In vitro toxic effects of different types of carbon nanotubes

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Abstract. We have carried out a comparative assessment of industrial single- and multiwalled carbon nanotubes (SWCNTs / MWCNTs) toxic effects in the cultures of macrophages (RAW 264.7) and bronchial epithelial cells (BEAS-2B). Lactate dehydrogenase (LDH) levels were measured directly in the medium. Intracellular reduced glutathione levels (GSH) were determined in culture lysates. Cell viability was determined by fluorescence method. RAW 264.7 were much more sensitive to the effects of carbon nanotubes (CNTs): MWCNT addition was not accompanied by a significant reduction in the viability of macrophages, but caused damage to cell membranes (release of LDH) with a dose and time-dependent oxidative stress. SWCNTs caused a significant decrease in viability and induction of oxidative stress. Improved darkfield microscopy revealed adsorption and accumulation of MWCNTs on the surface and inside the macrophages. Adding SWCNTs to BEAS-2B culture caused small statistically significant dose-and time-dependent decrease in viability and pronounced reduction in glutathione only at the highest concentration of nanoparticles. Bronchial epithelial cells BEAS-2B appeared to be much less susceptible to MWCNTs. The findings suggest that there are differences in the toxic action of different CNTs and there is a need for thoughtful approach to the assessment of nanomaterials toxicity.

1. Introduction

Single- and multiwalled carbon nanotubes (CNT), being ones of the most promising nanomaterials are becoming more and more common. The world is witnessing a steady increase in companies producing and using CNTs. At the same time the number of persons potentially exposed to the aerosol of carbon nanotubes at their workplaces increases as well [1, 2].

Accumulated data suggest a risk to human health upon occupational exposure to carbon nanoparticles. It has been suggested that nanosized particles are more dangerous than micro-particles due to the greater penetrating power, surface area and reactivity [6], whereas fibrous structure of carbon nanotubes may account for the pathogenicity inherent to mineral fibers (asbestos, silica) [3, 4, 5].

The aim of our study was a comparative evaluation of the industrial singlewalled and multiwalled carbon nanotubes toxicity in the cell cultures originating from pulmonary system.

2. Materials and Methods

2.1. Particles.
Single- and multiwalled carbon nanotubes used for experiments were produced using the catalytic vapor deposition. Characteristics of both carbon nanotubes types provided by producers are shown in the Table 1.

Table 1. CNT characteristics, provided by producers

Parameters	MWCNT	SWCNT
External diameter (nm)	8-15	1-2
Internal diameter (nm)	4-8	0.8-1.4
Length (um)	2 and more	3 and more
Total amount of impurities – catalysts leftovers (%)	up to 5	4-7
Bulk density (g/cm^3)	0.03-0,05	0.15
Specific surface area (m^2/g)	300-320	1100
Thermal stability in air (ºC)	up to 600	Up to 700

2.2. Dosage.
CNTs were sonicated in the presence of dipalmitoylphosphatidylcholine (DPPC) (0.01 μg/ml) to increase dispersion [7, 8] and visually were represented by tangles up to 5 μm and individual fibers. To assess the dose-dependent effects in the cultures, concentrations of 0.02, 0.2, 2.4 and 24 micrograms per cm^2 of the monolayer surface were selected (0.1, 1, 10, 100 μg/ml of medium respectively). These concentrations were used in previous studies on the comparative evaluation of the carbon nano-and microsized particles toxicity [9, 10, 11].

2.3. Cell cultures.
Two cell lines were taken for the study: RAW 264.7 transformed murine macrophages and immortalized normal human bronchial epithelium BEAS-2B. Cells were grown using MEM (Minimal Essential Media, «Gibco», USA) and DMEM (Dulbecco's modified Eagle medium, «Gibco», USA) media supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in the incubator (37 °C, 5% CO_2). Cells were seeded on 96-well plates at approximately 2,000 per well.

Lactate dehydrogenase (LDH) levels were measured directly in the medium as a marker of cell membranes damage [12]. Intracellular reduced glutathione levels (GSH) were determined in culture lysates using the ThioGlo® («Calbiochem», USA) reagent to evaluate indirectly the activity of free radical oxidation. Cell viability was determined by fluorescence method.

Part of the cells (RAW 264.7 exposed to MWCNT concentration of 0.2 $\mu g/cm^2$ after 2, 4, 6, 24 and 48 hours) was collected, washed off and analyzed using the improved dark field microscopy («CytoViva®», USA).

Statistical analysis was performed using the t-test in the Microsoft Excel 2010 software package.

3. Results

We had selected RAW 264.7 murine macrophages as they are the cells initiating and maintaining different types of immune responses in the case of contact with the foreign bodies [13]

Adding MWCNT to the RAW 264.7 culture caused no significant reduction in viability (Figure 1a), however it was accompanied by the increased concentrations of LDH in the extracellular medium after 48 hours at all doses (but not at 24 hours) (Figure 2a). Oxidative stress manifestations had a dose and time-dependent relationship (Figure 3a) - the higher the dose and exposure time (for the highest dose) the lower the levels of intracellular reduced glutathione.

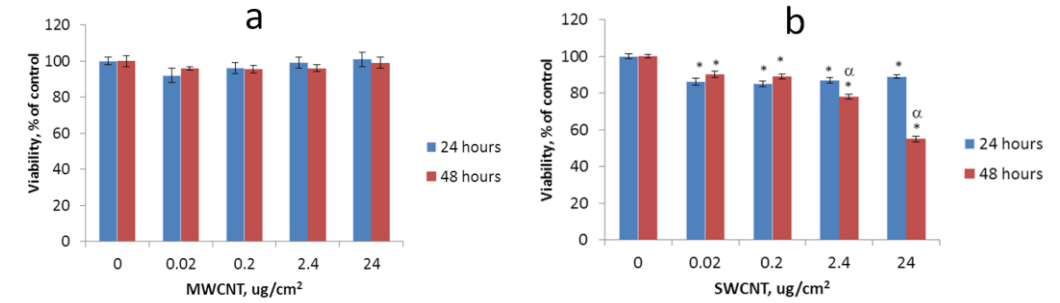

Figure 1. Viability of RAW 264.7 macrophages exposed to different types of CNTs. * - P <0.05 vs control, α - p <0.05 vs. 24 hours

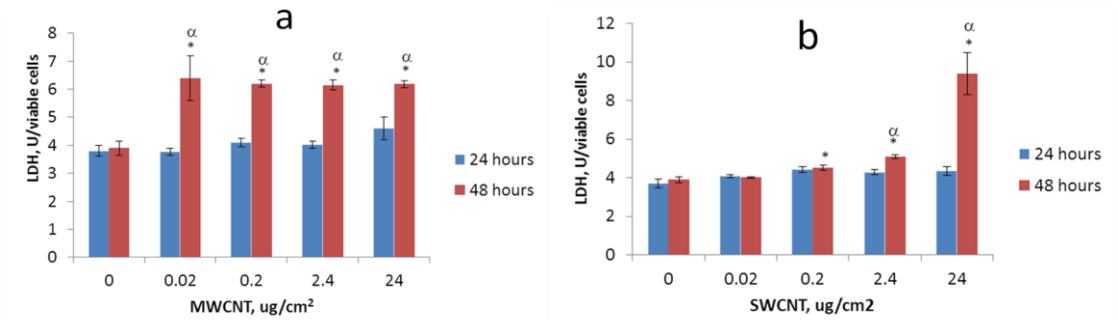

Figure 2. LDH leakage in the culture of RAW 264.7 macrophages exposed to different types of CNTs. * - P <0.05 vs control, α - p <0.05 vs. 24 hours

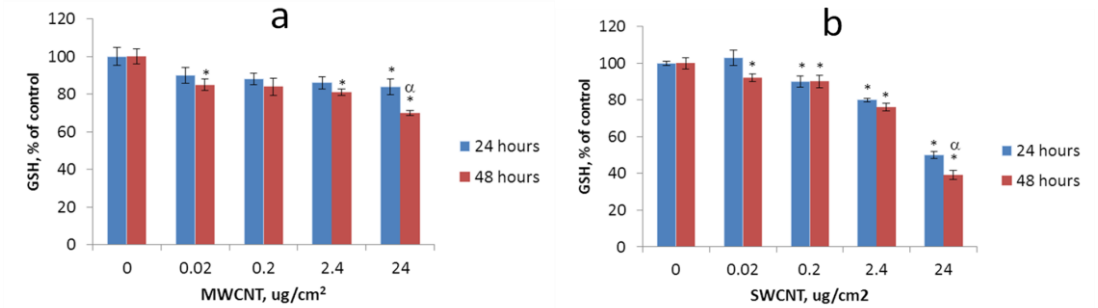

Figure3. Oxidative stress in RAW 264.7 macrophages exposed to different types of CNTs. * - P <0.05 vs control, α - p <0.,05 vs. 24 hours

Adding SWCNT to RAW 264.7 resulted in reduced viability (by 10-15%) at 24 hours after exposure, regardless of the dose. After 48 hours at 2.4 μg/cm^2 SWCNT viability decreased to 78 % and at 24 μg/cm^2- to 50% of the control cells (Figure 1b). Levels of LDH increased sharply after 48

hours at concentrations of 0.2, 2.4, and 24 μg/cm^2 (Figure 2b), which coincides with a noted reduction of viability in the same groups. Significant decrease in reduced glutathione levels was also found: the greater were the exposure dose and time, the lower GSH levels (Figure 3b).

Bronchial epithelium cells BEAS-2B, being the cells that play a major role in the evacuation of dust particles from respiratory tract, appeared a little susceptible to the introduction of MWCNT after 24 and 48 hours: there had been no significant reduction in viability compared to the control culture. The same results were obtained in the evaluation of cell damage and oxidative stress (data not shown).

Adding SWCNT to the BEAS-2B culture caused a small statistically significant dose- and time-dependent decrease in viability after 24 and 48 hours as well as marked reduction in the level of reduced glutathione at the highest concentration of 24 μg/cm^2 (data not shown).

Subsequent investigation of exposed RAW 264.7 cells by darkfield microscopy revealed adsorption and accumulation of MWCNTs on the surface and inside the cells. With increased exposure time number of CNT visually observed inside and outside the cells also increased (Figure 4).

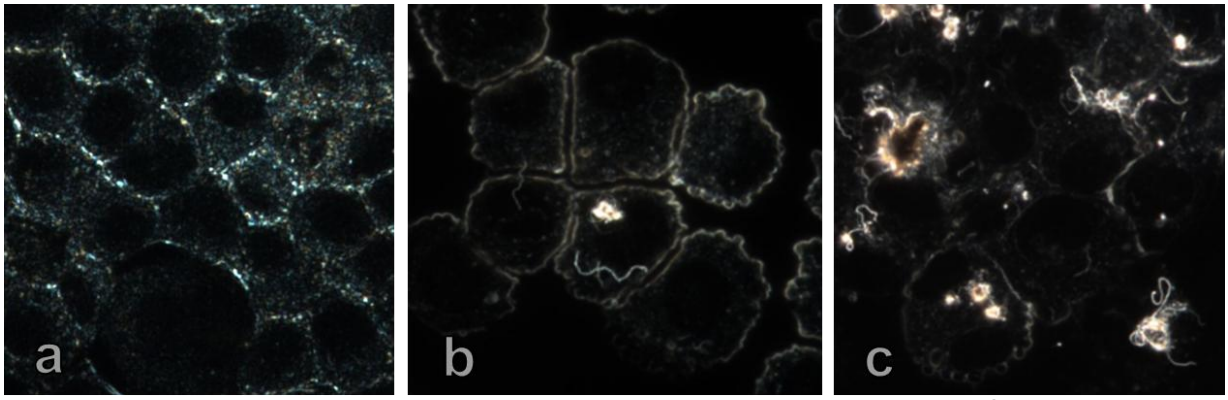

Figure 4. RAW 264.7 exposed to MWCNTs: **a** – control culture; **b** – 0.2 μg/cm^2, 2 hours; **c** – 0.2 μg/cm^2, 24 hours

4. Discussion

Obtained data suggest that the studied industrial SWCNT have a toxic effect, causing the induction of oxidative stress and cell death. At the same time MWCNTs administration, whereas not leading to a significant reduction in viability, cause damaging effect on the membrane of sensitive cells (macrophages). This complies well with the data obtained by other research groups (Hirano et al., Shimizu et al.) showing that MWCNT may associate with the membrane of macrophages and disrupt its integrity [14, 15].

Comparative studies of the SWCNT and MWCNT *in vitro* toxicity are few. Existing data have a multidirectional nature. Some researchers have found that SWCNT had greater cytotoxicity [11, 16, 17]. Some reported no acute toxic effects for both types of CNTs [18]. Others showed decreased viability when exposed to CNTs, but found no quantitative differences between single- and multiwalled nanotubes [19, 20]. Bacteria E.Coli, P.Aeruginosa and others were the most sensitive to the SWCNTs while MWCNT showed moderate toxicity [21]. According to our results, both SWCNT and MWCNT have toxic effects, but mechanisms and manifestations are different, which should be considered in the future in comparative toxicological studies.

One of the features of our study was to investigate macrophages response. Experiments *in vivo* suggest a significant role of the latter in the development of acute and chronic inflammation as well as profibrotic changes in the lungs upon exposure to CNT [9, 22, 23]. It was also shown that SWCNT are accumulated by macrophages to a much lesser extent than MWCNT [24]. Researchers studying genotoxic effects of CNTs in RAW 264.7 macrophages found signs of damage to the genetic apparatus at concentrations greater than 0.1μg/ml for SWCNT and above 1 μg/ml for MWCNT,

although cytotoxic effects appeared only at the highest doses (100μg/ml) [25]. Mechanisms of adverse effects remain poorly understood and studies using macrophage cultures are relatively scarce.

Our study has shown that CNT do have a toxic effect on the RAW 264.7 macrophages, unlike bronchial epithelium cells. It should be noted that we used immortalized leukemic macrophages in the experiment, which, however, are capable of phagocytosis, lysosomal activity, express and secrete most of the biologically active substances typical of normal macrophages, including various cytokines and mediators.

The experimental data obtained for one CNT type should be extrapolated on other CNTs produced by other companies and/or in other conditions with caution. Thus, there were found different responses to administration of MWCNT of differing morphology and length into the pleural cavity of rats - from minor local inflammatory response in case of short and tangled nanotubes to severe inflammatory response and granuloma formation for long rigid particles [26]. CNTs produced by catalytic vapor deposition also differ in catalysts content. Occupational exposure at primary manufacturers involves contact with both unpurified and purified material, while secondary production and handling implies contact with the already treated (and/or functionalized) carbon nanotubes. Investigators have already pointed out the role of contaminants - catalysts in the development of oxidative stress upon the cells' contact with CNT [18, 27]. In experiments on mice, purified SWNTs were less toxic to animals than samples containing residual catalysts, such as iron and nickel [28].

5. Conclusion

The macrophages (RAW 264.7) were much more sensitive to the effects of different types of CNTs than bronchial epithelial cells (BEAS-2B). SWCNT exposure resulted in the induction of oxidative stress and cell death. Addition of MWCNT did not cause a significant reduction in viability, but had a damaging effect on the cellular membranes of macrophages. The results show that it is necessary to develop *in vitro* or *in silico* models for the evaluation of various parameters of nanopaticles toxicity. Comparison of different CNT types should be based not only on the viability/cytotoxicity assessment, but also on specific adverse effects, such as oxidative stress, cell membranes damage and cytokine profiling. Furthermore, for regulatory documents it is necessary to classify nanoparticles not only by their chemical composition, but also by their morphological characteristics and properties.

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