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Carbon Nanotubes Exposure Risk Assessment: From Toxicology to Epidemiologic Studies (Overview of the Current Problem)

L. M. Fatkhutdinova[a], T. O. Khaliullin[a], and A. A. Shvedova[b]

[a] *Kazan State Medical University, ul. Butlerova 49, Kazan, 420012 Russia*

[b] *National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA*

e-mail: liliya.fatkhutdinova@gmail.com

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Abstract—Nanoscale size and fiber like structure of carbon nanotubes (CNTs) may determine high reactivity and penetration, as well as the pathogenicity of asbestos and other mineral fibers. Despite many in vitro and in vivo studies, the absence of full-scale data on CNT effects on human health clearly point out the necessity for epidemiological studies. Currently, several projects are initiated worldwide on studying health risks associated with the inhalation of industrial CNTs, including NIOSH-promoted research (United States), the European CANTES study, and the Russian CNT-ERA project. Studies comprising several successive steps, such as CNT exposure assessment in occupational settings, toxicological evaluation, and epidemiological observations, are critical for determining material safety and use criteria.

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INTRODUCTION

According to the EU definition of 2011 [1], carbon nanotubes (CNTs) are classified as nanomaterials. At the same time, based on the morphological parameters (length and aspect ratio), CNTs behave as fibers [73]. This dual nature of carbon nanotubes may determine their high reactivity and penetration [61], as well as the pathogenicity similar to asbestos and other mineral fibers [30]. CNT production is growing every year [4] due to their application in construction [97], machine engineering [17], electronics [15], power industry [19], space engineering [5], and biomedicine [75, 93]. Therefore, both specialists and consumers question the safety of CNT, as well as technological processes associated with CNT production and application. It would be rather unwise to assume that the innovative branch of nanoindustry is environmentally friendly. Failure to take action may slow down development and lead to financial losses of industrial enterprises. In particular, getting such products to the market without safety guarantees may damage the reputation and public image of a company, as well as cut off access to new markets. In addition, these innovative enterprises require assistance in solving such problems as registering new chemicals, product certification, the organization of a labor-protection system, and medical supervision over the personnel. Ways to obtain objective data on potential biomedical and ecological risks, as well as working out approaches to increase the efficiency of risk management, are very important for the sustainable development of this industrial branch [9].

EARLY HYPOTHESES AND FIRST EVIDENCE OF CNT TOXICITY

The toxic effects of CNTs have been studied since the early 2000s, when they were produced only in laboratories and during experimental manufacturing. Toxicological experiments were aimed at detecting susceptible organs and systems. Inhalation and dermal penetration were viewed as the main pathways CNTs could enter the human body [23, 55]. The design of experiments was based on such hypotheses of the interaction between nanoparticles and biological objects as oxidative stress and mechanical damage to cell structures. The first in vivo studies were performed on the toxicity of single-walled carbon nanotubes (SWCNTs) [44, 47, 50, 84, 91, 94]; but in a few years the focus shifted to multiwalled CNTs (MWCNTs) [32, 49, 57, 70, 88], because the latter were more commercially attractive.

In the experiment on mice [44], SWCNTs, carbon black (negative control), and quartz (positive control) were compared. In mice intratracheally instilled with 0.1–0.5 mg of nanotubes, the number of granulomas in the lungs was greater, and the inflammatory response was significantly stronger than those treated with quartz and carbon black. In [94], the pilot investigation was aimed at comparing the pulmonary effects produced by SWCNTs, quartz particles, and carbonyl iron particles in rats intratracheally instilled

with 1–5 mg/kg of the corresponding substance. Exposures to SWCNTs produced transient inflammation and tissue damage, as well as non-dose-dependent series of nonprogressive multifocal granulomas. The researchers came to the conclusion that these granulomas were evidence of a foreigh-object body reaction. In the aspiration experiment [84] (pharyngeal aspiration as a model of inhalation) on mice treated with SWCNTs at doses of 10, 20, 30, and 40 μg/mouse, a dose-dependent inflammation and fibrosis accompanied with alveolar wall thickening was found. At the same time, the materials taken for comparison (nanosized carbon black and silicon dioxide) did not cause alveolar wall thickening or granulomas. The inflammatory response was significantly weaker than at the same exposure doses. When comparing SWCNTs, carbon black, and vanadium oxide (pharyngeal aspiration, 2 mg/kg), it was found [47] that SWCNTs caused interstitial fibrosis, a significant increase in mRNA expression responsible for the platelet-derived growth factor, and the formation of carbon structures that bridge alveolar macrophages in situ. It could depend, according to researchers, results from the unique morphology of nanotubes and/or the presence of residues of metal catalysts. The bridges between macrophages were regarded as biomarkers of exposure. The important role of dispersion degree during exposure was shown in [50]. Researchers revealed that exposure to highly dispersed SWCNTs (pharyngeal aspiration, 10 μg/mouse) caused only interstitial fibrosis, whereas material with a low dispersion degree was characterized by fibrosis and granulomas.

In 2008, Takagi et al. [88] suggested using peritoneum as a pleura model. p53 +/– heterozygous mice (with a genetically inherited high risk of tumors) were administered intraperitoneally with 3 mg of MWCNTs or asbestos (crocidolite). In both cases, mesothelioma was induced, but the experiment was criticized by the scientific community because the dose was too high [24]. In the same year, Poland et al. [68] performed a similar comparative study of MWCNTs, asbestos, and ultrafine carbon black. The particles were injected into mice intraperitoneally at a dose of 50 μg (as opposed to 3 mg in [88]). Both asbestos and MWCNTs caused significant protein exudation and the formation of granulomas with cell aggregates. The researchers concluded that asbestos-like pathogenicity, which is assigned to CNTs, is achieved by the mechanism of the structure-activity relationship peculiar to asbestos and other fibrogenic fibers. In later experiments, the CNT translocation of the respiratory tract into the interstitium, subpleural space, and pleura was demonstrated [50, 70, 74]. Another feature of CNTs was their high biopersistence, i.e., a long period of stay in the pulmonary system as a result of inhalation or aspiration [13].

In vivo experiments on evaluating dermal toxicity have not been performed. However, the in vitro experiments of Monteiro–Riviere et al., revealed that MWNTs can penetrate into keratinocytes and stimulate an immune response, in particular IL-8 extraction [55, 83], as well as the high activity of free-radical oxidation and structural changes in keratinocytes (HaCaT culture) exposed to SWCNTs, which suggested the presence of dermal toxicity in the studied nanotubes.

The in vitro experiments were aimed at establishing the damaging effects of nanoparticles. In the first place, researchers were interested in oxidative stress and associated cyto- and genotoxic effects, the mechanical effect of nanotubes on cellular structures, and specific and nonspecific interactions with the receptors. Srivastava et al. 2010 [86] found oxidative stress and apoptosis in A549 cells (alveolar epithelium) exposed to MWCNTs, and the production of reactive oxygen species occurred, as was suggested by the researchers, via the cytochrome P450 system. Previously [78] the same mechanism of oxidative stress was shown in the cell culture of alveolar epithelium in response to SWCNTs. When studying the genotoxic effects of exposure to CNTs on RAW of 264.7 macrophages, the researchers detected some signs of damage to the genetic apparatus of cells at concentrations above 0.1 mg/mL for SWNTs and above 1 mg/mL for MWCNTs, although the cytotoxic effects appeared only at the highest doses (100 mg/mL) [53]. The toxicogenomic comparison of asbestos and MWCNTs in the experiment on human bronchial epithelial cells revealed changes in the expression of 12 common genes associated with mesothelioma and 22 common genes associated with lung cancer [42]. In studies [36, 37, 71, 80] it was found that MWNTs may associate with the cell membrane, including bronchial epithelial and macrophages, and disrupt its integrity, inducing the development of proinflammatory cytokines and cell death.

Therefore, oxidative stress and cytotoxic effects of CNTs have been shown for keratinocytes (see above), epithelial cells, and macrophages [17, 34, 36, 37, 71, 78, 80, 86]. Genotoxic effects and tumor induction were also observed [29, 41, 53, 76]. CNT interaction with specific receptors at the surface of cell membranes and/or organelles has not been identified, which may be due to the lack of any recognizable domains in nanotubes. It is most likely that there is no common toxic effect of CNTs, which would explain all of the observed effects. Further research is needed, including the application of modern cell and molecular techniques.

During this period, the first hygienic assessments of exposure to CNTs in the workplace were performed simultaneously with the toxicological experiments. They were based on indirect methods for determining impurities (catalysts such as nickel or cobalt) in the working area [14, 40, 48]. The results of measurements in conjunction with the data on electron microscopy of the samples demonstrated that the staff can contact CNTs.

It is noteworthy that the general restrictions for the initial phase of research were high exposure doses calculated for the easily registered biological response, the use of laboratory purified and/or modified samples rather than industrial CNTs, the poor relation of the exposure model in in vivo and in vitro experiments with real working conditions, and a lack of data on the actual exposure in the environment.

EVALUATION OF CNT TOXICITY IN THE CURRENT PERIOD

The later period of studies on CNT toxicity is characterized by some innovations. Complex inhalational units for CNT aerosolization with constant control over the number and size of individual particles were developed. Thus, it became possible to lauch chronic experiments for 1 year or more using low doses during the inhalation [51, 85] and confirm the specific profibrogenic effects of CNTs established during the acute and subacute experiments. In addition, the researchers began to pay more attention to extrapulmonary effects: systemic affection of the vascular bed [25], effects on the central nervous system [39], reproductive toxicity [3], and changes in the immune status [54]. It was proven that CNTs can induce neoplastic processes [28, 77, 82] and an allergic response [38, 64, 66]. In November 2014, the International Agency for Research on Cancer (IARC) classified MWCNT-7 (produced by the Mitsui ltd., Japan) as Group 2B: possibly carcinogenic to humans [31].

Significant changes took place in the hygienic evaluation of CNTs. The indirect methods for determining the content of CNTs in the environment (based on catalyst residues) were replaced by the direct calculation of elemental inorganic carbon, a component of carbon nanotubes [18, 52].

Toxicological experiments and the development of hygienic methods of research in the nanoindustry ensured the transition to the stage of epidemiological studies.

Currently, data on the effect of CNTs on the health of exposed individuals are insufficient, but there are several documented cases of CNT detection in the body of people affected due to a variety of reasons.

In particular, after the tragedy of 2001 in New York, a large number of rescue workers and persons providing assistance with debris removal and victim extrication had their pulmonary system affected by combustion products of building materials, fuel, and other substances [96]. High combustion temperatures contributed to the formation of tubular carbon nanostructures that were very similar to artificial CNTs. Subsequently, 12 891 people were examined during the screening program. Many of them had symptoms of respiratory lesions. In seven of the most severe cases, lifetime lung biopsy was performed. Four patients had CNTs, along with other dust particles in their bioptates, which were similar to carbon nanostructures found in dust samples at the crash site; they had such pathological changes such as cellular fibrosis, chronic bronchiolitis, and granulomas [35].

German researchers described a case of toner nanoparticles found in the peritoneum when screening the peritoneal cavity of a female office worker with complaints about persistant abdominal pain [90].

The studies of suspended particles in the air are of particular interest. During a long-term epidemiological cohort study performed in the 1990s on the basis of six cities in the United States, it has been shown that mortality from all causes, as well as cardiovascular and cardiorespiratory mortality, was significantly dependent on the content of dust in the air of cities [22]. More recent studies have confirmed the role of fine ($PM_{2.5}$, PM_5, PM_{10}) [69] as well as ultrafine (less than 1 μm) atmospheric particles in mortality from cardiovascular and pulmonary disease [16, 87, 95]. The accumulated experience of studying the effects of suspended particles in air on human health proves the need for field studies, including an assessment of exposure and the study of human health affected by nanoparticles.

In Taiwan, the first epidemiological study in the nanoindustry was performed in 2009–2010. It was based on 13 companies that produce or use nanoparticles of various types, including nanosized metals and their compounds, carbon nanoparticles (fullerenes, CNTs, and nanosized carbon black), and organic nanosized structures (dendrimers, liposome, etc.) [45]. During the study, the markers of cardiovascular and respiratory systems lesions, neurological functions, and immune-system state were evaluated. The results showed a decrease in the total antioxidant protection, an increase in the content of cardiovascular markers (C-reactive protein, and VCAM protein), and changes in heart-rate variability and cognitive functions. In the cross-sectional part of the study, functional changes in the respiratory system were not revealed, but there were signs of a decline in the respiratory functions during the prospective observation. The study was restricted by the absence of workers' subdivision according to specific types of nanoparticles.

Complex projects that combine a sequential assessment of CNT content in the environment, toxicological studies, and epidemiological observations are the most promising in terms of studying the effect of CNTs on health. Epidemiological studies involving the collection of biological samples in a relatively small population (for example, people working in enterprises that produce and apply CNTs) should be carefully prepared. First, it is necessary to determine potential outcomes and biological markers of exposure to nanoparticles. In this case, toxicological experiment most closely matching the real exposure scenario is preferred, including the application of the same nanoparticles which are found in the environment at

doses corresponding to the actual concentration along with the same ways of penetration. However, organizing this type of research is associated with numerous challenges, including the need to establish multidisciplinary teams, the development of methodological approaches for the transition from the CNT concentration in the external environment to the doses accumulated in the body, the selection of biomarkers of exposure and effect, and the relatively small (as of today) group of exposed persons as well as the difficult access to objects of the nanoindustry.

According to an international group of experts [72], the studies on toxicokinetics/toxicodynamics and noncarcinogenic effects of inhaled CNTs during the synthesis, processing, use, and disposal of the material should be considered the most important. At present, several projects on studying health risks associated with the inhalation of industrial CNTs are implemented: NIOSH-promoted research (United States), the Dutch CANTES study, and the joint Russian–United States CNT-ERA project.

In 2013, the launch of a small-format (about 100 employees) cross-sectional study to identify the relationship between exposure to carbon nanotubes and nanofibers and early changes in the pulmonary and cardiovascular systems was reported in the United States [27]. Currently, exposure in the workplaces is assessed in various ways to determine the relevant criteria for determining the content of CNTs and carbon nanofibers (CNFs) in the working environment [18, 26].

The study will include employees of at least ten enterprises producing and using various CNTs and CNFs. It is expected that the samples of blood and induced sputum will be taken from people under study. The selection of biological markers and survey methods was based on in vivo and in vitro toxicity studies of CNTs and CNFs; data from research on the pathophysiology of the tumor, fibrotic and inflammatory processes; and the results of studies of the medical and biological effects of non-carbon nanoparticles in humans [33, 45].

In 2014, the staff of several scientific institutions in the Netherlands and Belgium launched a study on early biomarkers of individual exposure to CNTs with a simultaneous hygienic assessment of the content of nanoparticles in the workplace air (CANTES). During the study, the content of elemental carbon in the air of an enterprise producing CNTs was assessed. Samples of blood, urine, and nasal and buccal epithelial cells were taken from the staff of the enterprise to assess a number of biochemical parameters and cytokine status. The results of the study have not yet been published in the form of articles, but the researchers reported increased levels of proinflammatory cytokines in the study group compared to the controls [92].

PROTOCOL OF THE RUSSIAN (CNT-ERA) STUDY ON CARBON NANOTUBES EXPOSURE AND RISK ASSESSMENT

Our study, which was started in 2011, includes hygienic, toxicological, and epidemiological stages.

At the initial stage of the study, we selected enterprises using the same type of reactors for the production of MWCNTs and performed a hygienic evaluation of the working places while determining the actual 8-hour TWA concentrations. Air sampling in the filters was carried out in the areas of contact with the aerosol MWCNTs followed by transmission electron microscopy to visualize nanotubes in the samples and determine the amount of elemental carbon by thermo-optical analysis.

Before planning the panel epidemiological study, a series of toxicological experiments [10, 11, 12] was performed during which the promising biomarkers of the effect were determined. The biological effects of industrial CNTs were studied in mice, cell cultures of macrophages, and cells of the bronchial epithelium. The exposure doses were selected based on the measured concentrations of MWCNTs in the air of the working environment, followed by the calculations of the deposited fraction in human lungs (according to the MPPD model [21]) and the accumulated deposited surface area doses (per 1 cm^2 of the alveolar epithelium). The exposure doses were determined based on the accumulated deposited surface area doses, taking into account the surface area of the alveolar epithelium in mice. Promising markers of profibrotic changes were selected, such as TGF-b and osteopontin (osteopoetin), which were later included in the scheme of epidemiological study CNT-ERA. In addition, based on the interstitium affection identified during the in vivo experiments, the biomarker panel was supplemented by Krebs von den Lungen-6 (KL-6) factor, a mucinlike high molecular weight glycoprotein, marking various interstitial lung diseases in humans [43, 89].

In 2014, Erdeli et al. [26] presented the results of inhalation studies in mice in which the calculation of the exposure doses was also based on the concentration of MWCNTs measured in the air of a number of enterprises. However, in contrast to our study, the US group studied enterprises producing and applying different types of MWCNTs. Thus, it was decided that the toxicological experiment would be based not on MWCNTs provided by the enterprises participating in the study, but rather the purified commercial sample purchased from the company. In addition, the researchers did not set out a specific task for selecting biomarkers, thereby being restricted to comparing the effects of different doses in relation to nonspecific indicators of local (in the lung tissue) inflammation.

At the present moment, at the Russian enterprises participating in the study is carrying out a panel inves-

tigation with the sampling of blood, nasal lavage, and induced sputum from the workers and a control group for further evaluating the content of fibrosis markers and systemic vascular effects. During the panel study, the biological samples from the same person are taken repeatedly. The advantage of this approach is that each participant is their own control. In case of high-precision methods, such a panel study provides qualitative and quantitative assessments of health risks even for a small number of participants [65, 79].

The successful completion of panel studies will allow us to organize a smooth transition to large-scale epidemiological projects. At the same time, it is necessary to consider the possibility of an international consortium to develop a single protocol of prospective study with the standardization of approaches to sampling, assessing industrial exposures, the selection of biological samples, and functional study methods. Applying the methods used in genomics, proteomics, and lipidomics will make it possible to identify specific changes in animals and humans that are not masked by nonspecific reactions.

An important component of the system for studying the risk of exposure to carbon nanotubes is the development of banks with biological samples taken from the workers in the course of observation with the possibility of delayed analysis as new hypotheses will appear.

MEETING THE CHALLENGES OF HYGIENIC STANDARDIZATION

In order to proceed to the assessment of risk in the workplaces and in the environment, we need data on acceptable levels of exposure to CNTs, but their development has been facing various difficulties, including the variety of CNT types, the complexity of their identification and quantitative evaluation in the environment, inadequate methodological approaches, and insufficient data on their biological effects.

One way to determine approximate safe exposure levels (ASELs) is to extrapolate the results of animal experiments based on the use of the lowest observed adverse effect levels with the application of risk-assessment methodology and establishment of the uncertainty factor. The first attempts to establish safe exposure levels (for MWNTs) were based on the results of subchronic inhalation experiments. The Nanocyl Company (Belgium), having assessed the risk and obtained an uncertainty factor of 40 based on the lowest observed adverse effect concentrations set out by Ma-Hock et al. [60] in the subchronic (90 days) inhalation experiments on rats [46], determined the no effect 8-hour weighted concentration for its MWCNTs (2.5 $\mu g/m^3$). Aschberger et al. [13] suggested the ASEL of 1 $\mu g/m^3$ for MWNTs taken by Ma-Hock [46], and 2 $\mu g/m^3$ for the Baytubes (Bayer MaterialScience, Germany) used by Pauluhn et al. [67] in a 13-week experiment based on the uncertainty factors of 50 and 25, respectively (obtained after the recalculation of the threshold levels of exposure and external respiration in rats and humans).

The Japanese National Institute of Advanced Industrial Science and Technology established the acceptable exposure level of 30 $\mu g/m^3$ for all types of CNTs [58, 59]. The ratio was set for a period of 15 years as a result of the inhalation experiment with MWCNTs (Nikkiso Co., ltd) in rats performed by Morimoto et al. with both SWCNTs and MWCNTs [56].

In 2010, the National Institute for Occupational Safety and Health (NIOSH, United States), having performed a quantitative risk assessment on the basis of the previous in vivo studies [44, 46, 67, 70, 81], found that the mean CNT concentration during the working shift (0.2–2 $\mu g/m^3$) upon exposure during the working time is associated with a 10% risk of respiratory diseases. However, taking into consideration the inadequate techniques for the detection and calculation of CNTs in the samples, the suggested recommended exposure level (REL) was 7 $\mu g/m^3$ (calculated as elemental carbon determined using the method of thermo-optic analysis) [62]. In April 2013, a new report was published by NIOSH which established the recommended exposure level for all types of CNTs of 1 $\mu g/m^3$. This was associated with an increase in the accuracy of procedures and, therefore, lower detection limits of CNTs in the samples [63].

In 2010, the Russian Federal Service for Supervision of Consumer Rights Protection and Human Welfare set out the ASELs for three nanomaterials, including SWNTs [2]. The drafters of the document were guided by the maximum allowable level of 0.01 fibers/m^3, which was suggested by the British Standards Institution in 2007, calculated as 1/10 of the maximum allowable level for asbestos fibers. Simultaneously, in 2010, the Russian Federal Service for Supervision of Consumer Rights Protection and Human Welfare published several methodic recommendations on a quantitative determination of nanomaterials, including carbon nanotubes [6, 7]. According to these documents, it is suggested to detect, identify, and calculate the number of CNT particles by transmission electron microscopy with contrasting by the salts of heavy metals. Infrared photoluminescence spectroscopy and infrared absorption spectroscopy can be used as additional methods of identification methods. It is suggested to take air samples using Krotov's apparatus with the deposition of aerosol particles in water. It should be noted that the opened Russian literature offers no data on the content of CNTs in air of the working place obtained using the methodology described above. Our results show that this approach does not allow an objective assessment of CNT content in the air, because it is extremely difficult to calculate individual nanotubes in the selected samples due to the rapid agglomeration of the particles.

Therefore, the recommended exposure levels for different CNTs in the world vary from 1 to 50 μg/m^3 (for 8-hour TWA concentrations). For comparison, the short-term exposure limit of carbon black in Russia is 4 mg/m^3; the 8-hour TWA concentration of carbon composite materials is 1 mg/m^3 [8]. Unfortunately, Russian experts have to rely on these values, for example, during toxicological evaluation of products.

CONCLUSIONS

1. In vivo and in vitro toxicological experiments allowed the presence of pronounced local profibrogenic effects produced by CNTs to be established on lung tissue. In addition, recent data suggest the potential possibility of extrapulmonary effects, such as systemic vascular changes and the affection of the immune status.

2. Under the conditions of insufficient data on the biomedical effects of CNTs, as well as the absence of reliable safety criteria for humans, it is urgent for assessing health risk to perform studies in exposed groups of people. Despite the small (at the current stage) number of exposed persons, a panel study with investigation of both local and systemic responses to CNT inhalation is the most favorable. Promising biomarkers are those of fibrosis and lesions of the cardiovascular system, as well as cytokines responsible for the development of inflammation and allergic reaction. This list will be enlarged with respect to in vivo and in vitro experiments. Thus, it is important to create a bank of biological samples (blood, urine, induced sputum, and buccal cells) to test new promising indicators.

3. Epidemiological studies should be carefully prepared. Complex projects combining a sequential assessment of CNT content in the environment, toxicological studies, and epidemiological observations are the most promising.

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