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CYTOTOXICITY OF DEXTRAN-GRAFT-POLYACRYLAMIDE/ZINC OXIDE NANOPARTICLES AGAINST DOXORUBICIN-RESISTANT BREAST CANCER CELLS

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The toxicity of drugs for chemotherapy and cell resistance to their action are the main obstacles in anticancer therapy. Advances in nanotechnology may offer an alternative to traditional methods of anticancer therapy and overcoming drug resistance. The study was carried out on doxorubicin-resistant MCF-7/Dox breast cancer cells and BALB/3T3 clone A31 as a model of normal fibroblasts with the use of Dextran-graft-polyacrylamide/zinc oxide (D-PAA/ZnO) nanoparticles. Cytomorphological analysis was carried out after cells staining with acridine orange. Immunocytochemical study of Ki-67, p53, Bcl-2, Bax, E-cadherin, N-cadherin, CD44 expression was done. Cytotoxicity of D-PAA/ZnO nanoparticles (EC_{50} = 2.2 mM) against MCF-7/Dox cancer cells but not against normal fibroblasts was demonstrated. The increased expression of proapoptotic proteins, E-cadherin, CD44 and decreased expression of proliferation-associated marker Ki-67 in cancer cells treated with D-PAA/ZnO was revealed. Cytotoxicity of D-PAA/ZnO NPs against MCF-7/Dox cancer cells can be potentially used for elaboration of new approaches to cancer treatment.

K e y w o r d s: zinc oxide nanoparticles, dextran-graft-polyacrylamide, doxorubicin-resistance, breast cancer cells, fibroblasts, cytotoxicity.

Cancer is one of the most deadly diseases in modernity. The main methods of cancer treatment are chemotherapy, radiation therapy and photodynamic therapy [1]. The methods are non-selective and have many side effects. In recent decades, nanotechnologies that offer alternative methods of cancer treatment have developed significantly. Nanoparticles of metals and their oxides are promising candidates for cancer diagnosis and treatment [2].

Zinc oxide nanoparticles (ZnO NPs) are widely used in various fields, including gas sensors, biosensors, cosmetics, optics, solar energy, drug delivery, etc [3]. ZnO NPs have vast implications for medical treatment, molecular identification and imaging. Attachment of nanoparticles to antigen-targeting ligands, such as antibodies, peptides, polymers, liposomes, etc. can target neoplasm antigens with significant similarity and specificity [4]. Zinc oxide NPs themselves can be the carriers for drug delivery due to their high biocompatibility [5].

Zinc is an important structural component of many enzymes and regulatory proteins of cells [6]. This trace element plays an important role in the formation of immunity and protection of cells from malignant transformation [7]. Zinc deficiency initiates cancer cell proliferation, increases the frequency of mutagenesis and decreases the level of p53 [8]. ZnO NPs have higher permeability and accumulation in cancer cells compared to other zinc-containing materials. One of the mechanisms of cancer cells inactivation is the generation of reactive oxygen species (ROS) [8,9]. ZnO NPs were effective against various cancer cells such as hepatocellular carcinoma, prostate cancer, non-small cell lung carcinoma, melanoma, cervix adenocarcinoma, head and neck squamous cell carcinoma, colon carcinoma, breast adenocarcinoma and glioma [10, 11]. Cytotoxicity is mediated by the penetration of zinc oxide nanoparticles into the cytoplasm, their dissociation into Zn^{2+}, the ROS production, which disrupt the functions of the endoplasmic reticulum, mitochondria, proteins

and damage DNA. These processes induce a caspase-dependent apoptosis pathway with a change in *Bcl2*, *Bax*, *chop*, and *p53* genes expression [12].

Free zinc nanoparticles tend to aggregate. Polymeric nanocarriers prevent this, facilitate targeted delivery and increase bioavailability [13]. Previous studies have proven the possibility of loading dextran-graft-polyacrylamide (D-PAA) copolymers with gold, silver nanoparticles and drugs [14-16]. The anticancer activity of the D-PAA/ZnO NPs against cancer cell lines was investigated previously [17, 18]

The aim of the research was to determine the sensitivity of the doxorubicin-resistant breast cancer cell line MCF-7Dox and fibroblasts BALB/3T3 clone A31 to the D-PAA/ZnO NPs nanosystem.

Materials and Methods

Synthesis of dextran-graft-polyacrylamide/zinc oxide nanoparticles. Dextran (Mw = 7.105 g·mol^{-1}) and Acrylamide for Dextran-graft-Polyacrylamide synthesis as well as precursors for ZnO NPs preparation (ZnSO4 and NaOH) were purchased from Sigma-Aldrich (USA).

Star-like copolymer Dextran-graft-Polyacrylamide (D-PAA) was used as a template for ZnO NPs *in situ* synthesis. The branched structure of copolymer provides high stability of nanosystems synthesized *in situ* [19, 20]. The copolymer was synthesized as it was reported in [20]. The molecular characteristics of copolymer: 5 grafted PAA arms to Dextran core; Mw = 1.57·10^6 g·mol^{-1}; Rg = 67 nm, and Mw/Mn = 1.81. D-PAA/ZnO NPs nanosystem by in situ synthesis: $ZnSO_4$ and polymer molecules were dissolved in water. 1 ml of $ZnSO_4$ (0.1 mol·ml^{-1}) was added to 5 ml of D-PAA (2·10^{-3} g·ml^{-1}). Then, in 20 min, 2 ml of 0.1 mol·ml^{-1} NaOH solution was added to D-PAA/$ZnSO_4$ mixture at steering.

Dynamic light scattering (DLS). DLS of samples was carried out using the NanoBrook Omni particle size analyzer (Brookhaven Instruments, USA) with 4 mW He-Ne laser at a wavelength of 534 nm. The scattering was measured at 173 degrees (backscattering). All samples were kept at 25°C for 5 min before measurements to achieve equilibrium. For DLS analysis, the algorithm implemented in Python 3.9 programming language was used to estimate particle size distribution (PSD).

In vitro cytotoxicity. The investigation was carried out on the doxorubicin-resistant breast cancer cell line MCF-7/Dox and fibroblasts BALB/3T3 clone A31. Cell lines were obtained from cell bank lines of human and animal tissues of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Science of Ukraine. Cytotoxicity tests were performed in 96-well plastic tablets (SPL, Korea). Cells were incubated in DMEM (Biowest, France) with L-glutamine, 10% fetal bovine serum (Biowest, France) and 40 mg/ml gentamicin (Biowest, France) at 37°C in a humidified atmosphere with 5% CO_2. 10,000 cells were added to each well and incubated for 24 h. DMEM was replaced with fresh medium containing different concentrations of D-PAA/ZnO NPs. Nanosystems were added to the incubation medium by the method of two-fold dilutions. Zinc concentration range was 0.05-0.000781 M. The cells were kept with ZnO NPs for 48 h. Untreated wells with cells (an equivalent amount of distilled water was added as a D-PAA/ZnO NPs solvent) were used as positive controls, and wells without cells were used as negative controls. Incubation medium was removed and 50 µl 0.5% of crystalline violet in 70% methyl alcohol was added to each well and stained for 10 min. Residual dye was removed by three times washing with distilled water. The optical absorption of the wells was measured using a tablet spectrophotometer (Labsystems Multiskan PLUS, Finland) at 540 nm. Calculation of the relative change in the number of cells was calculated according to the formula:

$$CD = (1 - D_s/D_c)\times 100\%,$$

where *CD* is a relative change in the cells number, %; D_s is the absorbance at 540 nm of an experimental sample; D_c is the absorbance at 540 nm of a control sample. Based on the results, "dose-effect" curves were constructed and EC_{50} were calculated according to the Hill equation [22]. The studies were repeated three times.

Cytomorphological studies. Acridine orange staining was used for morphological changes of cells. Cells were incubated with D-PAA/ZnO NPs for 24 h. Zinc concentration was EC_{50} calculated for MCF-7/Dox. Acridine orange stock solution 1 mg/ml was prepared. The final dye concentration in the staining solution was 0.002 mg/ml. Cells were stained for 1 min and washed for 1 min. All solutions were prepared based on saline. Fluorescence studies were carried out on an Olympus BX53 microscope (Tokyo, Japan) with luminescence block XCite Series 120 Q and the Olympus DP72 camera. Fluorescence excitation was carried out by blue light filter. All samples were tested under the same condi-

tions. The exposure time was 10 ms, the red:green ratio was 2:1. At least 10 fields of view were photographed for each sample. The study was repeated 5 times.

Immunocytochemical studies. Cells were incubated with D-PAA/ZnO NPs (EC50 for MCF-7/Dox) for 24 h. Anti-p53, anti-Ki-67, anti-Bcl-2, anti-Bax, anti-E-cadherin, anti-N-cadherin and anti-CD-44 antibodies (Thermo Scientific, USA) were used for determination of p53, Ki-67, Bcl-2, Bax, E-cadherin, N-cadherin and CD44. The cells were fixed with a mixture of methanol:acetone in the equivalent ratio at -20°C for 2 h. Cells were washed in phosphate buffer and incubated with antibodies for 1 h. UltraVision LP detection system (Thermo Scientific, USA) was used. Visualization was carried out using the DAB Quanto system (Thermo Scientific, USA). The level of protein expression was evaluated by the H-score method:

$$S = 0 \cdot N_0(\%) + 1 \cdot N_1(\%) + 2 \cdot N_2(\%) + 3 \cdot N_3(\%),$$

where S is the H-score index, N_0 is the number unstrained cells, N_1, N_2, N_3 are the numbers of cells with low, medium or high levels of protein expression, respectively [23]. The difference in protein expression between D-PAA/ZnO NPs treated and untreated cells was calculated using the formula:

$$D,\% = (1 - S_s/S_c) \times 100\%,$$

where D,% is a difference in protein expression, %; S_s is an H-score index for sample cells; S_c is an H-score index for control cells. The study was repeated three times.

Statistical analysis. Statistical data processing was carried out using the one-way ANOVA Scheffe test ($M \pm SD$; $P < 0.05$). Data were presented as mean and standard deviation ($M \pm SD$).

Results and Discussion

DSL of the D-PAA/ZnO NPs. DLS results are shown in Fig. 1. PSD for D-PAA in water solution demonstrates an average hydrodynamic radius (R_H) equal to 43 nm (Fig. 1, blue line). For the D-PAA/ZnONPs(SO_4^{2-}) nanosystem, two size fractions were registered (Fig. 1): R_H = 0.5-3 nm and R_H > 240 nm.

The fraction with R_H > 240 nm reviles the aggregation process in nanosystems, since individual D-PAA macromolecule are smaller (Fig. 1, black line). This fraction consists of polymer with incorporated ZnO NPs, which are not represented as separate peaks in PSD due to overlap with the most intense peak of polymer.

The ZnONPs were indentified by optical method, as it was reported in the previous work [17]. Physico-chemical properties and cytotoxicity of doxorubicin-containing nanosystems were reported in previous works [15, 24-26].

In vitro cytotoxicity. Previous studies have shown the absence of cytotoxicity of dextran-graft-polyacrylamide nanocarriers [15-17]. It is known that one of the obstacles to the use of anticancer drug is the acquisition of resistance by cells. Doxorubicin is DNA topoisomerase II inhibitor. This compound is used to treat various types of cancer, but the effectiveness of its use is significantly reduced after cells acquire resistance [27]. Nanomaterials with multiple mechanisms of action can solve this problem. A study was carried out on the doxorubicin-resistant breast cancer cell line MCF-7/Dox. The presence of doxorubicin (Dox) in a free state and in a complex with a D-PAA was slightly toxic to MCF-7/Dox cells (Fig. 2, *b*). The maximum cytotoxicity was 60-70% at a Dox concentration of 0.25 mM. EC_{50} of Dox for MCF-7 breast cancer cell lines is within 1 μM [28]. A high sensitivity of MCF-7/ Dox to the presence of D-PAA/ZnO NPs was revealed (Fig. 2, *a*). EC_{50} was 2.2 mM. Maximum cytotoxicity (close to 100%) was achieved at concentrations above 3 mM. Cytotoxic effects on fibroblasts were significantly lower (Fig. 2, *a*). Cytotoxicity of ZnO NPs depends on the method of synthesis, stabilizing agents, size and shape of nanoparticles [7, 12, 29]. The high cytotoxicity of nanoparticles of 20 nm (EC_{50} was 0.1 mM) to murine fibroblasts was shown, while nanoparticles of 40 and

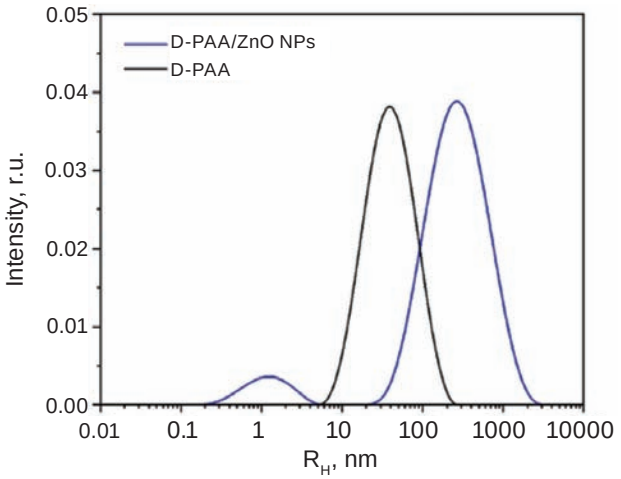

Fig. 1. Dynamic light scattering of dextran-graft-polyacrylamide/zinc oxide nanoparticles (D-PAA/ZnO NPS)

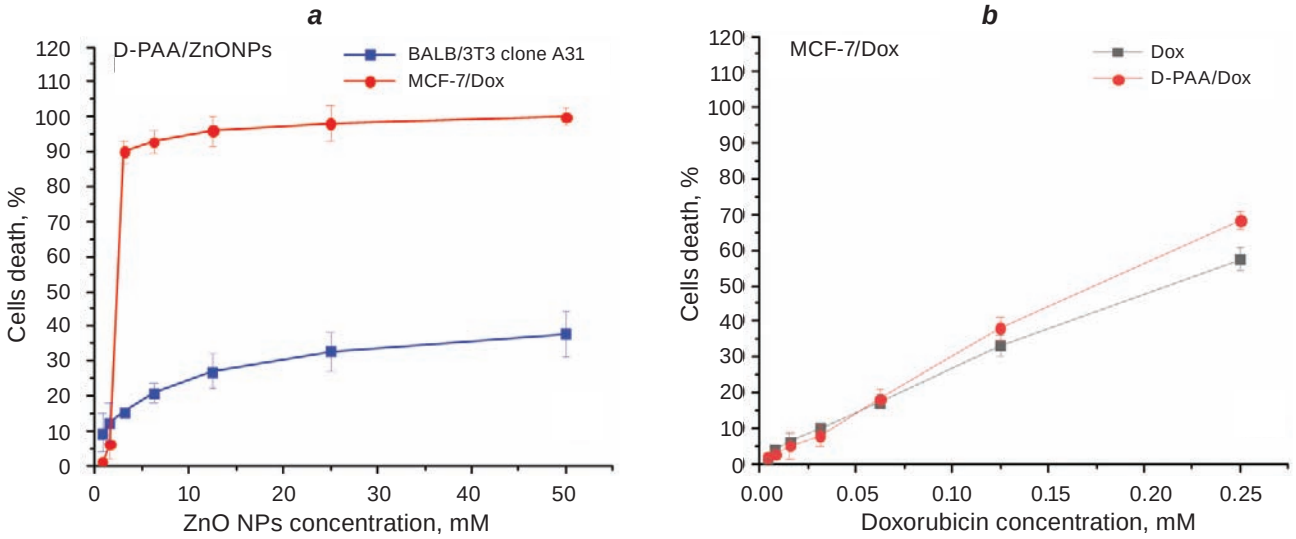

*Fig. 2. Survival of breast cancer MCF-7/Dox and fibroblasts BALB/3T3 clone A31 treated by dextran-graft-polyacrylamide/ZnO NPs (D-PAA/ZnO NPs) (**a**). Toxicity of doxorubicin (Dox) and D-PAA/Dox to MCF-7/Dox cells (**b**); (M ± SD, n = 3)*

80 nm had a much lower effect (EC_{50} was 1 mM) [30]. Other studies have shown selective cytotoxicity against myoblastoma cancer cells C2C12 and 3T3-L1 adipocytes PC-3 prostate cancer and WI-38 lung fibroblasts [31, 32].

ZnO NPs penetrate into the cancer and normal cells at different rates [33]. This is due to the high permeability of the cytoplasmic membrane of cancer cells to ensure intensive metabolism. ZnO NPs can accumulate in lysosomes [12]. Reduced pH to 4.5 promotes faster dissociation of nanoparticles and the release of a large amount of Zn^{2+}. A high concentration of Zn^{2+} promotes the accumulation of denatured and misfolded proteins [34]. Ubiquitin-dependent and other adaptive mechanisms are activated in cells. The accumulation of a large number of proteins with a broken structure and their aggregation initiates apoptosis [35]. We assume that these are determining factors in the different sensitivity of BALB/3T3 clone A31 fibroblasts and MCF-7/Dox breast cancer cells to the D-PAA/ZnO NPs.

Cancer cells drug resistance mechanisms include elevated metabolism of xenobiotics, enhanced efflux of drugs, growth factors, increased DNA repair capacity, and genetic factors (gene mutations, amplifications, and epigenetic alterations). For MCF-7/MDR (multi drug resistance) breast cancer cell line, an increased rate of drug efflux from the cells has been described [36]. This significantly reduces the effectiveness of tumor chemotherapy. ZnO NPs in an individual state and in a complex with nano-

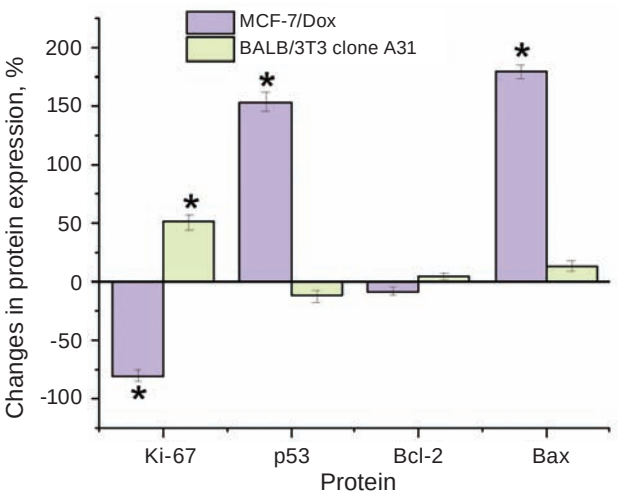

*Fig. 3. Expression of Ki-67, p53, Bax and Bcl-2 in breast cancer cells MCF-7/Dox and fibroblasts BALB/3T3 clone A31 treated with dextran-graft-polyacrylamide/ZnO NPs. Line "0" is basic protein expression (M ± SD, n = 3,*P < 0.05)*

carriers or drugs can significantly increase the effectiveness of treatment and overcome resistance to chemotherapy [37-39].

Thus, the breast cancer cell line MCF-7/Dox, resistant to doxorubicin, was sensitive to the presence of ZnO NPs. The results were proved the possibility of using D-PAA/ZnO NPs nanosystems as an alternative to chemical drugs for cancer treatment.

Immunocytochemical and cytomorphological studies. Expression of apoptosis and proliferation-associated markers p53, Ki-67, Bcl-2 and Bax were analyzed in MCF-7/Dox and BALB/3T3 clone A31 cell lines after treatment of D-PAA/ZnO NPs. A significant upregulation of pro-apoptotic p53 (at 1.5 time) and Bax (at 1.8 time) were found. At the same time, expression of the Ki-67 was downregulated (Fig. 3). Protein expression indicates that the D-PAA/ZnO NPs stimulated initiation of apoptosis in MCF-7/Dox breast cancer cell line.

The expression of p53, Bax and Bcl-2 proteins in the BALB/3T3 clone A31 cell line was not changed. Proliferation marker Ki-67 was upregulated by 50% (Fig. 3).

Cell morphology was evaluated after staining with acridine orange. There were no changes in the cell morphology of BALB/3T3 clone A31 fibroblasts after treatment by D-PAA/ZnO NPs for 24 h (Fig. 4, *1b, 2b*).

Spherical and red-stained cells were not detected. The shape of the nucleus was not disturbed. Incubation of MCF-7/ Dox cancer cells with D-PAA/ZnO NPs nanocomposites promoted the appearance of spherical cells and cells with a disturbed nuclear shape, which are morphological signs of apoptosis (Fig. 4, *2a*). In some cells, external protrusions of the cytoplasm (apoptotic bodies) were found.

The severity of apoptosis and proliferation markers as well as cytomorphological studies showed the selective cytotoxicity and proapoptic effect of D-PAA/ZnO NPs against MCF-7/Dox breast cancer cells. However, we do not exclude other regulated cell death modalities such as autophagy, necrosis or ferroptosis given the significant regulatory role of ROS signaling in these cell death modes.

The expression of E-cadherin, N-cadherin and CD44 was investigated. A significant upregulation of E-cadherin expression (more than 6 times) and downregulation of CD44 (at 40%) for MCF-7/Dox breast cancer cells were found (Fig. 5).

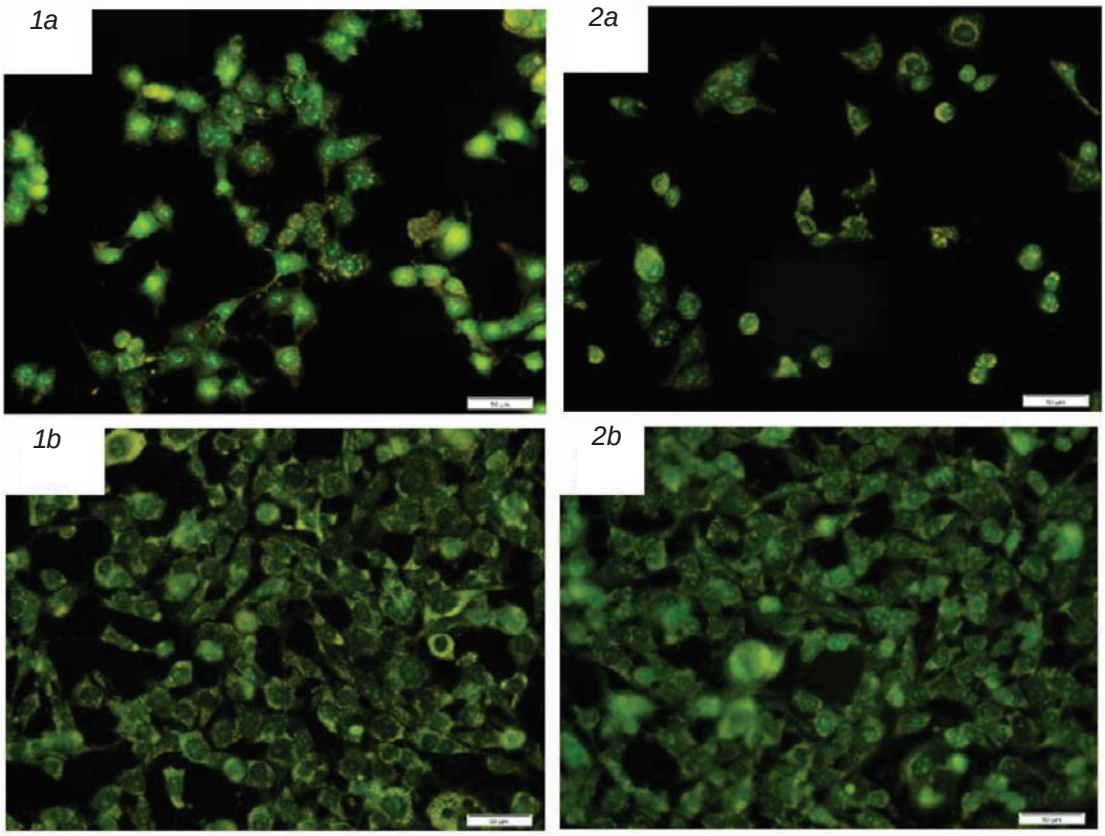

*Fig. 4. Changing of morphology of MCF-7/Dox breast cancer cells (**a**) and BALB/3T3 clone A31 fibroblasts (**b**) treated with dextran-graft-polyacrylamide/ZnO NPs. 1 – control, 2 – incubation with EC_{50} D-PAA/ZnO NPs for 24 h. (BALB/3T3 clone A31 was incubated at EC_{50} for cancer cells; staining with acridine orange 0.002 mg/ml in saline for 1 min; n = 5)*

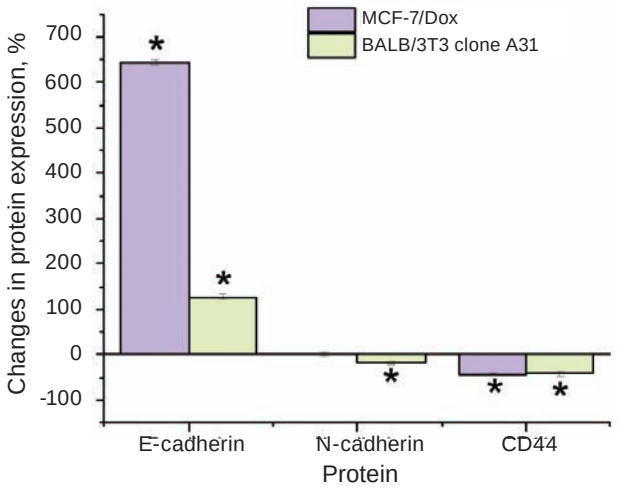

*Fig. 5. Expression of E-cadherin, N-cadherin and CD44 in MCF-7Dox breast cancer cells and BALB/3T3 clone A31 fibroblasts treated with dextran-graft-polyacrylamide/ZnO NPs. Line "0" is basic protein expression (M ± SD, n = 3, *P < 0.05)*

E-cadherin links with catenins forming the E-cadherin/β-catenin/α-catenin complex. Molecule interacts with the actin cytoskeleton, which stabilizes cell interaction, cell polarity and the integrity of epithelial tissue. Disruption of E-cadherin contributes to alteration of the intercellular junction, and increases cell migration ability, invasion and metastasis [40]. For breast cancer, E-cadherin expression is inhibitor of metastasis. Somatic E-cadherin inactivation is associated with an aggressive pattern of breast cancer [41, 42]

Similar changes in the CD44 expression were registered for BALB/3T3 clone A31 fibroblasts (Fig. 5). The functions of CD44 in cancer progression are quite controversial. The protein has many isoforms, which are markers of oncological transformation of cells [43]. The expression of E-cadherin was increased more than 2 times and N-cadherin was decreased by 18%. The functions of cadherins are the formation of intercellular contacts and adhesion to the intercellular matrix. The change in expression of these proteins in cancer cells after influence of D-PAA/ZnO NPs indicates an increase of adhesive properties and potentially decrease of metastatic potential.

Conclusion. Cytotoxicity of dextran-graft-polyacrylamide/zinc oxide nanoparticles (D-PAA/ZnO NPs) was tested *in vitro* on BALB/3T3 clone A31 fibroblasts and resistance of MCF-7/Dox breast cancer cells line to doxorubicin. The nanosystem was low-toxic to fibroblasts. Proapoptotic protein expression for cancer cells was increased after treatment with D-PAA/ZnO NPs. Increased expression of E-cadherin and CD44 was registered for MCF-7/Dox cancer cells after 24 h incubation with D-PAA/ZnO NPs. It indicates about increase of adhesion between cells and decrease of the tendency to metastasize. Thus, our findings indicate the selective cytotoxicity of D-PAA/ZnO NPs against MCF-7/Dox cancer cells and can be potentially used as an alternative to chemotherapy for cancer treatment.

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ЦИТОТОКСИЧНІСТЬ НАНОЧАСТИНОК ДЕКСТРАН-КО-ПОЛАКРИЛАМІД/ ОКСИД ЦИНКУ ПРОТИ ДОКСОРУБІЦИНРЕЗИСТЕНТНИХ КЛІТИН РАКУ МОЛОЧНОЇ ЗАЛОЗИ

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Токсичність хіміопрепаратів і стійкість клітин до їх дії є головними перешкодами в протипухлинній терапії. Нанотехнології пропонують альтернативу традиційним методам

протипухлинної терапії та дозволяють подолати стійкість до ліків. Дослідження проводили на резистентних до доксорубіцину клітинах раку молочної залози MCF-7/Dox і клоні A31 BALB/3T3 як моделі нормальних фібробластів із використанням наночастинок декстран-кополіакриламід/оксид цинку (D-PAA/ZnO). Проведено цитоморфологічний аналіз та імуноцитохімічне дослідження експресії Ki-67, p53, Bcl-2, Bax, E-кадгерину, N-кадгерину, CD44. Продемонстрована цитотоксичність наночастинок D-PAA/ZnO (EC_{50} = 2,2 мМ) проти ракових клітин MCF-7/Dox, але не проти нормальних фібробластів. Виявлено підвищену експресію проапоптичних протеїнів, E-кадгерину, CD44 і знижену експресію асоційованого з проліферацією маркера Ki-67 у ракових клітинах, оброблених D-PAA/ZnO. Цитотоксичність наночастинок D-PAA/ZnO проти ракових клітин MCF-7/Dox потенційно може бути використана для розробки нових підходів до лікування раку.

Ключові слова: наночастинки оксиду цинку, полімерні наноносії, резистентність до доксорубіцину, рак молочної залози, фібробласти, цитотоксичність.

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