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## Genotoxicity evaluation of multiwalled carbon nanotubes: in vivo studies in mice

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**Abstract.** The toxicity of carbon nanoparticles in recent years has attracted much attention due to their active use in industry, medicine and agriculture, which brings not only obvious benefits, but also potential danger to the environment and human health. Getting into the natural environment, bioaccumulating and bioconcentrating in them, carbon nanotubes pose a real threat to mammals, since due to their high migration ability they can penetrate the food chain of animals and humans. Therefore, the aim of this study was to study the genotoxicity of carbon nanotubes in in vivo experiments on laboratory mice. For this, based on the method of single cell alkaline gel electrophoresis assay, studies were conducted to determine the effect of aqueous colloidal solutions of multiwalled carbon nanotubes on genotoxicity towards various tissues in laboratory mice. During these acute and subacute in vivo experiments we have established that orally introduced multiwalled carbon nanotubes colloids damage deoxyribonucleic acid in various organs. In particular, 0.5 mg of multiwalled carbon nanotubes administered daily for 21 days have a prominent genotoxic effect. Thus, the obtained results have illustrated that multiwalled carbon nanotubes can potentially affect the genetic material in mammals, orally administered nanotubes increase the rate of nuclear deoxyribonucleic acid destruction.

### 1. Introduction

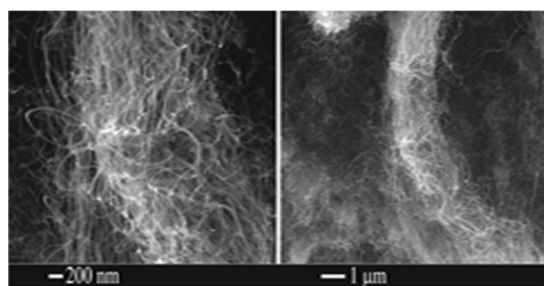
Nanomaterials with various properties have attracted wide attention in the modern world and are being increasingly manufactured and used. Carbon nanotubes (CNTs) represent a group of nanomaterials having a considerable potential as multifunctional building blocks and they are promising substances applicable in multiple areas of nanotechnologies: from nanocomposites to nanoelectronics, nanomedicine and nanobiotechnology. However, there is a growing body of evidence clearly indicating that CNTs require thorough evaluation of their potential harmfulness for humans and other biological systems. Though the medical applications of nanomaterials have attracted huge interest, the attention of the academic community is mostly focused on revealing the nanomaterials toxicity at the cell level. CNTs are known to penetrate the cell membrane and interact with intracellular molecules, resulting oxidative stress, impaired metabolism, and apoptosis [1]. Such topics as the toxic action of nanomaterials at the molecular level and genotoxicity in mammal cells have never been thoroughly studied [2,3]. Some data suggest that CNTs can accumulate in the endoplasmic reticulum in mice, and that CNTs produce free radicals, known as reactive oxygen species (ROS). For example, in [4] was



investigated the pulmonary health effects of 10 weeks of repeated oral or pulmonary exposures to multiwalled carbon nanotubes (MWCNTs) (4 or 40  $\mu\text{g}$  each week) in Apolipoprotein E-deficient mice. Intratracheal instillation of MWCNTs was associated with oxidative damage to deoxyribonucleic acid (DNA) in lung tissue and elevated levels of lipid peroxidation products in plasma, whereas the exposure only caused a modest pulmonary inflammation in terms of increased numbers of lymphocytes in bronchoalveolar lavage fluid. However, with subchronic exposure to BEAS-2B cell culture, small doses of multi-walled carbon nanotubes was shown high levels of intracellular reactive oxygen species (ROS) associated to MWCNT exposure. Nevertheless, an important proportion of these ROS levels seems to be associated to solubilized metals contaminants present in MWCNT. No primary DNA damage was obtained in the Comet assay although significant levels of chromosome damage were detected using the micronucleus assay [5]. In addition to the dose and exposure time, the physicochemical characteristics of MWCNTs affect the level of genotoxicity [6,7]. Thus, in the studies of S. Poulsen et al. the example of 10 different MWCNTs shows the dependence of their physicochemical characteristics on the manifestation of the genotoxic effect in the lungs and liver tissues [6]. R. Snyder et al. was investigated the potential of MWCNTs to impair mitochondrial gene expression and function in human bronchial epithelial cells. Mitochondrial DNA heteroplasmy and insertion/deletion mutations were not significantly affected by any treatment. It was conclude that carbon nanotubes cause mitochondrial dysfunction that leads to mitophagy in exposed BECs via a mechanism unrelated to its reported genotoxicity. In conclude that carbon nanotubes cause mitochondrial dysfunction that leads to mitophagy in exposed bronchial epithelial cells via a mechanism unrelated to its reported genotoxicity [1]. Thus, the question of the genotoxicity of MWNTs remains open. Therefore, the aim of this study was to study the genotoxicity of carbon nanotubes in *in vivo* experiments on laboratory mice.

## 2. Materials and methods

Taunit-M carbon nanomaterial (MWCNTs) manufactured by NanoTechCenter (Tambov, Russia) was used as a test material. Taunit-M is produced by the method of chemical vapor deposition of hydrocarbon in the presence of metallic catalysts (Fe) [8]. MWCNTs are hollow one-dimensional nano-sized cylindrical formations (figure 1). Typically, Taunit is represented by 1-1000  $\mu\text{m}$  agglomerations of entangled MWCNTs bundles.



MWCNTs Parameters	Values
Outer diameter	11-28 nm
Inner diameter	5-10 nm
Length	5-10 $\mu\text{m}$
Impurity content	<1-2%

**Figure 1.** SEM micrograph of MCNTs and their major parameters.

All the tests were carried out on outbred laboratory male mice at an age of 2 months and weight of  $20 \pm 1$  g. The animals were housed in plastic boxes with the floor area of  $200 \text{ cm}^2$  at a semi-natural light cycle of 12:12, at  $+22^\circ\text{C}$  and relative air humidity of 40-60%. The mice were handled in accordance with the Guide for the care and use of laboratory animals [9].

The MWCNTs colloids were blended for 20 min in distilled water using a Sonics VC750 ultrasonic homogenizer (300W, 23.7 kHz). The obtained solutions were administered forcefully orally using a medical syringe without a needle. The control group received distilled water. In the acute experiment the animals were divided into three groups of five mice. The first group received MWCNTs at 5 mg/day, the second group received 2.5 mg/day, the third group was used for control. The acute experiment lasted for 1 day. In the subacute experiment lasting for 21 days the experimental group received MWCNTs at

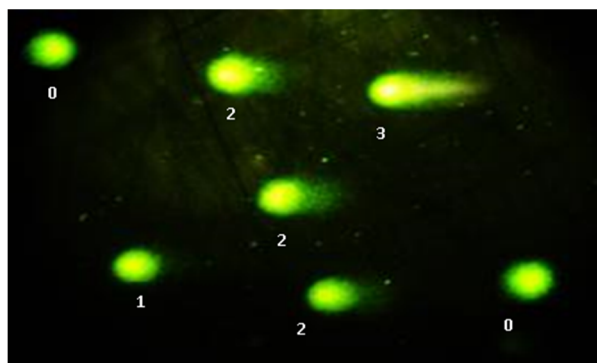
0.5 mg/day and the control group received distilled water. At the end of this period the animals were taken from the experiment.

Tissues from liver, seminiferous tubules, large intestine, brain, kidneys, lungs, as well as bone marrow cells and peripheral blood leukocytes were analyzed. The samples were prepared according to the Guidelines [10]. Tissue maceration was carried out at +4°C in phosphate-buffered saline (pH 7.5) containing 20 mM of EDTA-Na<sub>2</sub> and 10% of DMSO. The cell suspensions embedded in low-melting agarose were applied to glass slides at + 42°C. The proteins were lysed for 2 hours at +40°C (the lysis solution: 10 mM Tris-HCl (pH 10), 2.5 M NaCl, 100 mM EDTA-Na<sub>2</sub>, 1% Triton X-100 and 10% DMSO). Electrophoresis was carried out in a dark room according to the neutral variant of the method in tris-acetate-EDTA buffer (pH 8.9; 20 min; 1 V/cm). Ethanol-fixated and dried samples were stained with SYBR Green I. An epifluorescence-equipped microscope was employed for image analysis (figure 2). The data was processed using CometScore™ V. 1.5 software. The nuclei were ranged into four categories depending on the DNA destruction (figure 2). At least 100 nuclei were examined in each specific sample.

Kruskal-Wallis test was used to assess the DNA destruction values, it is sometimes referred to as index of DNA comet (IDC) and is calculated according to the formula:

$$IDC = (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4) / \Sigma$$

where  $n_0$ - $n_4$  is the number of DNA-comets of each category and  $\Sigma$  is the total sum of all the calculated DNA-comets [11].



**Figure 2.** Typical DNA-comet image of various stages of destruction of mouse bone marrow cell nuclei.

### 3. Results and discussion

The calculation results for the DNA comet indices of the cell nuclei collected from the experimental groups in the acute experiment are presented in Table 1.

**Table 1.** Values of the DNA damage indices in the acute experiment.

Organ ( $M \pm m$ )	MWCNTs 5 mg/day (a.u.)	MWCNTs 2.5 mg/day (a.u.)	Control (a.u.)
Liver	0.9±0.43	0.48±0.21	0.03±0.01
Seminiferous tubules	1.02±0.21*	0.11±0.01	0.18±0.08
Bone marrow	0.53±0.28	0.62±0.35	0.03±0.01
Large intestine	0.11±0.05	0.06±0.005*	0.003±0.001
Leukocytes	0.48±0.21	0.33±0.03*	0.07±0.01
Brain	0.67±0.34	0.0*	0.04±0.01
Kidneys	0.18±0.06*	0.06±0.002*	0.02±0.01
Lungs	0.45±0.16*	0.33±0.02*	0.06±0.01

$M$  is the group mean value,  $m$  is root-mean-square deviation

\* – significant difference (at  $p < 0.05$ ) compared to the control group

The calculation results for the DNA comet indices of the cell nuclei collected from the experimental groups in the subacute experiment are presented in Table 2.

**Table 2.** Values of the DNA damage indices in the subacute experiment.

Organ (M±m)	MWCNTs 0.5 mg/day (a.u.)	Control (a.u.)
Liver	1.85±0.26*	0.04±0.01
Seminiferous tubules	1.98±0.58*	0.22±0.08
Bone marrow	0.91±0.36*	0.05±0.01
Large intestine	2.5±0.33*	0.008±0.001
Leukocytes	1.85±0.61*	0.08±0.01
Brain	1.57±0.51*	0.06±0.02
Kidneys	1.55±0.20*	0.02±0.01
Lungs	1.15±0.63	0.07±0.02

*M* is the group mean value, *m* is root-mean-square deviation

\* – significant difference (at  $p < 0.05$ ) compared to the control group

According to the obtained data, administration of MWCNTs at the doses of 5 mg/day and 2.5 mg/day in the course of the acute experiment results in significantly increased values of damaged lung and kidney DNA compared to the control group. It is worth mentioning that the lower MWCNTs dosage of 2.5 mg/day leads, in addition to that, to increased IDC in the cells of the large intestine, brain and in leukocytes, which might be connected with the lower agglomeration levels of MWCNTs at lower doses and, consequently, their better distribution throughout the organism.

DNA destruction in leukocytes after exposition to MWCNTs has also been proven by the study of the human peripheral blood leukocyte culture. During the in vitro experiment the cells were exposed for 48 h to MWCNTs at the following concentrations: 12.5, 25 and 50 µg/mL. As a result, the increased level of the intracellular reactive oxygen species, registered throughout the experiment, has led to the cell damage and death, proliferation inhibition, DNA damage and inflammatory response [12]. In another study the authors have used the DNA-comet method to show the genotoxic activity of MCNTs at 2 µg/mL. Though it should be noted that under the same experimental conditions MWCNTs at the concentrations of 1, 5 and 10 µg/mL displayed only negligible effect on the DNA structure [13].

Subacute MWCNTs administration at a dose of 0.5 mg/day has a statistically significant adverse effect on the cell DNA in all the studied organs. The only exception is IDC in the lung cells, where the values are close to those of the control. In all the organs, except lungs, we have registered the statistically significant (at  $p \leq 0.05$ ) increase in the numbers of damaged DNA compared to the control. It is worth mentioning that in the large intestine the IDC value was the highest of all (2.5±0.33). It might be explained by the method of MWCNTs administration, as this organ is the target for the substances and their metabolites which get excreted through the digestive tract. The influence of MWCNTs on the cell DNA in various organs has been reported in a number of other research papers [1,6,13,14]. In particular, the genotoxic influence of MWCNTs on the bone marrow cells in in vivo experiments performed on lab mice has been proven in [13]. The values of % tail DNA were higher at all treatment doses (2, 5 and 10 mg/kg body weight) as compared to the negative control.

The oxidative stress is considered to be the main DNA-damaging mechanism. MWCNTs stimulate generation of the reactive oxygen species which are known to attack not only lipids, carbohydrates and proteins, but also the DNA structure [15]. Normally, antioxidant enzymes, such as SOD, GSH-Px, etc. reduce H<sub>2</sub>O<sub>2</sub> and superoxide radicals, thus saving polyunsaturated fatty acids from lipid peroxidation and providing additional protection to the cell membrane structure. However, excessive reactive oxygen species production disrupts the natural antioxidant system functions leading to certain sub-cellular damages, including protein denaturation, membrane deterioration and DNA damage [16-18].

#### 4. Conclusion

Thus, the obtained results have illustrated that MWCNTs can potentially affect the genetic material in mammals, orally administered nanotubes increase the rate of nuclear DNA destruction. The strongest effect has been observed during the subacute experiment. It is especially important to note that the genotoxic effect has been registered in all the studied organs, being a definite precondition for further pathologies. Though a number of papers show that MWCNTs display little acute and subchronic toxicity, nevertheless, the observed DNA damages might result in long-term toxic effects, for example, they might have adverse effects on the progeny of the exposed animals [18-20]. Because among MWCNTs potential negative effects, the potential for DNA damage is of paramount relevance, it is especially important to understand their mechanism of action. Besides, in the literature there is an assumption that direct MWCNTs have a more pronounced genotoxic effect than tangled MWCNTs [14]. The relationship between MWCNT properties and their genotoxic potential should further be investigated to better understand the mechanisms involved.

#### 5. Acknowledgements

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