Microencapsulated multicellular tumor spheroids as *in vitro* model to study cytotoxicity of stabilized docetaxel-loaded nanoemulsion

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INTRODUCTION

Taxanes (paclitaxel and docetaxel) are widely used for breast adenocarcinoma treatment. Paclitaxel, being a compound number one of a new class of microtubule stabilizing agents, has been hailed by National Cancer Institute (NCI) at the most advanced remedy for chemotherapy of the past 15-20 years (Singla et al., 2002). As for structure and properties, docetaxel and paclitaxel are close analogs. These compounds are able to stabilize cell microtubules preventing them from depolymerization, and thus promoting normal mitotic cell division. However, these compounds were found to possess very low water solubility and other unfavorable properties that limit their clinical application. A number of studies have been carried out to increase taxanes solubility, including chemical modification, co-solvency, emulsification, entrapment in micelles, microspheres, nanocapsules etc. (Singla et al., 2002; Hennenfent and Govindan, 2006). Among the most convenient systems for drug solubilization are stabilized oil-in-water nanoemulsions which are also the most efficient for hydrophobic compound encapsulation and which can be easily prepared.

In recent time *in vitro* models based on cell cultures, including multicellular tumor spheroids (MTS) have been became more important because of limiting the usage of animal models. MTS are 3D models based on animal cells; they can better mimic cell behavior in a small size solid tumor than the cells in a suspension or monolayer (2D) culture. MTS can be obtained by microencapsulation of tumor cells in alginate-chitosan microcapsules as described earlier (Markvicheva et al., 2003). This technique has several advantages over all classical methods, such as liquid-overlay, spinner flask and gyratory rotation systems, since it can provide the production of MTS with the desirable size within the narrow range. More over, some tumor cells can not form spheroids in suspension at all.
The aim of the study was to study cytotoxicity of stabilized docetaxel-loaded nanoemulsion using MTS generated in alginate-chitosan microcapsules as *in vitro* model. .

MATERIALS AND METHODS

Chemicals. Sodium alginate (medium viscosity), NaCl, $CaCl_2 \times 2H_2O$, EDTA were from Sigma, USA. Cultivation medium DMEM (PanEco, Russia); foetal bovine serum FBS (HyClone, USA); 3-4,5-(dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT-assay) (Serva, Germany) were used in the current research. Oligochitosan (Mw 4200 Da, deacetilation degree 87%) was kindly provided by Prof. A. Bartkowiak (Poland). Commercially available cytostatic agent Docetaxel (Beijin Lunarsun Pharmaceutical Co., Ltd.), soybean lecithin Lipoid-S100 (Lipoid), bovine serum albumin BSA (Sigma, USA), Soya oil from Glycine max (Fluka), 5% Dextrose, containing 0.026% NaCl and HCl (pH 3-4) as well as other reagents of special grade were used for docetaxel-loaded nanoemulsion preparation.

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Cell cultivation. In the study human breast adenocarcinoma cells MCF-7 were used. Cells were cultured in DMEM medium supplemented with 10% FBS in 25 cm^2 flasks (Corning Inc.) at 37ºC in a 5% CO_2 humidified atmosphere. The medium was replaced every 2-4 days.

Bioencapsulation of cells. Cell suspension was centrifuged (1000 rpm, 5 min) followed by removing a supernatant. Then 2 ml of sodium alginate solution (1.3% w/v) was added to cell precipitate (6-10×10^6 cells). To obtain Ca-alginate microbeads, this mixture further was extruded by peristaltic pump (0.5 ml/min) to $CaCl_2$ solution (0.5% w/v) using electrostatic droplet generator under following conditions: voltage 6 kV, needle diameter 0.3 mm, distance between the top of the needle and the surface of $CaCl_2$ solution – 1 cm. The obtained hydrogel microbeads were washed 3 timed with physiological saline and then were incubated with oligochitosan solution (0.1-0.3% w/v) at agitation for 5-10 min, in order to form an alginate-chitosan membrane on the microbead surface. Then microbeads were washed 3 times with physiological saline and were incubated in 50 mM EDTA solution for 10 min at gentle agitation, in order to dissolve the microbead core and to obtain alginate-chitosan microcapsules. Then microcapsules were again washed with physiological saline 3 times and once with DMEM.

Cultivation of bioencapsulated cells. Encapsulated cells were cultured in DMEM medium supplemented with 10% FBS in 25 cm^2 flasks (Corning Inc.) at 37ºC in a 5% CO_2 humidified atmosphere. To generate MTS with desired size the cells were cultivated for 2-3 weeks. The microcapsules size and the membrane thickness were determined by light microscopy.

Preparation of nanoemulsion. Crude emulsion containing docetaxel was formulated by stirring the water and oil phases when heating using a high-shear homogenizer (Heidolph, Germany). The stirring rate used for the manufacturing was 10000-13000 rpm. Further decrease of particle size was achieved by high pressure homogenization in APV Homogenizer (Germany) at 1800 bar for 4-5 times. The system was sterilized by filtration (Millipore, d= 0.22 μm) for the further *in vitro* studies.

Cytotoxicity study of docetaxel- loaded nanoemulsion. To study a cytotoxicity of nanoemulsion, the obtained MTS were incubated in DMEM medium in the presence of docetaxel-loaded nanoemulsion or commonly used formulation Taxotere at concentrations of 20, 300 and 1000 nM in 12-well plates for 48 and 72 hs. To analyze a total toxicity of emulsion, placebo emulsion (control 2) was used. Cell viability was determined by MTT assay.

RESULTS AND DISCUSSION

MCF-7 cells were microencapsulated using the electrostatic droplet generator as described above. The usage of the electrostatic droplet generator allowed us to generate MTS with a mean size of 400 μm. It is known that the optimal mean size of MTS is to be 300-600 μm. In the spheroids with the bigger size diffusion limitations for both oxygen and cell culture nutrients could occurred (Coutier et al., 2002). MCF-7 cells were cultured in alginate-chitosan microcapsules for 2-3 weeks and MTS based on MCF-7 cells with the desirable size were obtained (fig.1).

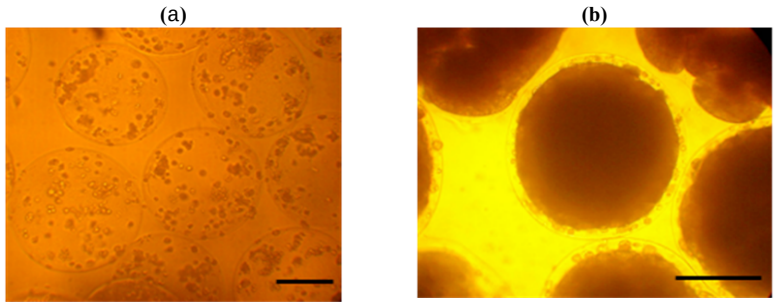

Figure 1: MCF-7 cells within the alginate-chitosan microcapsules immediately after microencapsulation (a) and multicellular tumor spheroids obtained in 28 days of cultivation (b). Scale bar shows 200 µm.

The cell size distribution is shown in figure 2. As can be seen from fig.2 after transferring the microcapsules from physiological solution into the culture medium the mean size increased from 300 µm (fig. 2 a) up to 400 µm (fig.2 b) which could be explained by swelling of the microcapsules.

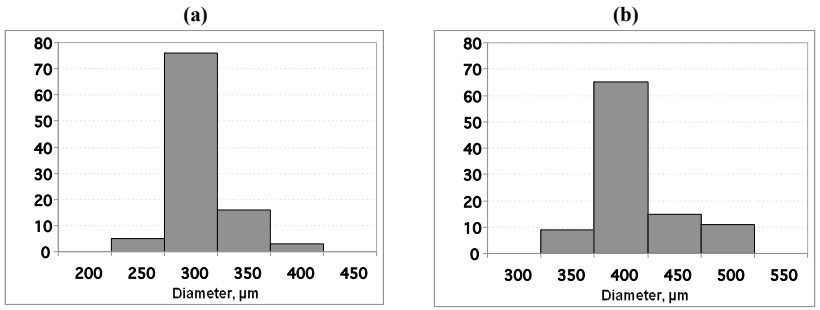

Figure 2: Cells size distribution in physiological saline (a) and in culture medium after 48 hs of incubation (b).

Stable docetaxel-loaded nanoemulsions (diameter bellow 200-300 nm) have been prepared and characterized (Uglanova et al., 2008).

The study of cytotoxicity of docetaxel-loaded nanoemulsion on the MTS compared to a monolayer culture and commonly used Taxotere formulation was performed. The lyophilized nanoemulsion was added both to a monolayer culture and to MTS. The number of viable cells was tested by MTT test after incubation of cells in DMEM with nanoemulsion at 3 various concentrations for 48 hs (fig.3) and for 72 hs. As can be seen in fig.3, cytotoxicity of all 3 tested samples of nanoemulsions was bigger that one of Texotere formulation. After 72 hs incubation MTS and monolayer culture with lyophilized docetaxel-loaded nanoemulsion and Taxotere, the decrease of cell viability was also observed. Thus, in the case of a monolayer culture, the numbers of viable cells were 25, 18 and

12% for samples containing lyophilized nanoemulsion at concentrations 20, 300 and 1000 nM, respectively. For MTS these values were higher, namely 33, 23 и 18%, respectively. Also a number of viable cells in both MTS and monolayer culture after the incubation with docetaxel-loaded nanoemulsion were less than in the case of Taxotere.

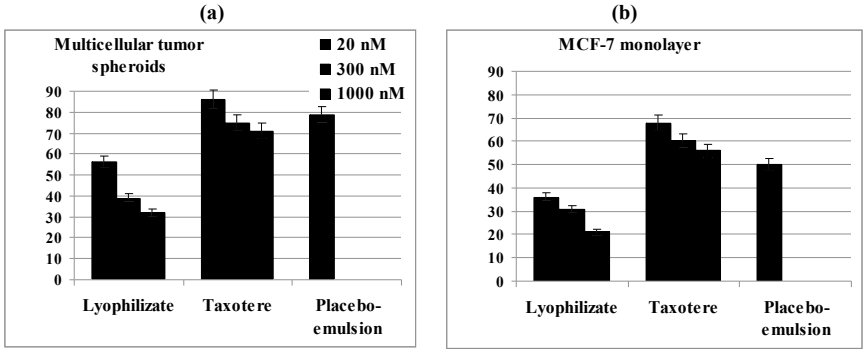

Figure 3: Cell viability of MCF-7 cells in MTS (a) and in a monolayer culture (b) after their 48 hs incubation with docetaxel-loaded nanoemulsions and Taxotere in the culture medium.

CONCLUSION

In vitro comparative study of the effect of docetaxel-loaded lyophilized nanoemulsion on MTS demonstrated higher cytotoxicity of the docetaxel-loaded nanoemulsion compared to Taxotere formulation. More over, MTS were more resistant to the nanoemulsion than the monolayer culture. Thereby microencapsulated MTS can be proposed as a more adequate *in vitro* model for anticancer drug screening.

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