± 6.56‡,§
P	0.06	17.017 ± 4.09†,‡,§	4.01 ± 1.99
	0.6	1.41 ± 0.55	5.75 ± 2.14‡
	6	4.62 ± 2.47	27.61 ± 6.41‡,§
FITC	0.06	0.16 ± 0.02	4.13 ± 2.54
	0.6	1.41 ± 0.55	5.75 ± 2.14
	6	4.86 ± 1.09	10.34 ± 2.42
L220	0.06	0.20 ± 0.12	0.68 ± 0.71
	0.6	2.34 ± 0.64	11.12 ± 2.78
	6	0.13 ± 0.03	7.68 ± 1.99

* Data correspond to one experiment done in triplicates ($n = 3$).
† $P < 0.01$ difference between concentrations for the same CNTs.
‡ $P < 0.01$ difference between time at the same concentration.
§ $P < 0.01$ difference between treatments.

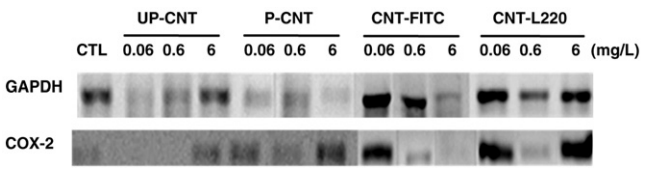

Figure 4. Effect of interaction with CNTs in COX-2 mRNA expression in MOs. MOs were incubated with different concentrations of CNTs for 24 hours. At term, total RNA was extracted, and COX-2 and GAPDH mRNA expression were analyzed by reverse transcription–polymerase chain reaction. Amplified products were detected by electrophoresis in 1% w/v agarose as described in the Methods section.

GAPDH and COX-2 amplification

To determine whether MOs are able to recognize and react against CNTs and/or added molecules, COX-2 messenger RNA (mRNA) expression was analyzed in MOs interacted with CNTs at 24 hours. Amplification of the GAPDH mRNA, a house-keeping gene, was detected in all interaction conditions. The expression of COX-2 mRNA, an inducible enzyme involved in the inflammatory process and in MOs activation,[28] was detected in MOs interacted with the CNTs (Figure 4) with higher intensity in MOs interacted with L220-CNTs (0.06 and 6.0 mg/L) as well as in P-CNTs in all analyzed concentrations.

Morphological analysis

To analyze if the interaction with different CNTs particles induced morphological alterations in MOs, cell morphology was analyzed at 24 hours; results showed that MOs interacted with UP-CNTs or P-CNTs (Figure 5, *C* and *D*, respectively) presented

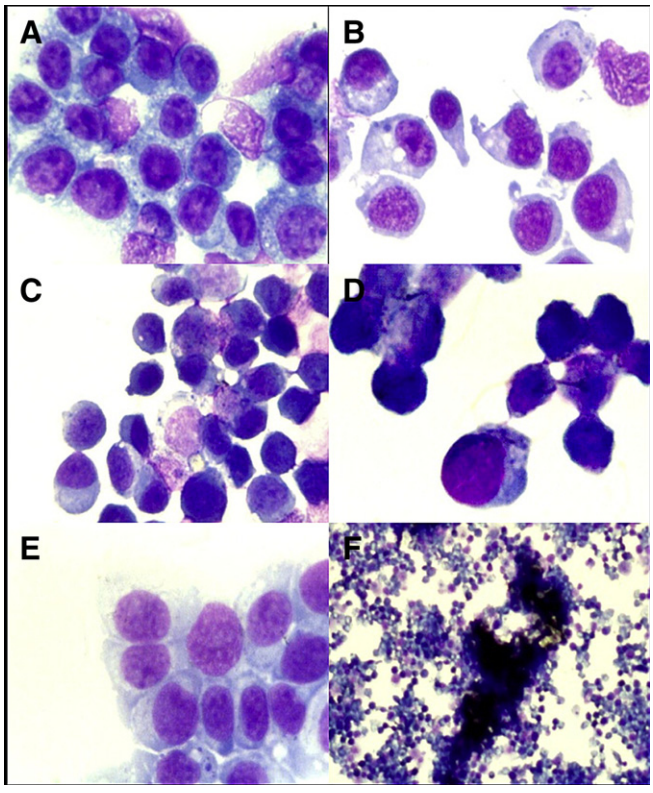

Figure 5. Photomicrographs of morphological changes detected in MOs interacted with CNTs. MOs were incubated with the different CNTs for 24 hours. At term, culture supernatant was removed, and cells were stained with Wright stain as described in the Methods section. Photomicrographs (original magnification 40×) show MOs as follow: **(A)** Control without stimulus; **(B)** interacted with L220-CNTs; **(C)** interacted with UP-CNTs; **(D)** interacted with P-CNTs; **(E)** interacted with FITC-CNTs. **(F)** MOs were interacted with L220-CNTs, original magnification 10×.

alterations characteristic of cell death. These alterations included a decrease in cell adhesion and in cytoplasm volume as well as shrinking and condensation of the cell nucleus. These alterations were not found in MOs without stimulus (Figure 5, *A*), which exhibited normal morphology. MOs interacted with L220-CNTs or FITC-CNTs (Figure 5, *B* and *E*, respectively) exhibited normal morphology, with the presence of cytoplasmic vacuoles that could contain the CNTs, in agreement with some reports.[29] The presence of pseudopodial and lamellipodial extensions of the membrane along the CNTs[30] has been reported, suggesting phagocytic events. However, under the resolution conditions available in this report, it was not possible to confirm or discount their presence. Further analysis by electron microscopy would be necessary. The formation of cellular aggregates in the presence of L220-CNTs was also observed (Figure 5, *F*), indicating a protein recognition by MOs.

Discussion

Results obtained in this study show that, in the case of CNTs, toxicity was dependent on the purification process of the CNTs as well as the molecule added. The order of magnitude of toxicity found was UP-CNTs > FITC-CNTs > P-CNTs > L220-CNTs. Several works have reported that the purification process eliminates residues on the surface of the CNTs and introduces certain charged groups on their surface, allowing higher stability in aqueous solution even though they might contribute to the cytotoxic potential.[10,12,25] Additionally, the purification procedure could also be important in toxicity; studies in which CNTs were purified with potassium permanganate solution or with concentrated sulfuric acid showed that these CNTs had a greater toxic effect[10] compared with studies in which purification was carried out with HNO_3 (ref. 31). However, these studies did not mention if a quantification of COOH groups was carried out. P-CNTs used in our study contained 9% of exposed COOH groups calculated as weight increment after purification,[22] and they showed lower toxicity than that described in previous studies. Furthermore, our results agree with those obtained by Vittorio et al.,[31] who also used P-CNTs prepared by the HNO_3 method, finding that they contained 8% of COOH groups. Additionally, semiquantitative analysis of elemental composition performed with SEM after purification showed a decrease in Fe content in P-CNTs when compared with the UP-CNTs, as well as an increase in O_2, results that suggest the introduction of oxidized groups (COOH, OH, CO) or Fe_2O_3 (ref. 32) over the CNT surface. Moreover, in SEM micrographs, the P-CNTs showed higher dispersion and lower folding when compared to UP-CNTs structures, an effect that, according to several investigators, is due to the presence of oxidized groups.[4,7] One of the most important influences on biocompatibility of materials is their dissolution properties. The toxicity effect should be evaluated as dependent on materials' solubility. Previous studies have evaluated CNTs in contact with cells and have found that unless they are functionalized, CNTs aggregate in the medium and do not remain uniformly in contact with the cells.[33-35]

On the other hand, functionalization is a process that allows the binding of chemical groups of interest on the surface of CNTs, contributing to diminish their toxicity.[36] Functionalization with FITC or with L220 was carried out on a linker N-Boc[22] together with the P-CNTs through a zwitterion ionic bond; this process allows higher functionalization yield. Nevertheless, in the case of L220, yield was very poor (5.28%); this could be due to steric obstruction of the protein, which is possible, because L220 has a very complex structure and the folding of the L220 on the CNT may be limited by the surface area thereof, therefore the amount of protein that could join the CNT would be low.The CNTs that were used in this trial exceed the conventional dimensions that other authors have used in their trials (about 500 nm long, and 40 to 50 nm in diameter); we consider that the dimensions of the CNTs we used provided an adequate surface so the L220 can be both efficiently functionalized and presented to MOs. However, more studies are required to confirm this fact. In fact, as shown in our results, the UP-CNTs and P-CNTs were more toxic than the f-CNTs, which is consistent with results reported by other authors.[37] Notwithstanding, results obtained with L220-CNTs showed less toxic effects when compared to other particles. These results suggest that despite the small amount of protein bound to CNTs, it was enough to reduce cytotoxicity and to allow stimulation of the MO.

On the other hand, FITC-CNTs presented a greater toxic effect on cell viability in comparison with other studies, in which no alteration in cell functionality had been observed, and which could be attributed to differences in the FITC used in both studies. Another possible explanation for this is the difference in length of the CNTs used in this study, which exceeded 120 μm and could have been damaging cell structures. Studies done by Sato et al[38] have demonstrated that when CNTs exceeded 20 μm in length, nanotubes cannot be phagocytized and cause direct damage to plasma membranes in cells from human acute monocytic leukemia THP-1 cells, as well as a greater inflammatory response when compared with shorter CNTs.[38] This principle could be applied to the results obtained with UP-CNTs, P-CNTs, or FITC-CNTs, but in the case of L220-CNTs, the decrease in cytotoxicity may be related to the presence of L220 functional groups that interact with receptors on the MOs.

The above results agree with those obtained in cell apoptosis, in which it was observed that the f-CNTs were associated with lower rates of apoptosis. Interestingly, the interaction time was particularly important, because a greater interaction time resulted in greater amounts of apoptosis. In the case of P-CNTs, interaction with 0.06 mg/L at 24 hours induced higher levels of cellular apoptosis, which might result from (i) a greater amount of viable cells that remain attached (Figure 2) and (ii) a greater dispersion of P-CNTs in the culture medium. However, at 48 hours the percentage of apoptosis decreased in comparison to 24 hours at the same concentration; this might be due to the presence of fewer viable cells, in that dead cells could be lost (unattached cells) as a result of the methodology used for the apoptosis assay. Similar results were reported by Bottini et al.[12] in Jurkat cells. In addition, Cui et al.[33] reported that HEK293 cells required 24 hours to secrete small proteins in response to interaction with single-walled CNTs. Decrease of apoptosis at 48 hours could result from an adaptation process or from a protective response to initial contact with P-CNTs. For this reason, some authors recommend performing biopersistence studies in in vivo models before CNTs can be used as adjuvants or in nanovaccines.[14]

Additionally, the fact that FITC-CNTs showed higher cytotoxicity but lower levels of apoptosis suggests that this type of CNTs may be causing cell death by a mechanism different from apoptosis.This is in agreement with other studies that have shown that necrosis is the main death mechanism in MOs interacted with CNTs.[32] Some authors point to the properties obtained from the chemical functionalization: solubility in water, high dispersion, and a low tendency to form aggregates—all of them relevant in cytotoxicity modulation.[37]

On the other hand, COX-2 is an inducible enzyme in cells, playing a role in inflammation such as MOs; in human and murine MOs, COX-2 expression is induced by lipopolysaccharides, interleukin 1, and phorbol esters.[28,31] Studies in vivo and in vitro have demonstrated that MOs present in lesions produced by *E. histolytica* expressed COX-2 (refs. 27,39).

The COX-2 mRNA expression detected in MOs interacted with P-CNTs could probably be attributed to the fact that these particles have a similar structure to bacterial lipopolysaccharides. This is due to the combination of the lipophilic portion of CNTs with COOH, OH, and CO groups created during purification. In this way, the P-CNTs could interact with CD14 receptors in the MOs, thus managing Toll-like receptor activation and inducing COX-2 expression.[28,40] The COX-2 expression observed in MOs interacted with L220-CNTs was expected, because L220 is a highly immunogenic protein[18] and could be recognized by Toll-like receptors of MOs and induce an inflammatory response.

Finally, results from the morphological analysis show that UP-CNTs and P-CNTs cause morphological alterations characteristic of cell death. This has already been reported by other authors,[10] and their results are in agreement with the apoptosis results of this study. Likewise, in MOs interacted with f-CNTs, alterations in the membrane are related to the formation of digitiform prolongations characteristic of endocytosis and cell phagocytosis processes already reported by Cheng et al.[28] This last result, along with cell proliferation, as well as the COX-2 expression of the MOs interacted with the L220-CNTs, suggest cellular activation and probably the initial activation stage of the MOs. Nevertheless, more studies are required to confirm this.

Therefore, it can also be concluded from this study that the use of L220-CNTs could have a great impact on the creation of a new nanovaccine for the prevention of amebiasis. Cell activation was observed without toxic effect in MOs interacted with these particles, even though L220 is a 220-kDa protein and, according to other works, conjugation of CNTs with proteins of high molecular weight (>80 kDa) can contribute to their higher cytotoxicity.[41] The use of CNTs in medicine has already resulted in a number of studies using diverse in vivo models. Several studies have demonstrated that f-CNTs with antigens from virus or parasites constitute a good synthetic vaccine formulation, because the immunogenic peptide conformation is well preserved.[42,43]

Studies are currently under way to determine the biopersistence and biodistribution of these particles in in vivo models.

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