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Original article

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New 3D *in vitro* models for assessing the toxicity of carbon nanotubes

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ABSTRACT

Introduction. In recent years, there has been interest in 3D cellular models that more accurately reflect *in vivo* conditions and can become an alternative to animal experiments in assessing the toxicity of nanomaterials. There is a need to develop 3D models of the human respiratory tract that can bridge the gap between traditional in vitro cell cultures and laboratory animals.

Material and methods. Mono- and co-culture 3D-models based on bronchial epithelial cells BEAS-2B and lung fibroblasts MRC5-SV40 have been developed. Pristine and purified from metal impurities TUBALL™ SWCNTs and Taunit-M MWCNTs were used as materials for the study. The range of concentrations studied included concentrations corresponding to actual occupational exposures (0.0006–100 μg/ml). To assess the cytotoxicity of CNTs in cell models, the level of lactate dehydrogenase (LDH) activity was determined after 72 hours of exposure.

Results. The cytotoxic effects of CNTs in 2D and 3D cell models manifested themselves in different concentration ranges: a three-dimensional model of bronchial epithelial cells turned out to be more sensitive to the effects of CNTs compared to a monolayer one, while in a spheroid model of fibroblasts a higher cytotoxicity threshold was noted for multi-walled carbon nanotubes compared to traditional cell culture. In three-dimensional cell co-cultures, a significant increase in LDH was observed starting at higher concentrations compared to monocultures.

Limitations. The present study was limited to the use of one type of cytotoxicity test when examining the effects of CNTs on cells of the respiratory system.

Conclusion. A method has been developed for three-dimensional cultivation of cells of the human respiratory system to simulate the interaction of epithelial and stromal cells of the lower respiratory tract. Traditional 2D cell models may underestimate or overestimate the toxicity of materials. Improved 3D *in vitro* models, closer in their properties and morphology to native tissue, are more reliable in determining toxic doses and targets.

Key words: *3D-model; nanomaterials; carbon nanotubes; in vitro; cytotoxicity*

Compliance with ethical standards. The study does not require the submission of a biomedical ethics committee opinion or other documents.

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Оригинальная статья

Introduction

The introduction of nanomaterials into various fields of industry and medicine is a current and promising task, however, to ensure successful and safe use, it is necessary to conduct a comprehensive assessment of their impact on the environment and human health. Carbon nanotubes (CNT), which have unique physicochemical properties, are the object of increased interest among researchers and enterprisers. These materials show high potential for applications in various fields, including the production of composite materials, construction, electronics and nanobiotechnology [1–3]. One of the key problems of hygienic support of production processes associated with the synthesis and use of CNT is the possible toxic effect on the cells of the respiratory system, since the inhalation route of entry in these circumstances is the main one.

In accordance with the 3R principles (reduction, refinement, replacement) in relation to experiments on laboratory animals [4], the transition from studies on *in vivo* models to advanced *in vitro* models reproducing real cell interactions in the body is relevant. *In vitro* experiments provide an opportunity to study the mechanisms of toxic action, but traditionally used monolayer cell cultures have significant limitations, including distorted cell morphology and lack of cell-cell interactions. In recent years, there has been increased interest in 3D cell cultures, which more accurately reflect *in vivo* conditions due to their microenvironment and functional organization that is close to real conditions. Advanced 3D cell models that approximate the properties and morphology of a living organism and have potential as an alternative to animal testing are emerging. The results of modern research indicate that existing 3D skin models for studying the toxic effects of harmful substances are at a high level of validation [5], while cell models of respiratory system still require further study.

Based on the structural organization, there are three main types of 3D cell culture models [6, 7]:

1) Scaffold base systems. Structures that mimic the extracellular matrix (scaffold) are used to create a native cellular microenvironment in this type of model. In this case, an attempt is made to most accurately simulate the three-dimensional spatial architecture of tissues.

2) Scaffold-free systems. In this case, aggregation and self-assembly of cells occurs naturally, without the use of any matrix. Examples of scaffold-free systems are spheroids and organoids that are cultured using techniques of hanging drop, magnetic levitation, and ultra-low adhesion surfaces.

3) Hybrid systems. This type of model includes a synthetic matrix and external physical supports that promote more complex interactions between cells and the extracellular matrix.

Scaffold-free systems are of greatest interest in the development of high-throughput approaches to toxicity studies, as they allow us to fill the gap between traditional 2D cell cultures and native tissue structures *in vivo* without the use of scaffolds and hydrogels. It is necessary to point out the differences between spheroids and organoids, which lie in the nature of the cells used [6]. Spheroids are three-dimensional clusters of cells that grow in the shape of a sphere, sticking to each other. Organoids are more complex 3D systems that can be cultured either scaffold-free or with scaffold, but their main distinguishing feature is the inclusion of embryonic stem cells, induced stem cells and adult stem cells and mimic the functioning of the organ.

3D cell models provide an alternative and potentially more effective platform for testing and assessing nanomaterial-induced toxic effects [8, 9]. In 2011, Movia D. et al. studied the cytotoxicity of SWCNT in 2D and 3D models of THP-1 macrophages [10]. It was shown that the decrease in cell viability and the secretion of proinflammatory cytokines were less pronounced in a 3D cell model compared to a monolayer, which demonstrates the importance of using tissue-like multicellular structures that are similar in properties to *in vivo* models. Hindman B. et al. used 3D models to study the fibrogenic potential of ultrananoparticles with different physical properties and crystalline silica [11]. Analysis was performed using 2D and 3D *in vitro* models consisting of WI38-VA13 human lung fibroblasts. CNT and silicon dioxide were used in this work; TGF-β1 protein was used as a positive control to induce fibroblast differentiation. CNT induced myofibroblast differentiation in both 2D and 3D cultures. However, 3D culture has enabled more detailed investigation of processes such as myofibroblast clustering, collagen deposition and remodeling, cell division, and matrix contraction, which are critical for understanding fibrosis *in vivo*. Kabadi P.K. et al. studied the toxic effects of carbon black, MWCNT and crocidolite asbestos fibers in 3D lung microtissue cultivated using a hydrogel and consisting of BEAS-2B bronchial epithelial cells, IMR-90 fibroblasts and THP-1 macrophages [12]. After exposure to nanomaterials, microtissue viability was evaluated, and tissue morphology and the expression of genes and proteins associated with inflammation and extracellular matrix remodeling were studied. The results of this study highlight the need for the use of 3D cellular models in predicting the long-term effects of nanomaterials on lung tissue: the effects obtained

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in 3D lung microtissues were similar to those shown in experiments on pulmonary toxicity in rodents. In another study, a 3D lung model consisting of A549 alveolar epithelial cells, MRC5 fibroblasts and THP-1 macrophages was exposed to two types of MWCNT at the air-liquid interface using the VITROCELL® Cloud system [13]. The authors demonstrated the initiation of proinflammatory reactions in a cell model in the absence of cytotoxic and profibrogenic effects. Also, spheroid models are of great interest in studying the interaction of CNT with tissues, since monolayer models provide a limited opportunity to assess the characteristics of the penetration of nanoparticles into different cell layers. It has been shown that CNT have a high rate of penetration into 3D cell structures [14], while the size, shape, aspect ratio, rigidity and surface composition of the particles play a major role in the level of absorption of CNT by cells and spheroids [15].

The aim of this work was to develop a 3D spheroid model of lung tissue, consisting of bronchial epithelial cells and human lung fibroblasts, for subsequent assessment of the cytotoxicity of industrial carbon nanotubes of various types.

Material and methods

Bronchial epithelial cells BEAS-2B (Cell Applications, Inc., USA) and human lung fibroblasts MRC5-SV40 (cell culture kindly provided by B.V. Chernyak, Research Institute of Physics and Biology named after A.N. Belozersky Moscow State University) were chosen as cell lines for 3D cultivation. The interaction of selected epithelial and stromal cells in three-dimensional co-culture mimics the processes occurring in native lung tissue, adequately reflecting the effects of harmful substances on the lower respiratory tract.

Ultra-low attachment 96-well with U-shaped bottom (Cell Floater, SPL Lifesciences, Korea) were used to form spheroids, which made it possible to simulate cell morphology and intercellular contacts close to *in vivo* conditions (Fig. 1, see on the colored sticker). Methods for cultivating 3D models consisting of one type of cells (monocultures of bronchial epithelial cells and monocultures of human lung fibroblasts) and two types of cells (joint cultures of bronchial epithelial cells and fibroblasts) were developed simultaneously. Monolayer cell cultures and spheroids were cultivated in DMEM medium (PanEko, Russia) in an incubator under conditions of 37 °C, 5% CO_2 and constant humidity.

Pristine and purified from metal impurities CNT were used as materials for the study: TUBALL™ SWCNT (manufacturer: OCSiAl group company, Novosibirsk) and Taunit-M MWCNT (manufacturer: NanoTechCenter LLC, Tambov). The range of concentrations studied was selected based on the analysis of non-toxic and toxic concentrations from the literature review, and also included concentrations corresponding to occupational exposures [16]. Plasma membrane integrity was assessed by testing the release of the enzyme lactate dehydrogenase (LDH) using the LDH Assay Kit (Abcam, UK) in the supernatant of cells exposed to CNT for 72 hours. In the experiment, 4 concentrations of the CNT were used (100, 50, 0.03 and 0.0006 μg/ml). Cells without CNT exposure were used as a negative control, and cells exposed to 1% Triton X-100 were used as a positive control for cytotoxicity. 50 μl of sample (2 μl of supernatant diluted with buffer to the required volume) and 50 μl of the reaction mixture were added to each well. Each sample was evaluated in triplicate. Optical density was determined on a Multiskan photometric tablet reader (Thermo Fisher Scientific, USA) at the wavelength specified in the instructions (450 nm). Measurements were taken every 10 minutes for 1 hour. LDH enzyme activity was calculated from the change in the amount of reduced nicotinamide adenine dinucleotide (NADH) between 20 and 50 minutes. The level of cytotoxicity (in %) was determined by the change in LDH activity in the samples and was calculated taking into account the results for the positive and negative controls using the given equation (1):

$$\text{Cytotoxicity (\%)} = \frac{\text{mean (cell exposed to CNT)} - \text{mean (negative control)}}{\text{mean (positive control)} - \text{mean (negative control)}} \cdot 100. \quad (1)$$

The obtained data was processed using Microsoft Excel 2016 and R software [17]. Differences were considered statistically significant at a significance level of $p<0.05$.

Results

3D models of monocultures and cocultures of selected cells of the human respiratory system were compact spheroids that maintained their morphology and viability throughout the entire incubation period.

In 3D model of bronchial epithelial cells BEAS-2B, a significant increase in cytotoxicity compared to the control was noted when exposed to MWCNT and SWCNT in the entire concentration range of 0.0006–100 μg/ml, including concentrations corresponding to industrial exposures – 0.0006 and 0.03 μg/ml (Fig. 2, *a*). Previously, in our publications we showed the results of assessing LDH activity in a monolayer culture of BEAS-2B cells at the same concentrations with similar exposure times [18]: in a 2D culture of bronchial epithelial cells, an increase in LDH activity was demonstrated upon exposure to pristine SWCNT and MWCNT only at a concentration of 100 μg/ml, and for purified SWCNT –

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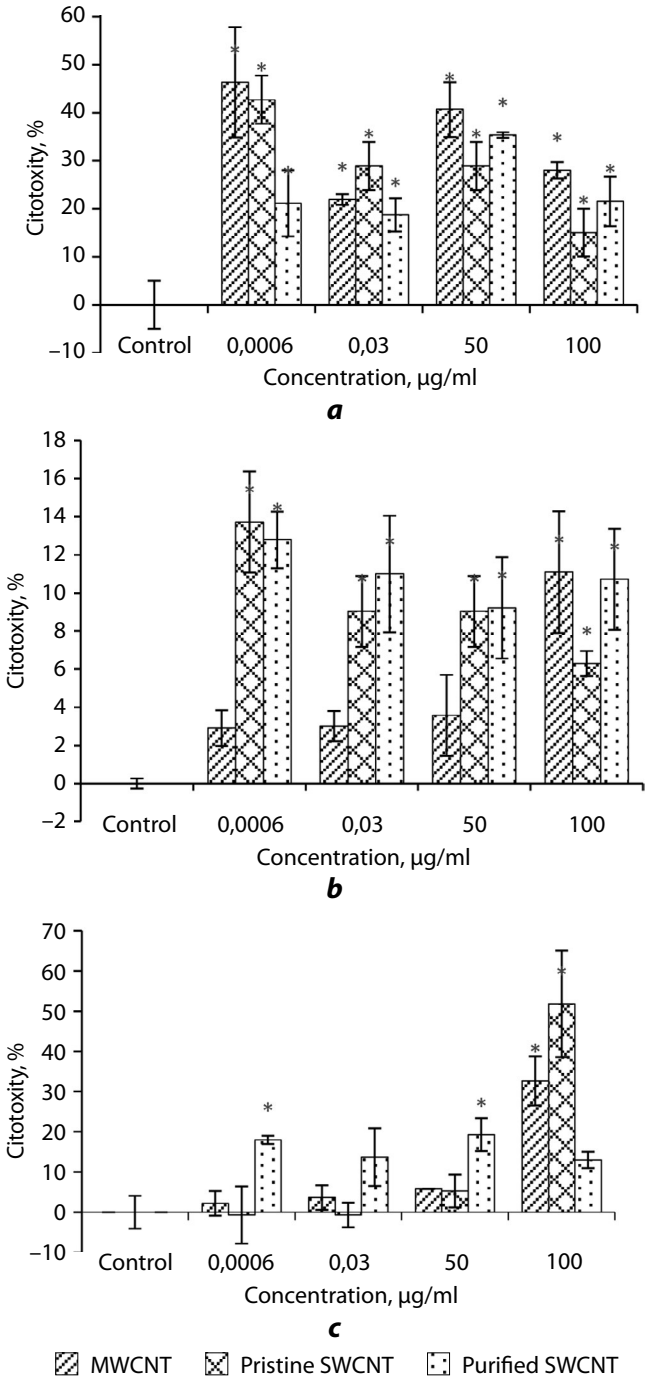

Fig. 2. Cytotoxic activity of the studied materials (according to the LDH test) after 72-hour exposure to spheroids: *a* – BEAS-2B bronchial epithelial cells; *b* – MRC5-SV40 lung fibroblasts; *c* – co-culture of BEAS-2B and MRC5-SV40 cells.
*$p<0.05$ compared to control.

at concentrations of 50 and 100 μg/ml. Thus, spheroids of bronchial epithelial cells turned out to be more sensitive to the effects of CNT compared to monolayer cultures.

For MRC5-SV40 fibroblast spheroids, an increase in LDH levels was shown upon exposure to purified and pristine SWCNT at all concentrations studied, however, for MWCNT, cytotoxic effects were noted only starting from a high concentration of 100 μg/ml (Fig. 2, *b*). Moreover, in a monolayer 2D culture of MRC5-SV40 fibroblasts, a significant increase in LDH was obtained at all tested concentrations of SWCNT and MWCNT (0.0006–100 μg/ml). Thus, when studying the cytotoxic effects of SWCNTs, 2D and 3D models of fibroblasts showed comparable results. For MWCNT, cytotoxic effects in the three-dimensional model appeared at significantly higher concentrations compared to monolayer cell culture, which may be due to differences in the penetration and accumulation of MWCNT depending on the spatial organization of cells.

In a co-culture of 3D BEAS-2B and MRC5-SV40 cells, CNT showed significantly lower cytotoxicity compared to experiments in 3D monocultures: an increase in LDH activity was noted for MWCNT and pristine SWCNT only at a concentration of 100 μg/ml (Fig. 2, *c*). It can be assumed that the interaction of epithelial and stromal cells and their exchange of signals leads to the formation of a microenvironment that is closest to native tissue and the activation of protective mechanisms when exposed to CNT. This pattern was not observed in the case of purified SWCNT, which may be due to the size of the agglomerates or other mechanisms of cytotoxicity, which requires further study.

Discussion

The obtained results demonstrate the significant influence of spatial architecture and morphology, as well as the composition of cellular models on the implementation of cytotoxic effects when exposed to carbon nanomaterials. However, the present study was limited to the use of one type of cytotoxicity test, whereas the use of additional tests may confirm the results and deepen existing understanding. Cultivating cells in 2D or 3D can determine the characteristics of their capture and deposition of CNs, as well as the activation of various signaling pathways in response to toxic effects. There is a need to study the mechanisms of toxicity involved in cells depending on the culture conditions (monolayer, three-dimensional with one cell type, three-dimensional co-culture).

Conclusion

A method for 3D cultivation of monocultures and cocultures of bronchial epithelial cells and human lung fibroblasts to simulate the interaction of epithelial and stromal cells of the lower respiratory tract has been developed. The principles of culturing 3D cell models for assessing the toxicity of carbon nanomaterials can be used to solve other problems, taking into account the target cells and the characteristics of the materials being studied.

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The results of this study suggest that traditional 2D cell models may underestimate or overestimate the toxicity of materials. Improved 3D *in vitro* models, closer in their properties and morphology to native tissue, are more reliable in determining toxic doses and targets. Such models can be recommended for use with other methods for assessing the toxicity of materials, including genotoxicity.

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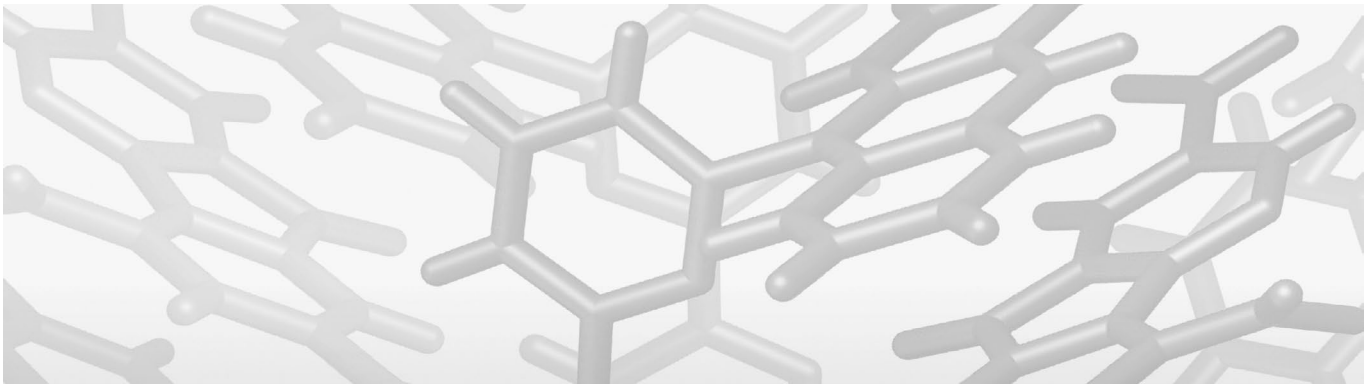

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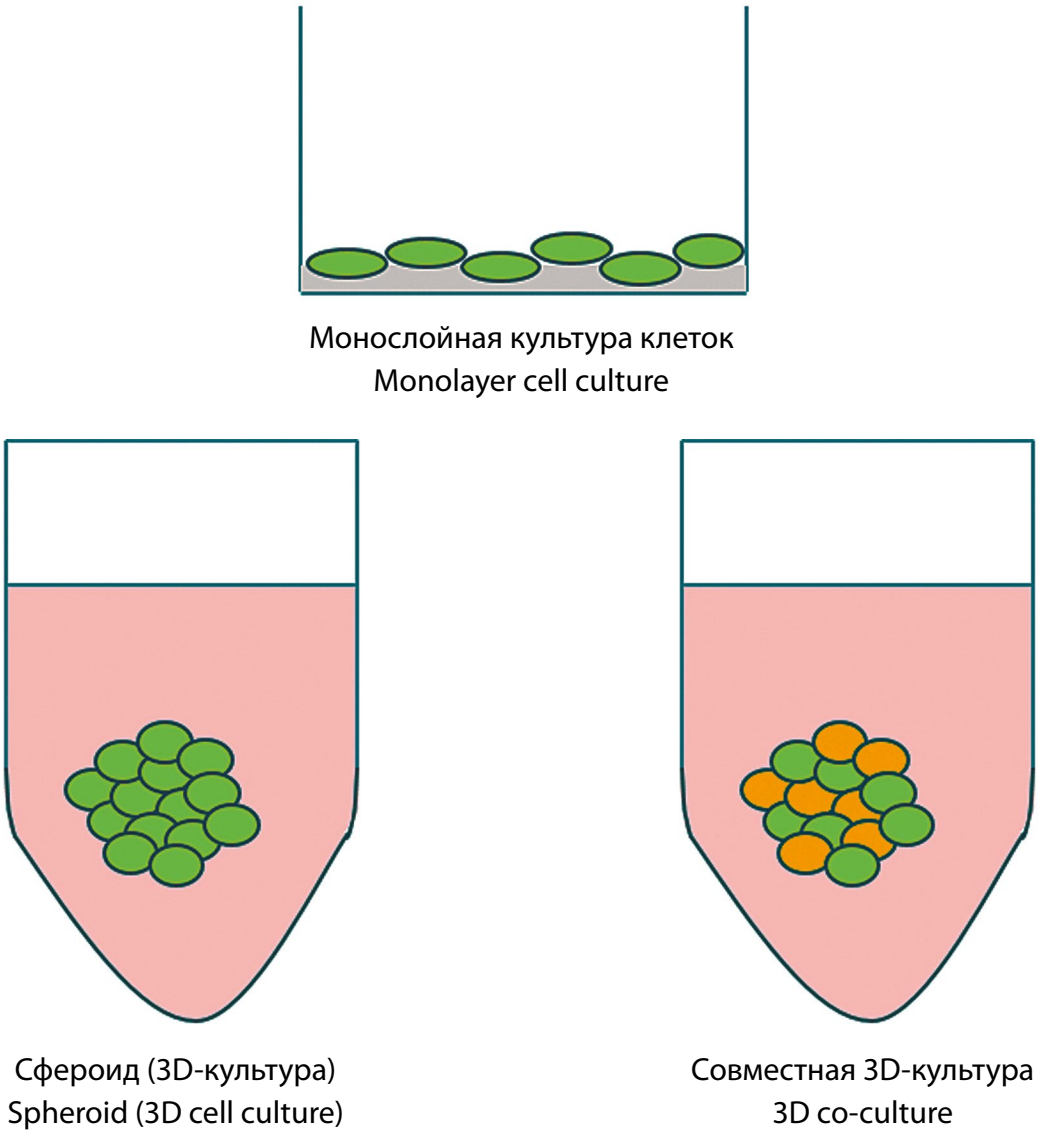

Рис. 1. Схематичное изображение монослойной культуры клеток и трёхмерной сфероидной модели монокультуры и совместной культуры клеток.

Fig. 1. Schematic representation of monolayer cell culture and 3D spheroid model of monoculture and co-culture of cells.