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EXPOSURE OF HELA CELLS TO LOW DOSE OF SINGLE WALLED CARBON NANOTUBES FUNCTIONALIZED WITH SINGLE STRAND DNA INDUCES NO SIGNIFFICANT TOXICITY

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ABSTRACT

Introduction. Single-walled carbon nanotubes(SWCNT) represents a promising material for the biomedical field. However, reports have appeared that suggest that toxic effects can appear following SWCNT exposure.

Aim. Present study aims to evaluate the *in vitro* effects following exposure to low dose of single walled carbon nanotubes functionalized with single strand DNA (SWCNT-ss-DNA) over HeLa cells.

Material and Method. Synthesis of the SWCNT (inductive heating method, acetylene, 850°C, Fe:Mo:MgO catalyst), followed by non-covalent binding of single strand DNA(ss-DNA) through sonication. HeLa cell line was exposed to 1mg/L ss-DNA-SWCNT solution with monitoring at 15 min, 24 hrs, 48 hrs.

Results. No toxic effects were observed at microscopic evaluation of cell morphology and at quantitative viability evaluation by means of MTT assay.($p>0.05$).

Conclusions. Limited dose exposure of HeLa cells to SWCNT-ss-DNA induces no cytotoxic effects.

KEYWORDS

carbon nanotubes, DNA, cytotoxicity, low concentration, *in vitro*

INTRODUCTION

Single-walled carbon nanotubes(SWCNT) represents a promising material for applications in various domains, according to recent literature. Proposed domains of interest include: robotics, electronics, or energetic fiels. Recently, applications of the nanomaterial have also been imagined for the biomedical domain. Various research groups have proposed inovative directions for SWCNT usage in medicine, among which the most important were: diagnosis/ therapy of neoplasma or infections, regenerative medicine/ tissue engineering, as well as immunotherapy(Feazell ,2007) (Lindberg ,2009) (Bharali ,2009)(Ajima ,2008) (Kang ,2007). (Liu ,2010) (Dhar ,2008) (Partha ,2009) (Khazaei ,2010) (Tripisciano ,2009). Although there are an increasing number of studies aiming to identify new applications of for SWCNT-based compounds, reports have appeared that suggest that toxic effects can appear following SWCNT exposure. Up to date literature brings contradictory evidences regarding their safety. Various characteristics of the nanomaterial have been suggested to determine changes in the intensity of toxic effects. Among them, physical and chemical features of the nanomaterial: cristalinity, purity, functional groups demonstrated an important modulatory effect in determination of their toxicity (Garza ,2008). Several *in vitro* studies have suggested that SWCNT induce toxic effects, with alteration of proliferation due to increased cytokine production (Zhang ,2007) as well as activation of reactive oxygen species (ROS) generation (Manna ,2005). There are, however, studies reporting no signs of in vitro toxicity following nanomaterial exposure. (Dumortier ,2006), therefore the issue of *in vitro* toxicity of SWCNT is still far from being elucidated. Present study aims to evaluate the *in vitro* effects exerted by exposure to low dose of single walled carbon nanotubes functionalized with single strand DNA over HeLa cells.

MATERIALS AND METHODS

Synthesis of the nanomaterial

Using a previousely published inductive heating method, we have performed the synthesis of the nanomateriaal (acetylene, 850°C, Fe:Mo:MgO catalyst -1.4:0.14:98.46 wt %)(Lupu ,2004) (Biris ,2006). After synthesis, nanotubes went through a purification process. For this step, HCl refluxing was performed using a Soxhlet device (HCl (1:1), 24 hrs). Following, the nanomaterial was washed (water, up to neutral PH) and dryied (120 °C, 12 hrs). Purity level of synthesized SWCNT was evaluated using thermo galvanometric (TGA) analysis. The test revealed a purity level of >98%.

Functionalization of SWCNT.

Binding of ss-DNA to SWCNT using non-covalent forces was performed using 40 mg SWCNT and 40 ml ss-DNA 0.1% solution (Sigma-Aldrich Chemicals GmbH - Germany)For the process, sonication for 15 minutes was used (Sonics, Vibra-Cell VC 505, 500 Watt, 20 kHz, ice-water bath, 30% amplitude). Following, the mixture went through a centrifugation process (Sigma-Aldrich, 1 hr, 4000g). At the end of the process, removal of undispersed

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SWCNT was performed. Optical absorption was assessed in both the collected supernatant and ss-DNA 0.1% solution (Jasco V-570 UV-vis-NIR Spectrophotometer, 1/40 dilution, 1 cm quarts cuvette). The estimative concentration of the prepared ss-DNA-SWCNT solution was 390 mg L^{-1}. Dilution was performed to obtain 1 mg/L concentrated solution.

HeLa Cell Culture and Exposure.

HeLa cell line (ATCC, Manassas, VA, USA) was grown using manufacturer protocols (25 cm3 plates in minimum essential medium, supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, humidified 5% CO2 incubator at 37°C). Monitoring of confluence was performed. When >70% confluence was observed, cells were split using PBS washing and tripsinization. For the experiments, the cells were cultivated to confluence on 96 well plates. The nanomaterial was administered to the cell cultures by adding to the culture medium and incubating for various periods of time (15 min. 24 hrs, 48 hrs) at 1mg/L concentration. Control cell line samples were constructed with medium exposure only and similar maintenance conditions. For each time point, triplicate samples were used for both control and test samples.

Toxicity Examination

Morphology of the cells was examined using a Motic A30 AE30 Binocular Inverted Microscope equipped with digital transfer unit. Proliferation assay was performed by means of MTT assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide]. In brief, at the end of exposure, cell culture medium was removed, and wells were washed with 200 µl Hanks Balanced Buffer Solution (HBSS+, #14025, Gibco). In the next step, cells were then incubated (3 hrs, 37°C, 5% CO_2) with 200 µl of 0.5 mg/ml MTT solution (#M2128, Sigma) in HBSS. MTT solution was consequently removed, and an extra 100 µl of DiMethylSulfOxide (DMSO, #D5879, Sigma) was added experimental wells. Optical density (OD) was measured (550 nm, with 655 nm reference). Cell viability was calculated as the % represented by the median of OD obtained for each condition from median of control samples.

Statistical Methods

Normality of continuous data was assessed by means of Kolmogorov-Smirnov Test. For testing of variance across time intervals Kruskall-Wallis test was used. Between group comparisons were performed by means of Mann-Whitney U Test for each time point. Dynamic assessment of viability in time for each group was evaluated by means of kinetic curve method, with area under the curve calculus(AUC). Between group differences in AUC levels were tested using a non-parametric method(Mann-Whitney U test). SPSS 17.0 (Chicago, Il, USA) statistical package was used for all data analysis.

RESULTS AND DISSCUSSIONS

Microscopic examination revealed no signs of cytotoxicity. The aspect of HeLa culture remained unmodified in terms of shape and dimension of the cell, quantity and color of the cytoplasm, presence, shape and dimension of the nucleus.(Fig.1)

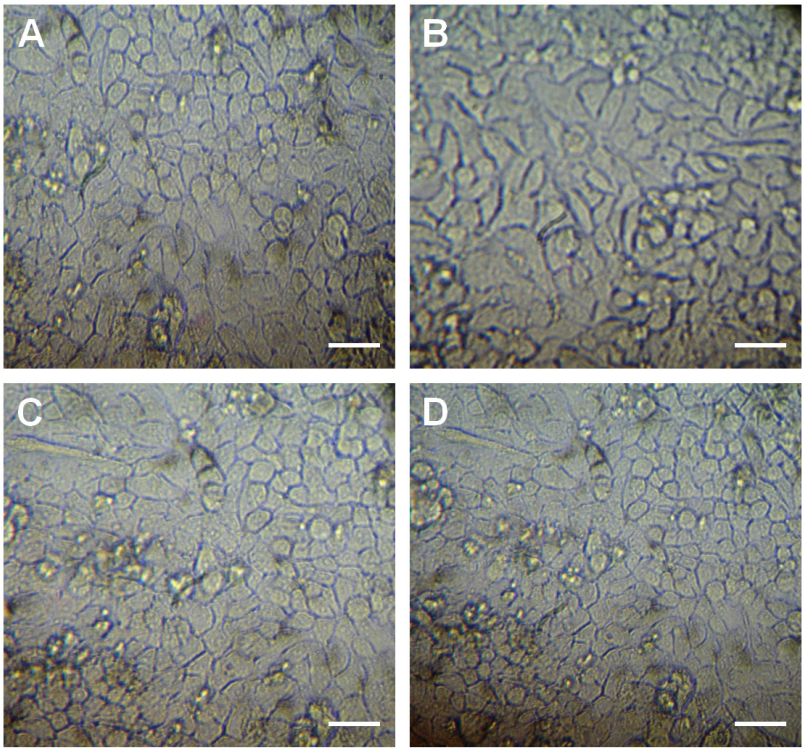

Fig. 1. Microscopic examinations of HeLa cell samples.

A. Control group, 24 hrs after exposure, B. Control group, 48 hrs after exposure, C. SWCNT group, 24 hrs after exposure, D. SWCNT group, 48 hrs after exposure.
Scale bar represents 50µm.

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Literature data on the *in vitro* toxicity of carbon nanotubes provides contradictory evidences regarding the existence of toxic effects following exposure. While some reports find no signs of cytotoxicity exerted by SWCNT alone (Dong ,2008), other reports find the nanomaterial to be both cytotoxic and genotoxic by inducing DNA damage and micronuclei. (Lindberg ,2009). The latter research finding is doubled by *in vivo* evidences on possible toxic effects of SWCNT following different types of administration, including pharyngeal aspiration and lung inhalation (Shvedova ,2005), dermal exposure(Murray ,2009) and intra periotoneal injection (Yang ,2008). Sources of variability could come from the various types of dispersion techniques, characteristics of the nanomaterial, but also from the concentration itself.

Previous studies suggest that the degree of sidewall functionalization highly modulates the intensity of toxic reactions. It has been stated that the higher the degree of sidewall functionalization, the less cytotoxic the SWNT sample. Also, the same research group suggests that sidewall functionalized SWNT samples induce significantly reduced cytotoxicity than surfactant stabilized SWNTs.(e.g. 1% Pluronic F108). (Sayes ,2006). Another research paper demonstrates the possible impact of carbon nanotube length, with a direct proportionality between length and toxic effects. Results, however, show less prominent effect of length range within *in vitro* as compared to *in vivo* experiments. (Sato ,2005). Results are concordant with our findings, since we have used a short- to- medium length range, sidewall functionalized nanomaterial. However, it has already been stated that toxic effects of nanomaterial are dose-dependent.(Nel ,2006), thus making the administration dose one of the most important determinant of carbon nanotubes' toxicity. Moreover, safe levels of exposure concentration should be unique for each type of nanomaterial.

For our chosen concentration (1mg/L), we have obtained no variance in viability levels across time for either of the two groups. Also, between- group comparisons for all time points did not reveal any statistically significant difference.(Table 1), thus suggesting the absence of ss-DNA-SWNCT-induce viability loss effects.

Table 1. MTT Assay results following exposure.

	Group	15 minutes	24 hrs	48 hrs	p value (across time variability)
Viable cells (%)	Control	86.3 (84.5-87.2)%	92.5(90.2-94.4)%	93.4(91.5-94.8)%	0.723
	SWCNT	84.8(83.5-85.4)%	90.6(89.7-93.3)%	92.5(90.2-93.6)%	0.851
p value (between-group comparisons)		0.894	0.717	0.913	

Results are expressed as median(95% CI).

Dynamics of the two groups in terms of viability assessment revealed no difference in AUC (95%) calculated values (controls: 898.5(879.1-901.2), SWCNT group: 884.2(872.3-899.5), p=0.912.) (Fig.2)

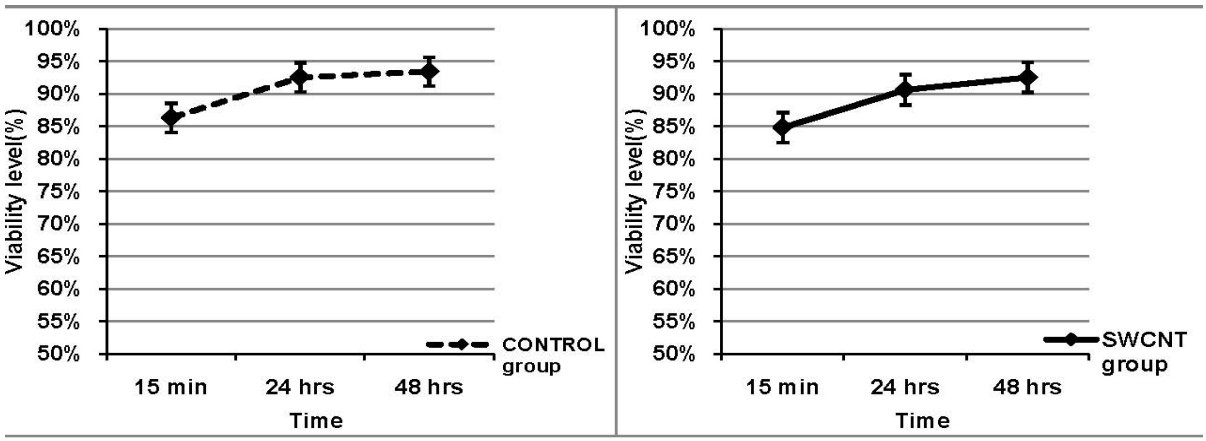

Fig. 2. Dynamics of HeLa cells'viability levels in the control group (left) and SWCNT group(right), respectively.
Points represents median level, error bars represent standard error.

It has been previously stated that another source for contradictory literature results is the interaction of the nanomaterial with various dyes utilized to assess toxicity. (Wörle-Knirsch ,2006). The study reports presence of SWCNTs interactions with some tetrazolium salts such as MTT but absence of such interactions with others (such as WST-1, INT, XTT).Such interactions could induce fake cytotoxicity reactions. For our own study, the low concentration we have used could generate reduced levels of such interactions thus explaining the absence of toxic reactions. Similar results have been published by other research groups, which strengthens our conclusions. A recently published report demonstrates lack of toxicity signs following SWCNT exposure as well as immune cells preservation.(Dumortier ,2006).

We are aware of the need for a complex pharmacological study with the inclusion of seriate concentrations. Athough it is mandatory for research to still be carried out for an exact concentration cut-off establishment, our results demonstrate that no toxicity is induced by 1 mg/L concentration.

CONCLUSIONS

Limited dose-exposure of HeLa cells to single walled carbon nanotubes functionalized with single strand DNA induces no visible and quantifiable toxic effects. Although higher doses of exposure may interfere with cell metabolism and viability, results demonstrate the safeness of small dose nanomaterial usage for biomedical purposes.

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