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ULTRASTRUCTURAL AND SOME FUNCTIONAL CHANGES IN TUMOR CELLS TREATED WITH STABILIZED IRON OXIDE NANOPARTICLES

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Aim: **To study the ultrastructure and some functional indexes of tumor cells treated with stabilized iron nanoparticles *in vitro*.** ***Methods:*** **3-[4,5dimethylthiazol-2-1]-2,5-diphenyltetrazolium bromide (MTT)-test, electron microscopy, polarography with applying of closed Clark's electrode.** ***Results:*** **It was shown that cultivation of cells with stabilized Fe_3O_4 leads to intracellular accumulation of ferromagnetic nanoparticles. The most active ferromagnetic uptake by cells has been observed after 24 and 48 h of incubation. The presence of ferromagnetic in cells led to altered mitochondrial structure that caused the decrease of oxygen uptake rate in the cells of all studied lines. Ferromagnetic released from the majority of cells via exocytosis or clasmacytosis after a certain period of time. The number of dead cells or cells with severe damage was moderate, so cytotoxic action of stabilized iron oxide nanoparticles was minimal toward the studied cell lines.** ***Conclusion:*** **the presence of ferromagnetic nanoparticles in culture medium led to alterations in mitochondria ultrastructural organization and decrease of oxygen uptake by mitochondria in sensitive and anticancer-drugs resistant cells.**

Key Words**: MCF-7 cells, A2780 cells, stabilized iron oxide nanoparticles, ultrastructure, mitochondria oxygen uptake.**

Nanotechnologies are one of the main directions of modern technological processes in medicine. It is predicted, that these technologies would be applied in oncology via synthesis of new nanodrugs with their target delivery to tumor cells. Methodology of antitumor drug delivery with application of nanotechnologies is the one that would allow overcoming some physical and pharmacological barriers to elevate the treatment efficacy of patients with cancer [1–3].

At present time the search for new technologies that on one hand will allow decreasing toxicity of drugs via generation and use of special constructions, particularly, liposomes, and on the other hand, will promote targeted delivery of drugs directly to tumor with the use of nanotechnologies, is being actively performed. Among constructive approaches to solve this task is the development of magnet-operated forms of anticancer preparations. This approach consists in general of a complex of cytostatic drugs with nano-sized particles of ferri-ferrous oxide that allows concentrating anticancer preparation in target area under influence of magnetic field.

Presently there are going trials of many nano-sized substances that possess ability for targeted delivery of anticancer preparations to malignant cells with simultaneous decrease of general toxicity [4]. It was shown that physico-chemical properties and biologic activity of substances in form of nanoparticles often significantly differ from substances in their standard physico-chemical state [5]. Particularly, metal nanoparticles reveal potent catalytic properties exerted in temperature differences up to hundreds of degrees, huge magnetic resistance, abnormally great magneto-calorimetric effect etc [6]. That's why oncologists pay special attention to possible use of nanoferromagnetics.

The first task for determining possible clinical application of nanoscale complexes in different branches of medicine, particularly in oncology, is study of biological effects which are able to cause toxic damage in cells after their contact with nanoparticles of different compositions.

Despite the clinical use of ferromagnetic nanoparticles as magnetic resonance imaging contrast agents [7, 8], yet there is insufficiency of experimental studies *in vitro* [9–11] about toxic effects and biological action of ferromagnetics on cells and influence of nanosized complexes containing ferromagnetic in liposomal form.

In several studies it was shown that toxic effect of nano-sized complexes on cells depends on material from which they were composed, their size, the presence of reactive groups on the surface of these complexes, their concentration, and cell histogenesis [3, 4]. Along with this it was demonstrated that the majority of nanosized complexes revealed their toxic effect on cells only at high concentrations (100–250 μg/ml) [12, 13].

The mechanism of penetration of coated and uncoated nanocomplexes is related to soluble phase of endocytosis that has been documented by elevated content of protein clarin in cell periphery [14–17] and by the data of study that showed that dextran-coated nanocomplexes are not receptor-dependent [17].

So, presently there are no final decisions concerning toxic effect and mechanism of biological action of stabilized ferromagnetics on malignant cells. Also, there is no data about ultrastructural and functional changes in tumor cells after the influence of ferromagnetics. That's why we have performed a study of ultrastructure and some functional in-

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dexes of tumor cells treated in vitro by stabilized iron nanoparticles.

MATERIALS AND METHODS

The objects of our study were wild type, cisplatin-resistant and doxorubicin-resistant human breast cancer cells (MCF-7 cell line) and human ovarian cancer cells (A2780 cell line). Original MCF-7 and A2780 cells were cultured in modified Dulbecco IS-COVE medium (Sigma, Germany) supplemented with 10% fetal calf serum ("Sangva", Ukraine) at 37 °C in 5% CO_2 atmosphere. The cells were passaged twice per week at the density of 2–4 x 10^4 cell/cm^2. The resistant variants (cisplatin-resistant MCF-7/DDP and A2780/DDP) and doxorubicin-resistant (MCF-7/Dox, A2780/Dox) cells were generated by culturing original cells in the presence of increasing concentrations of cisplatin or doxorubicin. Each two months, cell resistance was analyzed by proliferative method with the use of 3-[4,5dimethylthiazol-2-1]-2,5-diphenyltetrazolium bromide (MTT) method. At the time of experiment, the resistance degrees of MCF- 7 cells yielded 4 for cisplatin and 8 for doxorubicin, and 5 and 8, respectively , for A2780 cells .

We used magnetite (Fe_3O_4) synthesized by the method of electron-beam evaporation and Fe_3O_4 and NaCl deposition in vacuum. The stabilization of ferromagnetic was carried out by adding polydextrane-90 and phosphatidylcholine/cholesterol during dispersion. Size of stabilized particles was determined using laser correlation spectrometry. Iron concentrations (mg/ml) were measured by atomic-absorption spectroscopy [18].

The cells of studied lines were cultured with stabilized ferromagnetic at concentration of 100 μg/ml for 1, 2 and 3 days. For electron microscopy study, the cells were fixed for 1 h in 1.6% glutaraldehyde solution prepared in 0.1 M cacodylate buffer (pH 7.3). To remove glutaraldehyde, the cells were washed in 0.1 M cacodylate buffer for 16–18 h. To achieve optimal isotonic quality of fixing and washing buffers, cacodylate buffer was supplemented with saccharose (50 mg per ml). Post-fixation of the cells was done in 4% osmium tetroxide, with further dehydration in alcohols and placement in araldite by standard method [19]. Ultrathin slides prepared with the use of LKB-8800 ultratome, were contrasted with uranyl acetate and plumbous citrate and examined using electron microscope JEM-100B at accelerated voltage of 80 kV.

The rate of oxygen uptake by the cells was determined by polarographic method with the use of closed Clark's electrode. In polarographic well, the abovementioned medium for cell incubation saturated with oxygen, was placed. The temperature of incubation medium was 24 °C. Oxygen uptake rate was expressed in nanoatoms of O_2/min/1 x 10^6 cells. Statistical analysis of obtained data was performed using Student's t-test.

RESULTS

In our previous studies it was shown that acquirement of resistance to cisplatin and doxorubicin in human ovarian cancer cells of A2780 line and human breast cancer cells of MCF-7 line is accompanied by ultrastructural changes of the cells [20, 21]. For drug resistant cells the presence of well developed granular endoplasmic reticulum with long channels, and 2–3 Golgi apparatus loci was typical. Close to Golgi apparatus, a significant quantity of multivesicular bodies was always present. Resistant cells have a nucleus of irregular shape with invaginations and active nucleoli located near nuclear membrane that could be considered as a pattern of activated metabolism in the cells that acquired drug resistance. A lot of microtubules, and microfilaments in form of bundles of various thickness appeared in resistant cells of A2780 and MCF-7 lines.

So, A2780 and MCF-7 cells resistant to doxorubicin and cisplatin possess more developed cellular complex connected with elements of cytoskeleton, and complicated ultrastructural organization compared with wild type cells that evidences a higher degree of their differentiation.

After 1 day of incubation with stabilized dispersed Fe_3O_4 in culture medium one could observe the changes of ultrastructural organization in all studied cell lines. In cytoplasm phagosomes with ferromagnetic granules appeared (Fig. 1, *a*). Some part of phagosomes acquired more complex structure being united with lysosomes and transformed into phagolysosomes. Only in insignificant part of the cells no ferromagnetic was present in cell cytoplasm. The number of cells with necrotic patterns was insignificant, and was characterized by the presence of a large quantity of accumulated granules of ferromagnetic nanoparticles in the content of multiple phagolysosomes. We have detected that ferromagnetic nanoparticles were localized only in the cytoplasm of studied cells but not in cellular organelles. Meanwhile, according to the data of literature, TiO_2 nanoparticles were accumulated in cell mitochondria and nucleus [22].

Ultrastructural organization of wild-type cells in contrary to resistant ones was also practically not altered. In A2780/S and MCF-7/S cells one could observe just an elevation of endoplasmic reticulum channels number with a large quantity of ribosomes on the outer membrane and elements of Golgi apparatus (Fig. 1, *b*), while in the cells of resistant lines the increased quantity of cell organelles could be detected. Altered ultrastructure of all studied lines evidences functional loading of this cell organelle in response on the presence of Fe_3O_4 dispersion.

In all studied sublines the changes of cell shape detected by light microscopy examination, as well as altered ultrastructural organization have been observed. The cells acquired a more round shape with the formation of cytoplasm protrusions that promoted cell contact perturbation.

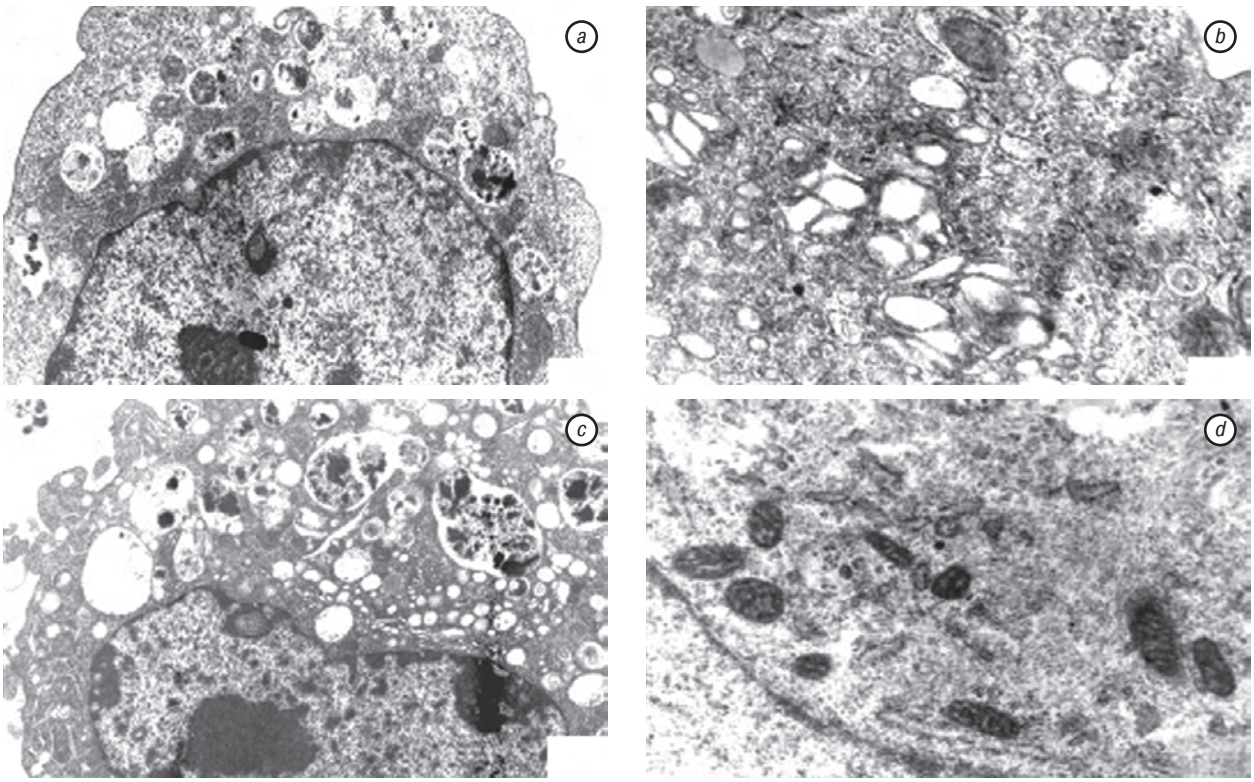

Fig. 1. Ultrastructure of tumor cells after 1 day of exposure to stabilized Fe_3O_4 dispersion: *a*, — sensitive to antitumor drugs A2780 human ovarian cancer cells, X10000; *b*, — sensitive to antitumor drugs MCF-7 human breast cancer cells, X10000; *c*, — resistant to doxorubicin A2780/Dox human ovarian cancer cells, X10000; *d*, — presence of single microfilament bunches in cytoplasm of sensitive MCF-7 human breast cancer cells, X20000

The most damaged organelles seemed to be mitochondria that possessed small dimensions with electron-dense matrix. These alterations of mitochondrial structure indicate a significant functional failure of these organelles and have been supported by the data of experiments on the rate of oxygen uptake by mitochondria. Altered structure of mitochondria may be caused by release of free radicals by iron present in nanoparticles that leads to inhibition of functional activity of mitochondria and some other cell organelles [23].

One of the characteristic features of resistant sublines was the presence of significantly larger numbers of phagosomes that were composed from conglomerates of electron-dense ferromagnetic granules of various forms and in different quantity (Fig. 1, *c*). It could be related to quicker accumulation of ferromagnetic in resistant cells compared with sensitive ones or with the functional state of resistant cells that possess more complex structural organization with the presence of significantly higher quantity of cell organelles that leads to rapid formation of complex phagolysosomes.

Intact resistant cells of A2780 and MCF-7 sublines are characterized by the presence of large numbers of microtubules, microfilaments, that form the bundles of different thickness localized near nucleus invaginations in A2780/Dox cells, or plasma membrane in A2780/DDP cells. So, drug resistant cells possess well developed cell complex associated with elements of cytoskeleton. However, after action of ferromagnetic cytoskeleton structural regularity became disturbed or the components of cytoskeleton underwent deorientation which is illustrated by the presence of only few small bundles of microfilaments localized in cytoplasm of studied cells (Fig. 1, *d*). It could be related to an appearance of phagolysosomes which, as it is known, move in cells along microtubules with the help of microfilaments and fibrilla. Phagosomes are bound to cytoskeleton filaments with the use of motor proteins (like myosin, kinesin, or dinein), which leads to ATP release and promotes transfer of phagolysosomes containing ferromagnetic, inside the cells [24]. Possibly, exactly the presence of large quantity of phagolysosomes leads to re-distribution of cytoskeleton elements that is required for cell functioning in altered conditions. Apart from this, ferromagnetic may cause alterations in cytoskeleton structure and function via direct influence on the structure of proteins that form cytoskeleton or indirectly affect cell functional state. Altered cell shape upon ferromagnetic action could also be caused by the reconstruction and the changed orientation of main elements and proteins of cytoskeleton, first of all, actin that is the main component of cell cytoskeleton and is responsible for cell shape, migration and distribution [25].

Ultrastructural study of cells treated with ferromagnetic for 2 days allowed to detect its elevated accumulation in the cells of all studied lines (Fig. 2, *a*). Ultrastructural patterns of cells were practically unaltered except for the formation of much higher numbers of phagolysosomes of larger size. It's necessary to note that in the cells of studied sublines further increase of apical part of cytoplasm could be

observed, which could be explained by larger total volume of nanoparticles accumulated by the cells. The structure of mitochondria remained altered especially in MCF-7/Dox cells (Fig. 2, *b*).

After 3 days of incubation of cells with ferromagnetic dispersion, a gradual decrease of its accumulation in the cells of all studied sublines has been detected (Fig. 3, *a*). Ferromagnetic has been removed from the cells by exocytosis of phagolysosomes in intercellular space, as well as by clasmacytosis (separation of small parts of cytoplasm containing large quantities of ferromagnetic) (see Fig. 2, *b*). At the third day of incubation all studied sublines showed significant number of blebs of various sizes with different quantity of ferromagnetic located on cell membrane. The number of blebs elevated along with the increase of incubation period with nanoparticles.

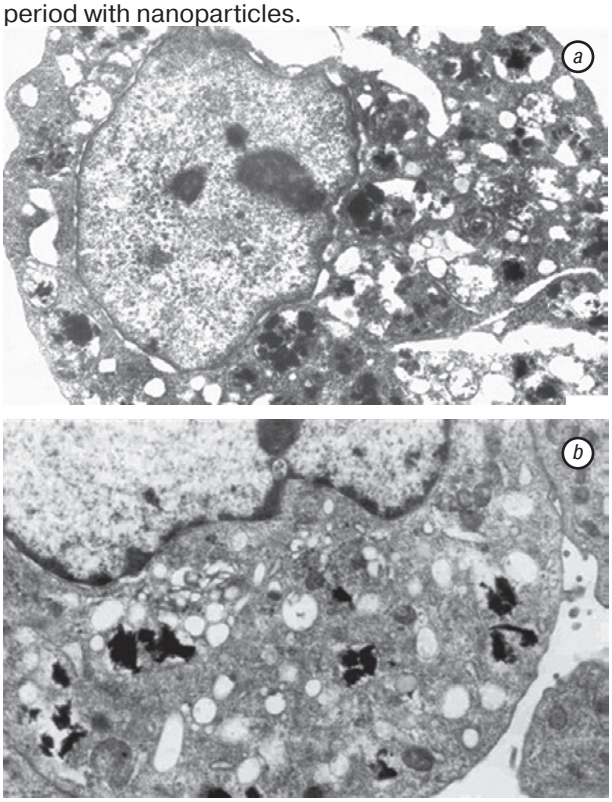

Fig. 2. Ultrastructure of tumor cells after 2 days of exposure to stabilized Fe_3O_4 dispersion: *a*, — resistant to doxorubicin human ovarian cancer A2780 cells, X10000; *b*, — resistant to doxorubicin human breast cancer MCF-7 cells, X10000

Among mechanisms of toxic action of small sized nanoparticles one should mention their ability to damage mitochondria that are indicators of cell functional state most sensitive to the action of damaging factors. It is known that among early signs of cell damage there are alterations of mitochondrial ultrastructure determined as formation of electron-dense mitochondria with weakly contoured crists, and their vacuolization. Exactly such structure of mitochondria was observed by us in the cells treated with nanocomplexes (Fig. 3, *c*).

After 8 days of cultivation of MCF-7 cells in standard medium that replaced ferromagnetic after 3-days incubation, such altered ultrastructure of mitochondria still could be observed in some cases.

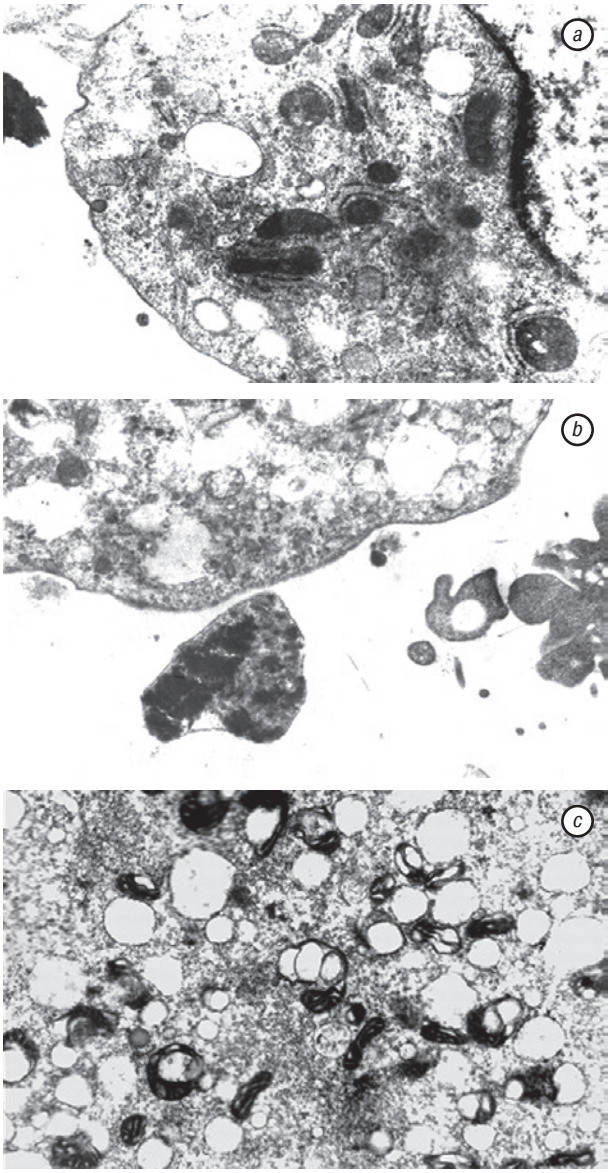

Fig. 3. Ultrastructure of tumor cells after 3 days of exposure to stabilized Fe_3O_4 dispersion: *a*, — resistant to doxorubicin human ovarian cancer A2780 cells, X10000; *b*, — sensitive to antitumor drugs A2780 human ovarian cancer cells, X10000; *c*, — severe damage of mitochondria structure in resistant to doxorubicin human ovarian cancer A2780 cells, X15000

It is known that 90–97% of oxygen in cells is metabolized by mitochondria [26]. Taking into account that many complexes of mitochondrial respiratory chain contain iron [27], we have studied an influence of stabilized nanoferromagnetic on the rate of oxygen uptake by MCF-7/S, MCF-7/ DDP and MCF-7/Dox cells. It has been revealed that intact MCF-7/Dox cells uptake oxygen somewhat more rapidly than original MCF-7/S cells ($0.05 < p < 0.1$; Table). This fact is in accordance with our earlier data obtained on rat tumor cells [28]. Interestingly enough, 48 h incubation with ferromagnetic resulted in decreased rate of oxygen uptake by the cells, but at different degree in different cell sublines (see Table). After incubation with ferromagnetic the rate of oxygen uptake by MCF-7/S cells significantly decreased (by 33%; $p < 0.05$), while in MCF-7/DDP and MCF-7/Dox cells it decreased by 10 and 21%, respectively. This fact evidenced the re-

sistance of MCF-7/DDP and MCF-7/Dox cells not only to some cytostatics, but also to some other agents, for example, to ferromagnetics of certain composition and size. These data are important for further studies of effective antitumor nanocomposites designed for use *in vivo*.

Table. Oxygen uptake rate in MCF-7/S, MCF-7/ DDP and MCF-7/Dox cells after their incubation for 48 h with ferromagnetic stabilized with dextran and phosphatidylcholine (n = 5). Oxygen uptake rate was expressed in nanoatoms of O_2/min/1 x 10^6 cells

Treatment	Cell line		
	MCF-7/S	MCF-7/DDP	MCF-7/Dox
Control	10.8 ± 0.2	10.3 ± 0.7	12.2 ± 1.0
After 48 h incubation with ferromagnetic	7.2 ± 0.4*	9.3 ± 1.2	9.6 ± 0.7

Note: *difference is significant compared with respective control ($p < 0.05$).

In conclusion, it has been shown in our study that cultivation of cells with stabilized Fe_3O_4 dispersion leads to intracellular accumulation of ferromagnetic nanoparticles. The most active ferromagnetic uptake by wild type cells have been observed after 24 h and 48 h of incubation. Resistant cells accumulated iron more slowly and its maximal concentration was registered after 48 h. The presence of ferromagnetic in culture medium has led to altered mitochondrial structure which caused the decrease of oxygen uptake rate. Also, the decrease of the oxygen uptake rate by the cells that may also promote cytotoxic damage upon treatment with stabilized iron occured. In the large majority of cells after 72 h of incubation ferromagnetic was released from nanoparticles via exocytosis or clasmacytosis. One could detect such ferromagnetic particles in intercellular space. After 72 h in significant number of cells no ferromagnetic particles could be observed. The number of dead cells or cells with severe damage was moderate enough, so cytotoxic action of nanoparticles of stabilized iron toward studied cell lines was minimal. The results of our study allow us to conclude that the presence of ferromagnetic in culture medium leads to alteration of ultrastructural organization and decreased oxygen uptake by mitochondria in the cells sensitive and resistant to anticancer drugs.

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