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MECHANISMS OF THE CYTOTOXICITY OF CARBON NANOTUBES

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Probable mechanisms of cytotoxicity of a total fraction of multi-walled carbon nanotubes using spin probe methods (in human erythrocytes and rat hepatocytes) and electrical breakdown (in mouse oocytes) have been studied. Erythrocyte membrane damage induced by carbon nanotubes is developed in time. After two-day exposure at 279 K the erythrocyte samples with carbon nanotubes concentrations of 10, 50, 100, and 200 μg/ml showed a part of damaged erythrocytes of 4, 10, 16, and 25%, respectively. A 4 h exposure of rat liver homogenate with carbon nanotubes at 273 K resulted in a significant decrease in liver homogenate mitochondrial activity. The study of the processes of oocyte electroporation has shown that probable mechanisms of nanotube cytotoxicity are associated with an increase in membrane conductivity, alteration of surface potential, formation of defects within the membrane, and as a consequence, with an increase in membrane permeability. In addition to their capability to affect membrane structural changes, the nanotubes have a capacity to inhibit delicate electrochemical processes in cells.

INTRODUCTION

A carbon nanotubes (CNTs) cytotoxicity still remains one of the key issues and is of current interest up to the present time, since practical use of these nanostructures with their unique properties in such fields as biotechnology, molecular biology and medicine may be complicated because of carbon nanoparticles possible adverse effect on subcellular and cellular structures as well as organs and tissues of living organism. The available data regarding pulmonary toxicity, skin irritability, cytotoxicity, biocompatibility, environmental impact as well as the matters related to CNTs therapeutic action are conflicting and do not give a clear view of harmlessness and safety level of such nanomaterials for living organism [1–5]. In spite of conflicting data about cytotoxicity of CNTs with different size, geometry and chirality, it becomes clear that the problem on their harmlessness is first and foremost solved at the cellular and membrane level. In turn, this requires a combined application of high-performance and sensitive research methods with ability not only to detect interaction between CNTs and plasma membranes, and between those and cell organelle membranes but to study the structural changes in a cell induced by CNTs, track intracellular dynamics of CNTs distribution between cell organelles, and to evaluate the parameters of membrane integrity and activity of subcellular structures.

Up to now, it remains unclear how does a cellular membranes mechanical damage induced by CNTs contribute in their cytotoxicity and what is a contribution of more delicate CNT influence on biochemical processes in subcellular organelles such as endoplasmic reticulum, mitochondria and nucleus. If the fact that DNA and cell nucleus are influenced by CNTs has been multiply proved experimentally [6, 7], then nowadays there is no evidence of such influence on the activity of mitochondria, inside the cell though they play a key role in a cell viability. First of all, it can be put down to limited possibilities of physical methods used when investigating such complicated biological systems as cells in the presence of sufficiently large and cumbersome, from the molecular biology point of view, carbon nanomaterials. In order to study the mechanisms of CNT cyto-

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toxicity at the molecular and membrane levels we proposed to utilize two biophysical methods: spin probes one and electrical breakdown of the cells. The method of spin probes is successfully used in molecular biology and pharmacology for a long time. It can be judged about microviscosity and polarity of probe microenvironment, conformational changes in proteins and membranes, lipid fluidity and membrane integrity as well as about redox activity in the cells and tissues by electron paramagnetic resonance (EPR) spectra of the stable nitroxyl radical (probe) being introduced into a suspension of cells or proteins [8]. Spin probe method makes it possible to study the opaque solutions and viscous suspensions of biological objects and to investigate not only cell suspensions but also biological tissue samples of skin, liver, kidney, muscular and nerve tissue as well.

An electrical breakdown method is based on the phenomenon of electroporation common used in biotechnology in recent years [9–13]. The basic theoretical prerequisites of this approach are as follows.

When a cell is placed into the uniform electric field, there is a sharp rise in the membrane potential. Exceeding a certain critical value of membrane potential (being about 0.2–1.0 V by some authors estimates) causes local rearrangements of a lipid bilayer and a sharp elevation of electrical conductivity of the cell membrane due to appearance of short-time defects of a through pore type (hydrophobic or hydrophilic). If a hydrophilic pore radius exceeds a certain critical value, there will be an irreversible electrical breakdown of membrane followed by damage and lysis of the cell [9–11].

A value of critical voltage can serve as an important parameter characterizing the structure of a membrane and a degree of its integrity. In many works, it was shown that even small oscillations in membrane structure caused by external chemical and physical factors had an effect on the critical voltage value [13].

Taking into account high electrical conductivity of nanotubes and their ability to bind up with membranes, that as a whole allows us to consider the CNTs to be a potential intermediary, conductor of the current between external and internal membrane surfaces, we proposed the use of the electrical breakdown method to obtain independent supplementary data on affinity of CNTs to membranes, and membrane conductivity and permeability change under the influence of the nanotubes. Furthermore, such data would make it possible to evaluate the portions of conducting (metal) nanotubes and non-conducting (semiconductor) ones in total fractions of CNTs produced by different process.

An objective of our work was to study mechanisms of CNT cytotoxicity by spin probes. For this, there was tracked the integrity of human blood erythrocyte membranes and rat liver homogenate mitochondrial activity in the presence of suspensions of CNTs without hepatocyte cells damage. An attempt was made using an electrical breakdown method to obtain independent data on the affinity of CNTs to membranes of oocytes (mouse eggs), change of oocyte membranes conductivity and permeability under the influence of nanotubes. Such combined approach enabled us to define the basic probable mechanisms of cytotoxicity of the nanotubes.

EXPERIMENTAL

In the present work, the multi-walled high purity CNTs were used. Those were produced on a pilot plant designed by Chuiko Institute of Surface Chemistry of the National Academy of Sciences of Ukraine and TMSpetsmash Limited Company (city of Kyiv) by catalytic pyrolysis of unsaturated hydrocarbons [14]. The characteristics of CNTs were as follows: internal diameter, 1–2 nm; external diameter, 10–40 nm; ash content, less than 0.4%; and nanotube content, more than 95%.

The human blood erythrocytes were obtained from the erythromass washed from plasma and from the stabilization solution by centrifugation in physiological saline (pH 7.2). Rat liver homogenate was prepared by the procedure described in [15].

As a paramagnetic probe a water-soluble iminoxyl radical: 2,2,6,6-tetramethyl-4-oxo-piperidine-1-oxyl (TEMPON) was used [8]. The research objects were mouse oocytes at the stage of metaphase II of meiosis obtained from 6–8 week-old female mice; line CBA, by a standard procedure [16]. For the purpose of the determination of oocyte membrane stability in solutions we employed an electroporation system [9–13].

The stable CNT suspensions were prepared as follows. To the water suspension of CNTs (100 mg per 100 ml of water) we added 0.1 ml of heparin solution (5000 Units per 1 ml) which was

then ultrasound treated, froze and after defrosting centrifuged at 10 000 rpm for 15 min. For each experiment the two samples of the erythrocyte suspension have been prepared, each of 0.9 ml. One sample (control) was exposed during appropriate exposure time at the appropriate temperature without CNTs and the same was done with another sample (test) but with CNT suspension. After exposure, 0.1 ml of the spin probe standard solution and widener (potassium ferricyanide) were added to each sample, and then EPR spectra were registered using a radiospectrometer (Brucker ER 100 D, Germany). Thus, a cell aging during exposure was accounted. The similar experiment has been conducted using hepatocytes suspension. Erythrocytes were in contact with CNTs at 279 K for two days, and the liver homogenate was in contact with those at 273 K for 4 h.

An electrical conductivity of mouse oocytes was determined using apparatus including a single rectangular pulse generator, from which output voltage was applied to microelectrodes made of a glass capillary-sealed thin gold filament. Oocytes being in a drop of the solution, they have been placed between the microelectrodes (Fig. 1).

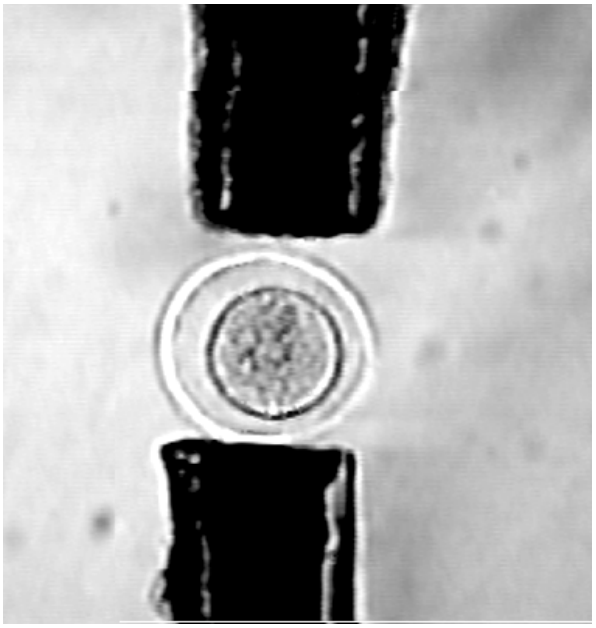

Fig. 1. Microimage of the mouse oocyte fixed between microelectrodes

Hardware was developed at the laboratory of molecular and cellular biology of the Institute of Livestock Farming of Ukrainian Academy of Agriculture Sciences. Pulse voltage with the linearly increasing amplitude and duration of 50 μs was applied to microelectrodes. Specific conductivity was indirectly measured (by using oscilloscope) while measuring the voltage amplitude of calibration resistor in series connection with the microelectrodes, according to [17]

$$G = U_r / R(U_{output} - U_r)K,$$

where G – specific conductivity of the cell, μS/cm; U_{output} – voltage of the generator; U_r – calibration resistor voltage; R –calibration resistor resistance, kΩ; $K = L / S$ – constant of the device; L – distance between microelectrodes; S – cross-section area of the electrode.

RESULTS AND DISCUSSION

Previously, a quantitative express method has been proposed and developed by Ivanov [18] for studying the integrity of isolated cells and those of tissue samples. The method makes it possible the determination of the amount of native (intact) cells in the sample after some kind of influence using spin probes.

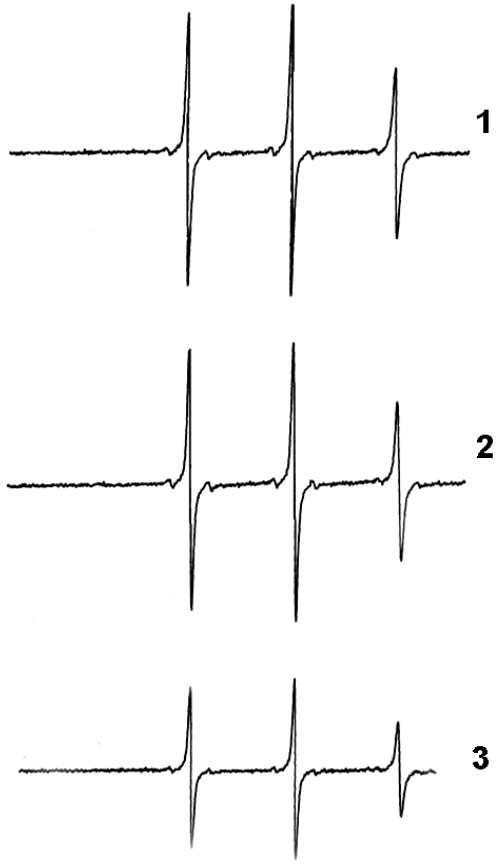

Fig. 2. EPR spectra of the spin probe in the cytosol of donor blood erythrocytes after a two-day incubation at 6°C at various concentrations of CNT: *1* – control; *2* – 10 μg/ml; *3* – 200 μg/ml

In present work, we employed this method to assess CNTs influence on the erythrocyte membrane integrity. In our experiments, it was demonstrated that when the erythrocyte suspension was mixed with the CNT suspensions at concentrations of 10, 50, 100 and 200 μg/ml followed by a 10 min exposure, no change in EPR spectra was observed. This confirmed the erythrocyte integrity to be intact in all the experiments. And there was the only experiment (when the concentration of CNTs was 200 μg/ml) when the signal intensity of EPR had decreased by 12–15%, and that indicated membrane damages appeared in a significant amount of erythrocytes.

After 2 days the amount of damaged erythrocytes has been already more than 25% (Fig. 2). This is evidence that the damage of the erythrocyte membranes under influence of CNTs is developing in time. The result obtained is in compliance with the conclusions made by authors in works [1–5] that a disintegration process of different cells had a progress within a period from some hours to some days. It was found that even lower concentrations of CNTs had a damaging effect on erythrocytes. Thus, after 2 days the CNT suspensions at concentrations of 10, 50, and 100 μg/ml had damaged 4, 10, and 16% of erythrocytes, respectively (Fig. 3).

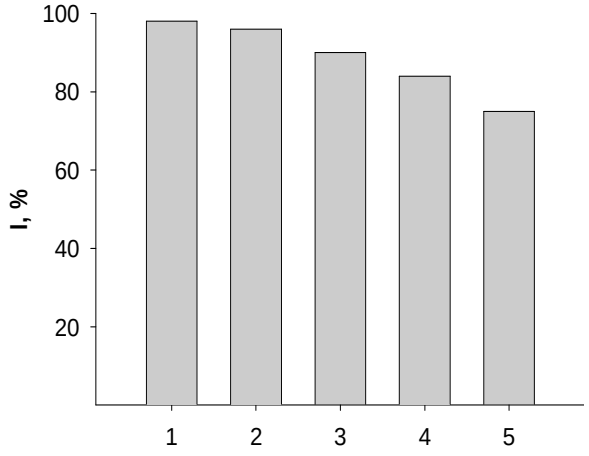

Fig. 3. The influence of the incubation of donor blood erythrocytes with CNTs at different concentrations on the intensity of the central component of EPR spectrum of a paramagnetic probe (1): *1* – control; *2* – 10 μg/ml; *3* – 50 μg/ml; *4* – 100 μg/ml, *5* – 200 μg/ml

A slow dynamics of the erythrocytes under the influence of CNTs seems not to be tied to the mechanical nature of the erythrocyte membrane damage, its consequences being very rapidly developed. Obviously, the ability of CNTs to manifest the semiconducting and metallic properties depending on their structure and environmental conditions should be taken into account [19]. We suggested that nanotubes can substantially influence the biochemical processes occurred in mitochondria, neurons, and cardiac hystiocytes etc. which are related to electron or charge transfer. In this respect, we used a modification of the spin probe method where the activity of the electron transfer chain (respiratory chain of mitochondria) can be predetermined without mitochondria extraction from hepatocytes [20] by the reduction rate of water-soluble probe (1) introduced into a suspension of the cells having mitochondria (hepatocytes). A probe reduction (1) to non-paramagnetic hydroxylamine is carried out by using a strong antioxidant, coenzyme Q_{10} of the mitochondrial respiratory chain. Hereby, the intensity of the EPR spectrum is exponentially decreased with time. By taking the logarithm of the intensity we obtain the straight lines with a slope ratio to X-axis to be in proportion to the constant of the probe reduction rate and therefore to the activity of mitochondria being inside the hepatocytes.

Figure 4 shows the data on the influence of a CNT suspension at a concentration of 200 μg/ml on the constant of the spin probe reduction rate (1) in the suspension of hepatocytes (mitochondrial activity). The probe reduction rate was assessed by the intensity rate fall of spectral lines of EPR probe being in the liver homogenate.

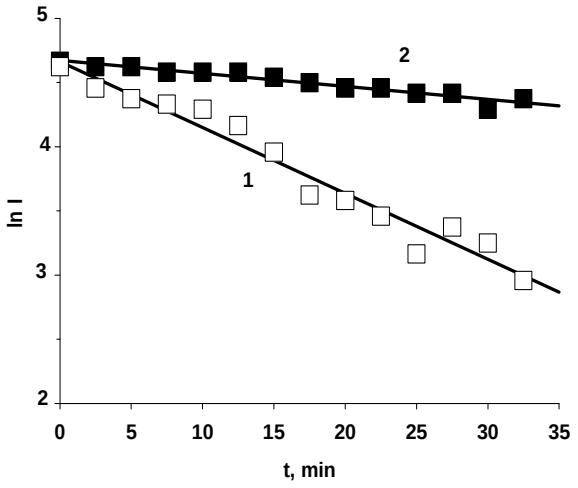

Fig. 4. A spin probe reduction in the liver homogenate after a 4 h incubation period: □ – control; ■ – at CNTs concentration of 200 μg/ml

It can be seen from the figure that the presence of CNTs in hepatocyte suspension after a 4 h exposure results in a several times inhibition of the electron transfer chain in mitochondria (i.e. substantial decrease in mitochondrial activity of the cells). Thus, a mechanism of CNT cytotoxicity for hepatocytes consists not only in plasma membrane overcoming and rupture but in a subsequent inhibition of respiratory chain of hepatocyte mitochondria.

The data obtained entitle us to suggest that the proposed method of cytotoxicity research (spin probes) is an unique possibility for obtaining of important information regarding the mitochondrial activity without cells damage during their extraction (as usually done by biochemists), and also the influence of conducting nanotubes on intracellular electrochemical processes. It might be that the inhibition mechanism is related to mechanical damages of mitochondria as subcellular structures. Perhaps also, that an interaction of conducting nanotubes with mitochondria (shunting) results in a partial redistribution and leakage of respiratory chain electrons onto high conducting nanotubes, that in turn causes the decrease in the mitochondrial electron flow and decrease in reduction rate of a nitroxyl radical (probe).

The study of the mouse oocyte membrane stability in the presence of CNTs was carried out as follows. The oocytes were exposed in a 0.3 M solution of saccharose, and the dependence of oocyte conductivity on the electrical field applied in certain range of values not exceeding some critical value (3 kV/cm) was determined. This was control. Then, the same cells were transferred into a 0.3 M solution of saccharose containing nanotubes (10 μg/ml) and exposed to electric pulse with increasing amplitude. That treatment was performed so that nanotubes settled to the thick oocyte membrane have rapidly diffused into the inner layers of the oocyte membrane due to the reversible electroporation (an opening of small pores in membrane and their resealing). In that event, the use of an electric pulse with relatively low amplitude values results in a reversible electroporation effect. At the same time, substances from the intracellular water enter the cell.

The effect described is in common practice in current biotechnology to include exogenous macromolecules, particularly, enzymes and DNA, into target cells. In our work, we used this phenomenon to make an attempt to introduce the nanotubes settled at the surface of membrane into deeper layers of membrane in order to investigate their influence on the electrical conductivity of mouse oocytes and the state of a membrane [9–13].

It should be noted that a large membrane of mouse oocytes is thick and in contrast to other membranes of cells the transmembrane transfer of CNTs in case of oocytes, evidently, requires a long period of time.

Then an oocyte was triply washed from the nanotubes solution in order to be completely free of the nanotubes being in the solution or those slightly bound up with the membrane surface. After that, the electrical properties of oocytes were studied once again in a 0.3 M solution of saccharose.

As a result, the relationships of calculated cell specific conductivities on field strength between the electrodes were plotted and the following regularities were obtained (see Fig. 5).

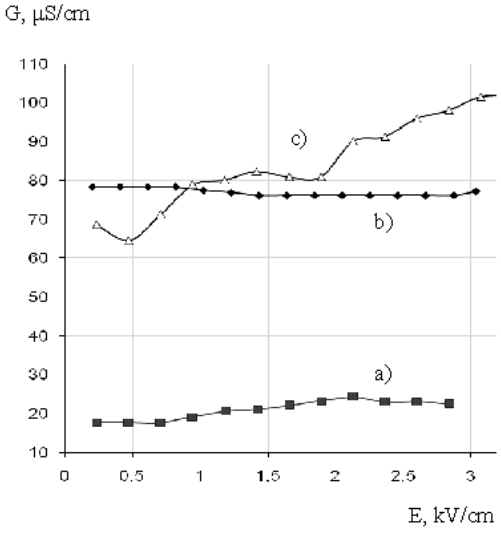

Fig. 5. The relationship between mouse oocyte specific conductivity and the applied electric field under three types of exposure conditions (*a*) in a 0.3 M solution of saccharose; (*b*) in a 0.3 M solution of saccharose with nanotubes content; (*c*) in a 0.3 M solution of saccharose after washing from nanotubes

Slightly increasing linear conductivity-field strength relationships were characteristic of the oocyte exposed to an electric pulse in a 0.3 M solution of saccharose (bottom curve *a*). Conductivities were in the range of 15–25 μS/cm. At the same time, multiple small spikes of the conductivity were recorded indicating a reversible elec-

troporation of the membrane. After the oocyte has been transferred to a solution of saccharose with nanotubes content (10 μg/ml), the absolute values of oocyte conductivity were nearly 4 times as much as those previously recorded (middle curve *b*), and this indicated a sharp increase in the cell conductivity (80–90 μS/cm) seemed to be a result of conducting nanotubes have been bound to a membrane. After the removal of nanotubes from the solution, the electrical properties of the oocyte have been cardinally changed, as it shown in Figure presented (top curve *c*). A conductivity relationship looks like a linearly increasing curve in the range of conductivity values of 60–100 μS/cm with marked multiple spikes which indicate that the conductivity is further increased and a lot of pores are forming and resealing in a membrane (reversible electroporation).

A main conclusion that could be done from experiments is as follows: the method used has a high sensitivity to electroconductive properties of nanotubes confirmed by a sharp increase in conductivity of the oocyte cell after adding the nanotubes. At the same time, the increased cell conductivity remains high if nanotubes are diffused into membranes or cells. In the absence of nanotubes, a value of conductivity was a few times less. Some theoretical and practical opportunities follow from this, namely: an opportunity of evaluating the portion of conducting nanotubes in different total fractions, a comparative evaluation of electroconductivity of complexes of nanotubes with proteins, DNA, and polymers introduced into a solution by a parameter of the cell electroconductivity which should allow developing the novel scientifically established nanomaterials.

We consider that the data obtained by the method of electrical breakdown of oocytes give us a supplementary information on probable mechanisms of influence of total fractions of CNTs on membranes, i.e. show the membrane-binding capacity of the conductive CNTs, their capacity to significantly increase the cell electroconductivity by changing the surface potential, and as a consequence, to increase a cell membrane permeability.

At the same time, the parameters of the cell specific conductivity in the presence of CNTs may serve as a test for evaluation of a portion of conductive nanotubes in a total fraction of CNTs by comparison with nanotubes obtained by other technologies.

Similar investigations are very promising in the comparative study of cell conductivity in the presence of modified nanotubes since conductivity of modified nanotubes is notably decreased. With this, an opportunity appears to study the influence of the chemical modification of nanotubes or properties of their polymeric coating on the electoconductive properties of CNTs provided stable nanotube-polymer hybrids are formed.

CONCLUSIONS

It has been demonstrated by a spin probes method that erythrocyte membrane damage under the influence of CNTs is developed with time: introduction of a CNT suspension at concentrations from 10 to 200 μg/ml does not immediately result in erythrocyte membrane damage, but after a two-days period of exposure at 279 K to CNTs at concentrations of 10, 50, 100 and 200 μg/ml the samples showed percentage of erythrocytes damaged as 4, 10, 16, and 25%, respectively.

Liver homogenate exposure to CNTs for 4 h at 273 K results in an approximately 5 fold decrease in mitochondrial activity (inhibition of the chain of electron transfer in mitochondria) of the liver homogenate. The data obtained show that cellular damage under the influence of CNTs not only implies structural changes within the cell but can inhibit delicate intracellular electrochemical processes.

It has been shown by the electric breakdown of the mouse oocytes that there is a nearly 4 fold increase in the oocyte conductivity provided CNTs are binding to the membrane. An increase in voltage applied to the cell with subsequent oocyte washing from nanotubes in the solution results in the reversible electroporation, i.e. forming the apparent pores in the cell membrane and their subsequent resealing.

The data obtained suggest that mechanisms of cytotoxicity of nanotubes, except their influence on the structure, are related to a sharp increase in the membrane conductivity of the cell, further changing the membrane surface potential, forming the defect germs in the membrane, and as a consequence, increasing the membrane conductivity what makes a membrane barrier ineffective to a certain extent.

The degree of increase in cell conductivity when nanotubes are binding to them may serve as

a test for evaluation of a portion of conducting (metal) nanotubes in the entire fraction of nanotubes produced by various technologies as well as for evaluation of the electroconductivity of modified nanotubes and their hybrids.

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Механізми цитотоксичності вуглецевих нанотрубок

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З використанням методів спінового зонда (в еритроцитах людини і гепатоцитах щурів) та електричного пробою (в ооцитах миші) вивчено вірогідні механізми цитотоксичності нефракціонованих багатошарових вуглецевих нанотрубок. Показано, що пошкодження мембран еритроцитів під дією вуглецевих нанотрубок прогресує з часом. Після дводенної експозиції при 279 K зразки еритроцитів з концентрацією вуглецевих нанотрубок 10, 50, 100 і 200 мкг/мл показали частку пошкоджених еритроцитів 4, 10, 16 і 25% відповідно. Експозиція гомогенату печінки щура з вуглецевими нанотрубками при 273 K протягом 4 год призводить до значного зниження активності мітохондрій. Вивчення процесів електропорації ооцитів показало, що можливі механізми цитотоксичності нанотрубок пов'язані зі збільшенням провідності мембран, зміною поверхневого потенціалу, з виникненням дефектів у мембрані і, як наслідок, збільшенням проникності мембран. Як доповнення до здатності нанотрубок викликати мембранні структурні зміни, вони також можуть пригнічувати тонкі електрохімічні процеси в клітинах.

Механизмы цитотоксичности углеродных нанотрубок

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С использованием методов спинового зонда (в эритроцитах человека и гепатоцитах крыс) и электрического пробоя (в ооцитах мыши) изучены вероятные механизмы цитотоксичности нефракционированных многослойных углеродных нанотрубок. Показано, что повреждение мембран эритроцитов под действием углеродных нанотрубок развивается во времени. После двухдневной экспозиции при 279 K образцы эритроцитов с концентрацией углеродных нанотрубок 10, 50, 100 и 200 мкг/мл показали долю поврежденных эритроцитов 4, 10, 16 и 25% соответственно. Экспозиция гомогената печени крысы с углеродными нанотрубками при 273 K в течение 4 ч приводит к значительному снижению активности митохондрий. Изучение процессов электропорации ооцитов показало, что возможные механизмы цитотоксичности нанотрубок связаны с увеличением проводимости мембран, изменением поверхностного потенциала, образованием дефектов в мембране и, как следствие, увеличением проницаемости мембран. В дополнение к способности нанотрубок вызывать мембранные структурные изменения, они также могут ингибировать тонкие электрохимические процессы в клетках.