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Comparative characteristics of various fibrous materials in *in vitro* experiments

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Abstract

Aim. Comparative assessment of the effect of fibrous materials on cell cultures RAW264.7 and BEAS-2B.
Methods. The effects of various fibrous materials — single-walled carbon nanotubes of two types (SWCNT-1 and SWCNT-2), differing in morphological characteristics, and chrysotile asbestos as a positive control — was assessed on two cell lines macrophages RAW 264.7 and human bronchial epithelium BEAS-2B cells. The studied materials' concentration range for experiments on cells was selected taking into account the SWCNT content in the air of the working area and the subsequent modeling of SWCNT deposition in the human respiratory tract. Suspensions of the studied materials were prepared based on cell culture media by ultrasonication. Cytotoxicity assessment after 48 hours of incubation was performed by using the MTS colorimetric assay. The expression level of apoptosis markers was assessed by immunoblotting using the corresponding monoclonal antibodies. Visualization of SWCNT-1, SWCNT-2 and chrysotile asbestos in BEAS-2B cell cultures was carried out by improved dark-field microscopy.
Results. According to dark-field microscopy, all the studied fibrous materials were found on the surface or cytoplasm of the cells. SWCNT and chrysotile asbestos did not have a direct cytotoxic effect in the MTS assay and did not induce apoptosis according to the results of Western blotting in cell cultures of RAW264.7 macrophages and BEAS-2B bronchial epithelium. In the cells of the bronchial epithelium (BEAS-2B) that showed greater sensitivity, a slight increase in the expression of pro-apoptotic protein PARP, which was more pronounced for shorter SWCNT-2, was revealed.
Conclusion. Both types of SWCNTs, despite the differences in morphological characteristics, demonstrated similar effects in *in vitro* experiments; this result, with its further verification, can have an important practical application in justifying approaches to determining the safety criteria for single-walled carbon nanotubes as a class of nanomaterials of the same type.
Keywords: single-walled carbon nanotubes, RAW264.7 macrophages, bronchial epithelial BEAS-2B cells, dark-field microscopy.

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Background. The unique physical and chemical properties of single-walled carbon nanotubes (SWCNTs) facilitated their usage in various fields, including composite materials, construction, nanoelectronics, nanobiotechnology, and bionanosensors [1–3]. The global market for nanomaterials, including SWCNTs, is growing annually [4]. The transition to large-scale production of SWCNTs determines the need to assess possible medicobiological effects, including the study of the effect of these materials on human health, including production conditions [5]. Moreover, understanding the methods by which the properties of new nanomaterials determine their interaction with cells, tissues, and organs is a paramount task to substantiate approaches to safe use [6].

Considering such advantages of *in vitro* experiments in humans (absence of experiments with animals), relatively low cost, and high capacity, these studies have significant potential in assessing toxicity, including nanomaterials [7].

Most studies of SWCNTs have focused on the analysis of their toxic effects on the respiratory sys-

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tem, as the lung is the most likely the target organ following inhalation of substances. In *in vitro* experiments, the effect on the respiratory system is most often modeled on cultures of bronchial epithelial cells BEAS-2B and human alveolar epithelial cells A549. Studies have shown a decrease in the viability of BEAS-2B cells and signs of oxidative stress upon exposure to SWCNTs [8–10]. By contrast, researchers reported the absence of cytotoxicity upon incubation of the human alveolar epithelial cell line A549 with SWCNTs [11–14].

A study of the effects of exposure to SWCNTs on monocyte-derived macrophages [15] and human monocyte-derived macrophages [16] did not reveal a direct cytotoxic effect [15]; however, the macrophages suppressed the absorption of apoptotic cells [16]. On alveolar macrophages RAW 264.7 [10, 16–19], SWCNT exposure results in increased toxicity [10, 16, 18, 19], synthesis of transforming growth factor-β [10, 17], dose-dependent decrease in cell viability [10, 17], and the ability of SWCNTs to penetrate through cell membranes into the cell nuclei [20].

Contradictory results can be caused not only by differences in test conditions but also by the physicochemical characteristics of the SWCNTs, which necessitates a comparative study of the biological effects of different SWCNTs.

In this study, a comparative assessment of the effects of different SWCNTs was performed. The doses were selected by taking into account real industrial exposures as well. Chrysotile asbestos was used as a control material (positive control), which was chosen due to the morphological characteristics of chrysotile asbestos that has a fibrous structure similar to SWCNTs.

Materials and methods. Considering the predominantly inhalation route of SWCNT [21], RAW 264.7 cells were selected for the study, which are transformed into murine macrophages (cells of the immune system including the respiratory tract), as well as immortalized cells of normal human bronchial epithelium BEAS-2B (cells of the lower respiratory tract).

SWCNT-1 and SWCNT-2, which are not purified from metallic impurities, with different morphological characteristics were used in this study. SWCNT-2 are shorter and thinner (length, 100–1000 nm versus > 5 μm for SWCNT-1; average diameter, 0.8–1.2 nm versus 1.6 nm for SWCNT-1), with a larger specific surface area (400–1000 m^2/g versus 410 m^2/g). The experiment also used a sample of crushed chrysotile asbestos from the Bazhenovskoye deposit (Middle Urals) with a length-to-width ratio of 3:1 and length greater than 5 μm (provided by the Yekaterinburg Medical Scientific Center for the Prevention and Healthcare of Industrial Workers).

Concentrations of the study materials were selected based on the preliminary data on the concentration and dispersion of SWCNTs in the air of the working area of an SWCNT-1 manufacturing enterprise. SWCNT-1 concentrations in the air of the working area at various production sites of the manufacturing enterprise were significantly lower than the reference level proposed by the USA National Institute for Occupational Safety and Health [22], i.e., 1 $μg/m^3$ (REL NIOSH), which is widely used for the approximate assessment of the occupational risk in the absence of national recommendations.

The size distribution of SWCNT-1 particles was assessed using transmission electron microscopy of filters made of mixed cellulose ethers, for which air samples were taken (at least 210 L at a rate of 2–4 L/min) at several workplaces of the enterprise, where, according to preliminary data, the contact with SWCNT aerosol is possible. Transmission electron microscopy was performed using a Carl Zeiss Libra 120 microscope (Germany) with the Carl Zeiss AxioVision computer program. According to transmission electron microscopy data, the size of SWCNT agglomerates ranged from 0.3 to 3 μm.

To calculate the number of particles and mass of the deposited fraction (deposition) of SWCNT aerosol in the human respiratory tract, the multiple-path particle dosimetry model was used [23]. When calculating the deposition, a conservative scenario was used, in which the SWCNT concentration in the air of the working area was taken as 1 $μg/m^3$, which corresponded to REL NIOSH and was many times higher than the SWCNT-1 levels in the air of workplaces. In addition, as input parameters of the model, data on the dispersed composition of the aerosol and 8-h exposure were used, which corresponded to the work shift duration. The Yeh–Schum symmetric model was used to calculate deposited fractions of aerosol in human lungs [23].

The obtained fraction and number of particles deposited in the human respiratory tract were used to calculate the deposited dose of SWCNT in human lungs over 25 years of work (250 shifts per year) with subsequent recalculation per 1 cm^2 of human alveolar epithelium and calculation of the required surface doses and concentrations (taking into account the area and volume of the plate well) for introduction into cell cultures. The calculated deposited dose in the lungs was 2000 μg, which corresponded to the concentration of 0.0006 μg/ml. Taking into account our calculations and literature data [8–20], the concentration range for the study

was 0.0001–10 μg/ml, including a wide range of nontoxic and potentially toxic SWCNT doses.

Suspensions of the study materials were prepared using Dulbecco's modified eagle medium (DMEM, Gibco, Sigma-Aldrich, D1145-500ML, Waltham, MA, USA) and bronchial epithelial cell growth medium (BEGM; Sigma-Aldrich, Germany). The choice of the culture media for dispersing SWCNTs and chrysotile asbestos was justified by the cell lines RAW 264.7 (American Type Culture Collection, Manassas, VA, USA) and BEAS-2B (Cell Applications, Inc., San Diego, CA, USA).

The suspensions were prepared under aseptic conditions (in a biobox) by ultrasonic treatment using a Sonic Vibra Cell Sonicator (Sonics & Materials, Inc., CT, USA) apparatus with operating parameters of 750 W, 20 kHz, 40% amplitude, pulse 5/6, and duration of 30 min. Initially, SWCNT suspensions were prepared at an initial concentration of 0.1 mg/ml, which were used to obtain the necessary dilutions for experiments. The quality control of the dispersion obtained was performed by the dynamic light scattering method using a Zetasizer Nano-ZS analyzer (Malvern Instruments, Great Britain), and the distribution of the agglomerate sizes in suspensions was assessed.

During grinding, packaging, or processing, materials (SWCNT and chrysotile asbestos) can be contaminated with bacterial endotoxins (lipopolysaccharides are the main structural components of the outer cell wall of gram-negative bacteria), causing various side reactions when exposed to biological objects [24]. Thus, the prepared suspensions were additionally tested for the presence of bacterial endotoxin using the LAL reagent (Endosafe KTA, series K2422L, Charles River Laboratories, MA< USA) using the turbidimetric kinetic method [25]. The preparation and evaluation stages of the resulting suspension are described in more detail in our previous study [26].

The cultivation of cell lines was performed in a CO_2 incubator (LAMSYSTEMS ILM-170, Miass, Russia) under standard conditions of 37°C and 5% CO_2. The cells were inoculated into an appropriate culture medium (RAW 264.7 macrophages in DMEM, BEAS-2B bronchial epithelial cells in BEGM) with the addition of fetal calf serum (15% for DMEM and 5% for BEGM), as well as penicillin–streptomycin–L-glutamine (PANEKO, Russia).

Cytotoxicity and apoptosis were assessed on RAW 264.7 and BEAS-2B cells 48 h after the addition of suspensions of the test materials, and SWCNT and chrysotile asbestos were visualized on BEAS-2B cells.

Cytotoxicity was assessed using the colorimetric MTS test (Promega, WI, USA), based on the assessment of the metabolic activity of cells, which reflects their viability [27]. In this technique, living cells with normal metabolism synthesize NADP-H-dependent[1] cellular oxidase-reductase enzymes that can reduce the tetrazolium dye into insoluble formazan, which has a purple stain, while weakened or dead cells do not have this ability. The optical density is proportional to the number of living cells in the wells.

In the experiment, 18 concentrations, in the range of 0.0001 to 10 μg/ml, were investigated. The MTS test results were evaluated by comparing the optical density in the experimental and control wells. To achieve significance, each concentration of the material was examined in triplicate. Optical density was measured using a Multiskan flatbed photocolorimeter (Thermo Fisher Scientific) at a wavelength of 492 nm.

The expression level of proteins serving as markers of apoptosis was assessed by immunoblotting (western blot analysis). Cell extracts were obtained by lysis of cells in a radioimmunoprecipitation buffer with the addition of protease and phosphatase inhibitors. Samples (30 μg) were separated in 4%–12% polyacrylamide gel and transferred to a nitrocellulose membrane by wet transfer. Incubations with primary monoclonal antibodies poly-(ADP-ribose)-polymerase (PARP)[2] (Life Technologies, CA, USA) and a cleaved form of caspase-3 (Cl.Caspase-3, Cell Signaling, MA< USA) were conducted at 4°C for 16 h.

Anti-actin antibodies (GenScript Biotech, NJ, USA) were used as a control for the protein load in the study samples. Secondary antibodies (rabbit or mouse) conjugated with peroxidase (Santa Cruz Biotechnology, Inc., USA) were added at a concentration of 1:1000, after which the membranes were incubated for 1 h at room temperature and visualized by chemiluminescence using the gel documenting system Fusion Solo (Vilber Lourmat, France).

Doxorubicin, a type II DNA topoisomerase inhibitor[3], was used as a positive control for apoptosis. For this study, the material concentrations of 2.5 μg/ml, 0.04 μg/ml, and 0.0006 μg/ml were used (SWCNT-1, SWCNT-2, and chrysotile asbestos).

Dark-field microscopy was used to visualize SWCNT-1, SWCNT-2, and chrysotile asbestos in BEAS-2B cell cultures. Microscopic examination of cell cultures was performed on an Olympus BX51 microscope (Japan) equipped with a Cy-

[1] NADPH: reduced nicotinamide adenine dinucleotide phosphate

[2] PARP: poly-(ADP-ribosyl)-polymerase

[3] DNA: deoxyribonucleic acid

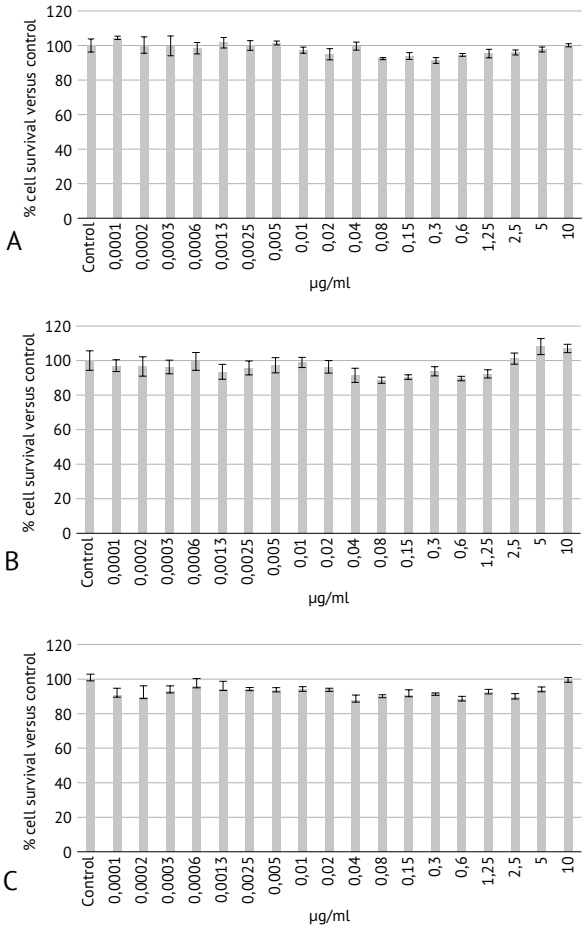

Fig. 1. Cytotoxic activity of the test materials against RAW 264.7 cell lines; mean value ± standard deviation of cell survival in the MTS test after 48-h exposure of the test materials: A, SWCNT-1; B, SWCNT-2; C, chrysotile asbestos; $p > 0.05$ for all comparisons with 0 μg/ml in control

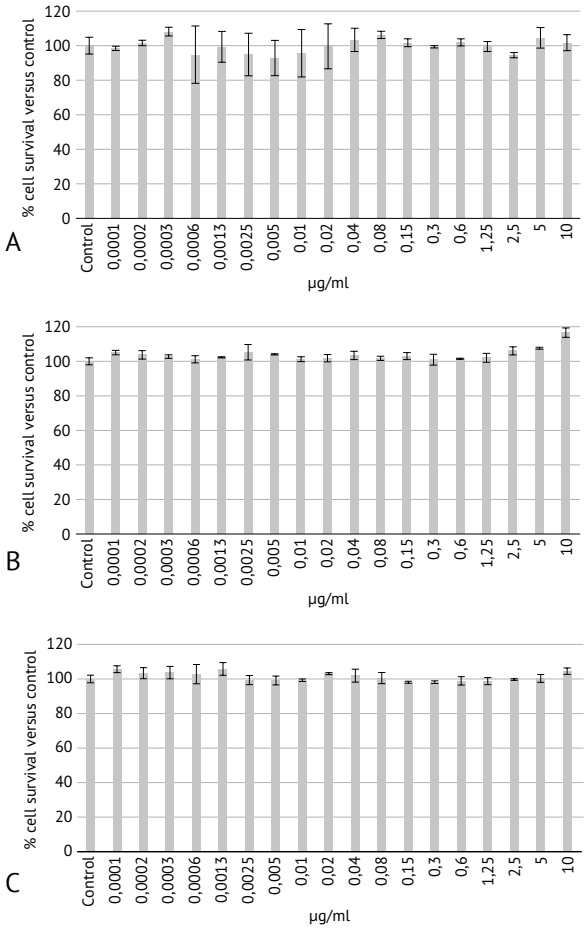

Fig. 2. Cytotoxic activity of the test materials against BEAS-2B cell lines; mean value ± standard deviation of cell survival in the MTS test after 48-h exposure of the test materials: A, SWCNT-1; B, SWCNT-2; C, chrysotile asbestos; $p > 0.05$ for all comparisons with 0 μg/ml in control

toViva® dark-field condenser for visualization in a dark-field mode with oil immersion [28, 29]. Dark-field images were acquired using an Olympus semi-apochromatic objective (× 100, variable numerical aperture 0.6–1.3), a Dage xL CCD camcorder, and Exponent 7 (Dage-MTI) software. Dark-field microscopy enables the visualization of nanosized particles in cells [28, 29].

Data obtained were processed using Microsoft Excel 2007 software. To assess the significance of differences between samples, Student's *t*-test was used. Differences were considered significant at *p* lower than 0.05. Values are presented as arithmetic mean ± standard deviation.

Results and discussion. According to the dynamic light scattering data, agglomerates up to 1000 nm in size prevailed in suspensions of SWCNT and chrysotile asbestos prepared using DMEM and BEGM.

The results of the MTS test 48 h after exposure to SWCNT-1, SWCNT-2, and chrysotile asbestos revealed a high survival rate of RAW 264.7 and BEAS-2B cells and the lack absence of a direct cytotoxic effect (Figs. 1, 2). Cell survival rate under the influence of all test materials was not lower than 87% compared with those of the control and did not differ significantly from the control.

Analysis of the expression of apoptosis markers by western blotting in RAW 234.7 and BEAS-2B cells showed the lack of an apoptogenic effect in comparison with the effect of the positive control (doxorubicin) for SWCNT-1, SWCNT-2, and chrysotile asbestos (Figs. 3, 4). Moreover, on bronchial epithelium BEAS-2B cells, the expression of the cleaved form of PARP increased in comparison with the negative control (cell medium) (Fig. 4), which was more pronounced for SWCNT-2.

The analysis of BEAS-2B exposed to a concentration of 2.5 μg/ml (the highest concentration at which the ability of the study materials to induce apoptosis was assessed) by improved dark-field microscopy revealed the adsorption and accumulation

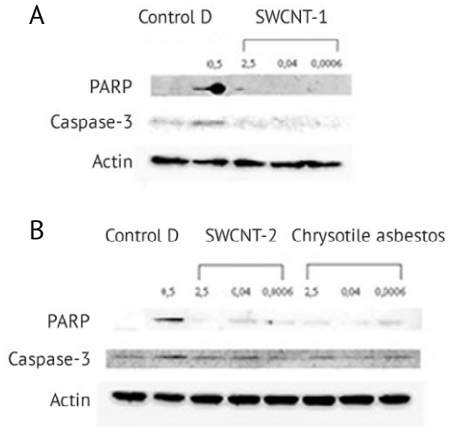

Fig. 3. A representative western blot on RAW 264.7 cell line (48-h incubation): A, SWCNT-1; B, SWCNT-2 and chrysotile asbestos. The materials were used at concentrations of 0.0006, 0.04, and 2.5 μg/ml. Apoptosis markers were cleaved forms of caspase-3 and poly-(ADP-ribose)-polymerase (PARP). Actin represents the level of protein in the samples. doxorubicin (D), 0.5 μg/ml

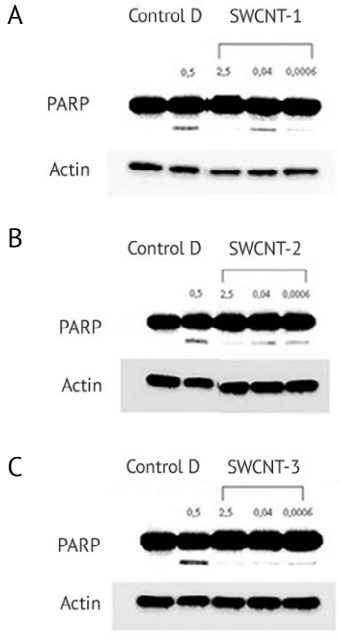

Fig. 4. A representative western blot on BEAS-2B cell line (48-h) incubation: A, SWCNT-1; B, SWCNT-2; C, chrysotile asbestos. The materials were used at concentrations of 0.0006, 0.04, and 2.5 μg/ml. Apoptosis marker was a cleaved form of poly-ADP (ribose) polymerase (PARP). Actin indicated the level of protein in the samples; doxorubicin (D), 0.5 μg/ml

of all study materials (SWCNT-1, SWCNT-2, and chrysotile asbestos) on the surface and inside of cells (Fig. 5, arrows).

In the analysis of the comparative toxicity of SWCNT-1, SWCNT-2, and chrysotile asbestos, all studied materials did not show direct cytotoxicity in the MTS test in the selected concentration range (0.0001–10 μg/ml) in relation to both cell cultures. In other studies using cell cultures BEAS-2B and RAW 264.7, SWCNTs at a concentration range of 0.8–6.6 μg/ml demonstrated cytotoxicity [9, 30], i.e., dispersed in the potentially toxic Pluronic F12® medium [31]. In the present study, materials were dispersed in a biocompatible culture medium [26]. Bronchial epithelial cells were more sensitive to the action of both SWCNT types than macrophages RAW264.7, as the expression of the signaling protein PARP was increased in the BEAS-2B cultures for SWCNT-1 and SWCNT-2.

Another study revealed the expression of genes for signaling proteins of apoptosis (caspase 3/7, bax) under SWCNT concentrations of 2.5 and 25 μg/ml [32]. The selected concentration range of chrysotile asbestos did not have a pro-apoptotic effect on the study cells, which may be due to the lower specific surface area of chrysotile asbestos than that of SWCNTs [33].

Both SWCNT types, despite their differences in morphological characteristics, demonstrated approximately the same effects. This result, with its further verification, can have an important practical application because data enables the assumption of the presence of common mechanisms that determine the interaction of SWCNTs with biological systems and, accordingly, common approa-

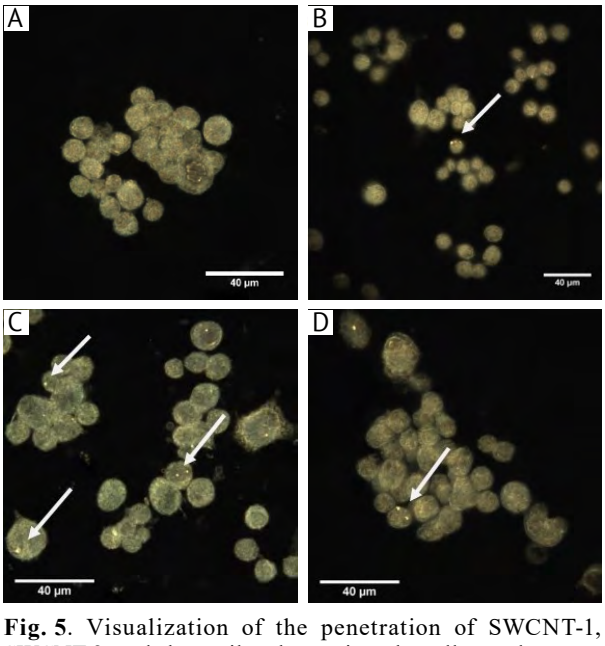

Fig. 5. Visualization of the penetration of SWCNT-1, SWCNT-2, and chrysotile asbestos into the cell cytoplasm under dark-field microscopy. BEAS-2B cells exposed to the test materials at a concentration of 2.5 μg/ml (48 h of exposure): A, control (BEGM); B, SWCNT-1; C, SWCNT-2; D, chrysotile asbestos

ches to determining the safety criteria for SWCNTs as a class of nanomaterials.

Conclusion. The results of this study revealed that the study materials did not exert a direct cytotoxic effect on the MTS test and did not have an apoptotic effect against RAW264.7 macrophages and BEAS-2B bronchial epithelial cells according to the results of the western blot. In bronchial epithelial cells that showed greater sensitivity, the expression of the signaling apoptotic protein PARP, which was more pronounced for shorter SWCNT-2, slightly increased.

In this study, the doses determined based on a conservative scenario were used, which provided for modeling at a concentration of 1 μg/m^3, which is many times higher than the actual content of SWCNTs in the air of the working area (conservative scenario). Further investigations of the cellular reactions in a wider range of doses and an increased exposure time can help in identifying the mechanisms of action of different SWCNTs to determine biomarkers that can predict possibly the triggering certain pathways of cell damage and death.

CONCLUSIONS

Both SWCNT types, despite their differences in morphological characteristics, demonstrated similar effects in *in vitro* experiments. This result, with its further verification, can have significant practical application in substantiating approaches to determining the safety criteria for SWCNTs as a class of nanomaterials.

Author contributions. G.A.T. performed the review of the literature on the research subject, as well as cell cultivation, testing (MTS, western blot) on cells, statistical data processing, plotting, and generalization of the results obtained. P.D.D. was involved in cell culturing, conducted the tests (MTS, western blot) on cells, and summarized the results obtained. A.M.D. developed the methods for preparing suspensions of materials for inclusion into the cells. G.F.G. conducted the tests (MTS, western blot) on cells, processed the statistical data, and plotted the figures. N.N.Kh. performed the dark-field microscopy of cells and summarized the results obtained. R.F.F. developed the methods for visualization of nanomaterials in cells (dark-field microscopy). S.V.B. organized the research on cells and summarized the results obtained. L.M.F. was the research supervisor, developed the research plan and design, reviewed the literature on the research subject, created the research aims and objectives, and summarized the results obtained.

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Conflict of interest. The authors declare no conflict of interest.

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