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Sustained Release of the Antitumor Drug Paclitaxel from Poly(3-Hydroxybutyrate)-Based Microspheres

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Abstract—The development of sustained release formulations based on biodegradable polymers is a promising trend in modern pharmacology. Polyhydroxyalkanoates (PHA) attract increasing attention due to their biodegradability and high biocompatibility, which make them suitable for the development of novel drug dosage forms. We have produced poly(3-hydroxybutyrate) (PHB)-based microspheres loaded with the antitumor drug paclitaxel and investigated morphology, drug release kinetics and the effect of these microspheres on tumor cells in vitro. The data on the kinetics of drug release, biocompatibility and biological activity of the biopolymer microspheres in vitro have demonstrated that the studied system of prolonged drug release had lower toxicity and higher efficiency compared to the traditional dosage forms of paclitaxel.

***Keywords*:** poly(3-hydroxybutyrate), paclitaxel, microspheres, sustained release, antitumor.

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INTRODUCTION

The development of novel (bio)polymer-based drug dosage forms is one of priority directions of medicine and pharmacology. There is increasing evidence that drug preparations produced as traditional drug formulations (solutions, tablets, ointments, etc.) for treatment of oncologic, cardiovascular, and infectious diseases do not exhibit all therapeutic potential of the included drug substances and do not eliminate their toxic and negative side-effects. For example, most chemotherapeutic agents used in oncology are characterized by high toxicity, which causes severe complications and decreases the life quality of patients. These include liver, kidney, and hematopoietic stem cell injuries, impairments of blood coagulation, anorexia, etc. Moreover, drug dosage forms containing a drug substance may be also toxic. For example, one of the most widely used antitumor agents, taxol, is a drug dosage form, in which the drug substance, paclitaxel, is included into the polyhydroxyethylated castor oil-based drug form known as cremophor. Paclitaxel is a water-insoluble plant alkaloid exhibiting potent antiproliferative and antitumor effects associated with microtubule disassembly in mitosis. As most cytostatic drugs paclitaxel is characterized by very high toxicity and numerous side effects. However, in the traditional drug formulation not only the drug substance, but also its base, cremophor, may induce unwanted effects, for example, severe allergic reactions [1].

Use of biocompatible polymers as a base for drug formulations may not only eliminate many side and toxic effects but also add some novel characteristics such as prolonged effect of the drug. Gradual release of drug substance from biopolymer microparticles provides prolonged maintenance of necessary concentrations of an acting substance in the body or, locally, in certain organ or tissue. This removes necessity of frequent repeated administration of the drug substance, decreases toxicity and side effects of drug substances and increases their stability and efficiency due to a uniform rate of drug release and its effective consumption. If a biodegradable polymer is a base of the drug formulation it is completely degraded after drug substance release and biodegradation products are then excreted from the body. Now the development of polymer-based drug formulations (PBDF) mainly involves synthetic biodegradable polymers widely used in medicine: polylactides, polyglycolides and their copolymers [2, 3].

Although PBDF possess certain advantages they may also have some limitations including toxicity of a polymeric matrix, biological incompatibility, unwanted side effects of biodegradation products, need for implantation and subsequent extraction of polymeric forms and also higher cost of PBDF compared with traditional drug formulations [4]. Use of

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synthetic polylactides, polyglycolides and their copolymers as PBDF may be also complicated with possible development of chronic inflammatory tissue reaction that can be developed after implantation of these polymers [5–9]. In many cases drug release kinetics from microspheres based on these polymers or rates of their degradation are also unsatisfactory.

We have successfully used bacterial poly-(3-hydroxybutyrate) (PHB) as the antitumor PBDF alternative to polylactides, polyglycolides and their copolymers [9–11]. Earlier we demonstrated that PHB is characterized by high biocompatibility and in vitro hydrolytic and enzymatic degradability in vitro and biodegradability in mammalian tissues in vivo [5, 6, 12]. Importantly, tissue reaction on PHB implantation was lower than that of polylactide [12]. Using this biopolymer we have also developed PBDF containing various drug substances: dipyridamole, indomethacin, levofloxacin, rifampycin, and others. The developed PBDF demonstrated sustained uniform release of corresponding drug substances over several months. It was also demonstrated that drug substance release occurs due to combination of two processes: diffusion of the drug substance for the polymer matrix and PHB biodegradation. In addition, varying conditions of polymer preparation and its molecular mass it was possible to change the rate of drug substance release from the polymer base [7–9, 13, 14]. At the present time, biotechnologically obtained biodegradable and biocompatible polymers (PHB and its copolymers) are actively investigated for subsequent use in medicine and pharmacy for the development of medical devices and drug formulations [10, 11].

Thus, the aim of this study was to develop PHB-based microspheres containing the antitumor drug paclitaxel, to investigate their morphology and kinetics of drug release from these microspheres, biocompatibility and biological activity in vitro using a human breast cancer cell line in comparison with the traditional drug formulation of Paclitaxel.

MATERIALS AND METHODS

Materials

Poly (3-hydroxybotyrate) (PHB) used for preparation of biopolymer microspheres was obtained during microbial synthesis using the *Azotobacter clorococcum 7B* strain as the PHB producer [14]. Paclitaxel was obtained from Biomol (USA), chloroform from Ekos-1 (Russia), polyvinyl alcohol (PVA) was obtained from MP Biomedicals (USA).

Preparation of PHB Microspheres

18 PHB microsphere samples have been prepared by the method of single-step emulsification and solvent evaporation as described earlier [4]. The method was adopted for paclitaxel encapsulation. A solution of paclitaxel and PHB of molecular mass 255 kDa (1 : 4) in 8 mL chloroform was gradually added to 100 mL of 1.5% PVA in distilled water (w/v) under stirring, which was carried out at 1000 rpm for 2 h using a mechanic stirrer RZR 2021 (Heidolph, Germany). After complete evaporation of the organic solvent microspheres were separated by centrifugation at 4400 rpm for 6 min using a 5702 R centrifuge (Eppendorf, Germany) and then they were washed three times for complete removal of the emulsifier and paclitaxel from the sphere surface. These microspheres were then dried in a thermostat at 37°C.

Study of the Size of PHB Based Microspheres and Paclitaxel Content in Them

A mean diameter and standard deviation of the prepared microsphere samples were determined by microphotographs obtained by means of a light microscope Biomed 1 Var. 2 (Biomed, Russia) with a digital ocular MYscope 300M (Webbers, Taiwan).

Paclitaxel content in microspheres was determined spectrophotometrically after their dissolution; light absorbance was measured using a DU-650 spectrophotometer Beckman Coulter (USA) (absorbance maxima at 242 and 278 nm). Results were compared with a control solution of PHB in chloroform and with calibration curves generated using solutions of PHB and paclitaxel in chloroform.

Kinetic study of Paclitaxel Release from PHB-Based Microspheres

The experiment on paclitaxel release from microspheres in vitro was carried out at 37°C in a TS-1/20 thermostat (Russia) in 25 mM potassium phosphate buffer, pH 7.4, containing a small quantity of the emulsifier (0.05% Triton X-100, v/v): four samples of microspheres (20 mg in 4 mL of buffer were mixed using a BioSan shaker at 330 rpm. During the study of paclitaxel release at certain time intervals microspheres were separated from buffer by centrifugation at 14000 rpm using a 5702 R Eppendorf centrifuge and a 4 mL-portion of fresh buffer was added. The content of paclitaxel in the buffer was determined spectrophotometrically using the Beckman Coulter DU-650 spectrophotometer; results read versus phosphate buffer were compared with the calibration curve generated using alcohol-aqueous solutions of various concentrations of paclitaxel. Remaining content of paclitaxel in microspheres was also determined spectrophotometrically after their dissolution in chloroform.

Microscopy

A pilot study of microspheres and their shape was carried out by light microscopy using a microscope Biomed 1 Var. 2 (Biomed) equipped with a digital ocular MYscope 300M (Webbers). Microphotographs

were obtained by the method of scanning electron microscopy (SEM) in electron and ion emissions using microscopes FEI-SMA-QUANTA 200 and SMA QUANTA FEG.

Study of Microsphere Interaction with a Cell Culture in vitro

Microsphere biosafety in vitro was investigated using a MCF-7 human breast cancer cell culture. Cells were cultivated using methods described by Freshni [5]. The effect of a tested agent on cell survival was calculated according to the formula N% = (number of cells in the experiment/the number of cell in control) × 100. Cell survival was evaluated by the MTT-test as the most demonstrative method in experiments with cultures of tumor cells [15].

Various concentrations of microspheres dispersed in a cultivation medium were added to cultivated cells. Before this experiment dried microspheres were sterilized at 100°C for 10 min. The microspheres were tested in quadruplicate parallel assays at the following concentrations: 3 μg/mL, 10 μg/mL, 30 μg/mL, 100 μg/mL, 1 mg/mL, and 3 mg/mL equivalent to 0.3 μg/mL, 1 μg/mL, 3μg/mL, 10 μg/mL, 30 μg/mL, 100 μg/mL, and 300 μg/mL of paclitaxel, respectively. A suspension of empty biopolymer microspheres (3 mg/mL) without the drug substance were used as a negative control. A solution of pure paclitaxel (3 μg/mL) without biopolymer microspheres was used as a positive control. Measurements were carried out every 24 h for 72 h.

RESULTS AND DISCUSSION

Using the method of direct (single-step) emulsification [4, 6] we obtained PHB-based microspheres with encapsulated paclitaxel with the mass of the drug substance in the polymer of 10 ± 1%. The diameter of microspheres was 41 ± 6 μm.

Figure 1 shows photographs obtained by means of light microscopy of microspheres with encapsulated paclitaxel.

For more detailed study of microspheres and the structure of their surface we have used electron scanning microscopy with electron and ion emission (Figs. 1b, 1c). The surface of the resultant spheres was rough with well defined polymer fibrils. Cross-section of a microsphere by an ion beam (Fig. 1d) demonstrated that the microsphere lacks cavities and it is rather homogeneous over the whole cross-sections and only small defects could be seen.

During the next stage of this study we have investigated kinetics of paclitaxel release from 6 microsphere samples of 41 ± 6 μm in diameter. Figure 2 shows a kinetic profile of paclitaxel release into phosphate buffer from PHB microspheres. One can see that on early steps the time-dependence of the drug substance is approximated by a degree function, while at later stages it is described by a linear function with high R^2 value ($R^2 > 0.95$). Similar behavior was also observed for the kinetic profile of microspheres with dipyridamole [6] with exception of the first phase of drug substance release from the polymer matrix, known as the burst effect, which was higher in the case of dipyridamole. Such difference may be obviously attributed to higher affinity of paclitaxel to PHB (due to hydrophobic interactions between the polymer and the drug substance) compared to dipyridamole.

The first stage of drug substance release from microspheres was characterized by predominance of diffusion processes between water buffer and the drug substance, while on later stages diffusion is replaced by hydrolytic degradation of the polymer base thus explaining the linear rate of drug substance release.

In order to confirm existence of degradation of polymer microspheres we have made microphotographs of the investigated system at various stages of decomposition (Fig. 3, on the top—light microscopy, on the bottom—scanning electron microscopy).

Thus, we have demonstrated in vitro gradual hydrolytic destruction of PHB-based microspheres with encapsulated paclitaxel. Degradation of the polymer matrix may be clearly distinguished on day 30 of microsphere incubation, and on day 90 degradation is the dominating process. Thus, it is reasonable to suggest that the final linear stage of drug substance release from microspheres is mainly associated with polymer base degradation, while the initial phase of the release is determined by diffusion of the drug substance from the polymer microsphere.

During the final step of this study we have investigated interaction of PHB-based microspheres with encapsulated antitumor drug substance paclitaxel with the MCF-7 human breast cancer cell culture.

The tumor cells were cultivated with various concentrations of microspheres containing paclitaxel, with empty polymer microspheres (lacking the drug substance) or with the traditional drug formulation of paclitaxel with cremophor, taxol. The results of these experiments are presented as the dependence of cell survival on duration of incubation with microspheres (Fig. 4).

These results indicate: first, polymer microspheres are biocompatible with MCF-7 cells; second, the degree of cell growth suppression directly depends on concentration of added microspheres. Figure 4 shows that empty microspheres did not influence growth and proliferation of cells, while various concentrations of microspheres containing the drug substance caused a concentration-dependent decrease of these parameters. The traditional drug formulation of paclitaxel initially caused potent inhibition of cell proliferation, however, later inhibition of cell growth became smaller. The latter may be associated either with utilization of traditional drug formulation of paclitaxel by

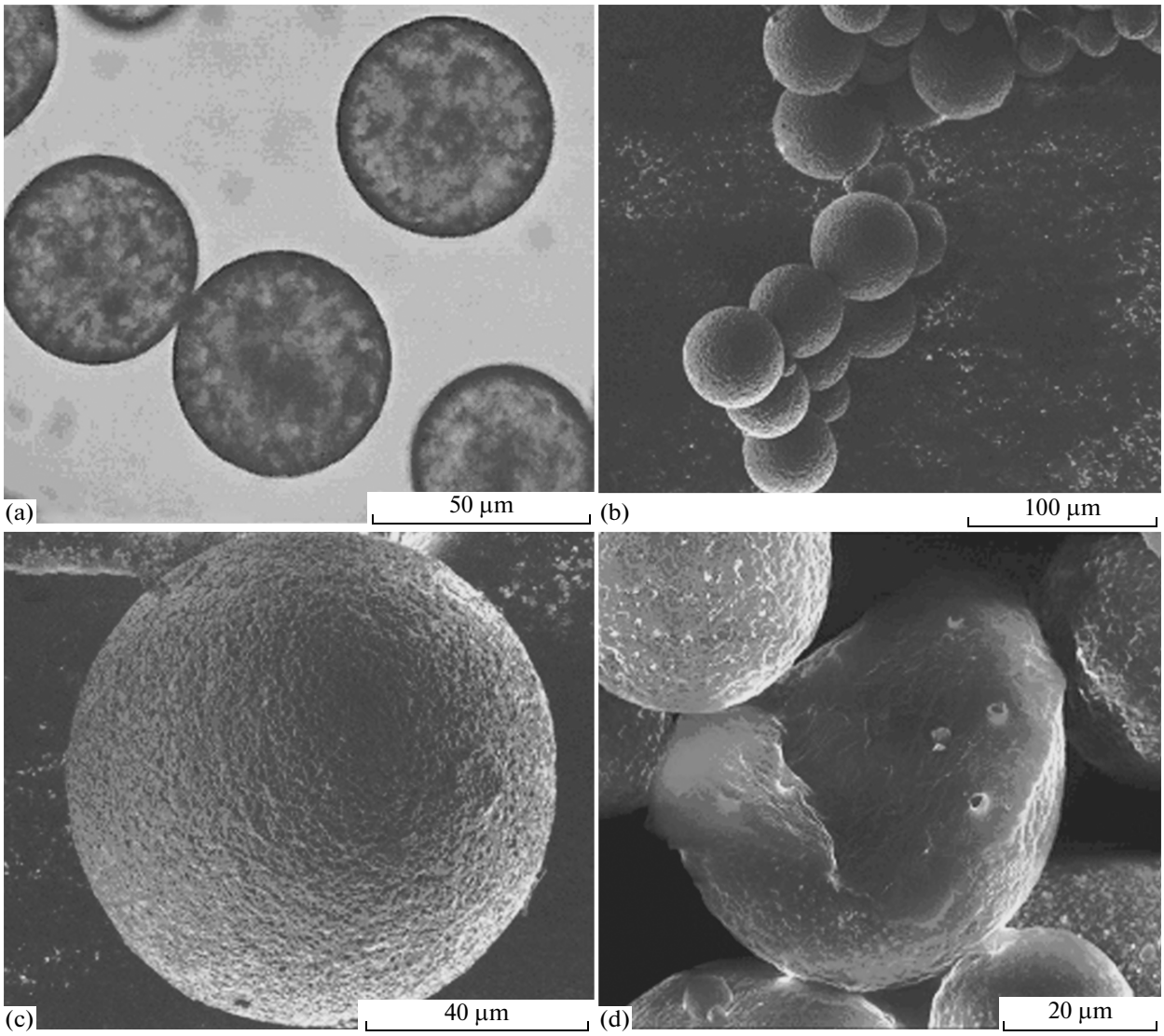

Fig. 1. PHB-based microspheres with encapsulated paclitaxel (a: light microscopy, magnification ×100). Electron microscopy, ion emission. Microspheres with paclitaxel (b, c), miscrosphere cross-section (d).

cells or its degradation in the medium resulting in a time-dependent decrease of its acting concentration accompanied by subsequent increase of the cell growth. The biocompatible polymer microspheres prepared and used in this study obviously provide constant release of paclitaxel into the cultivation medium and promote maintaining of constant acting concentration. This increases microsphere efficiency compared with the traditional cremophor-based drug formulation.

Results of these studies are consistent with results of studies of other prolonged antitumor systems based on microspherese prepared using copolymers of polylactides and polyglycolides. Efficiency of polymer based drug systems was investigated in vitro using various cell cultures. It was demonstrated that microspheres with encapsulated antitumor drug substances caused prolonged and effective suppression of growth of tumor cells. Inhibition of growth of tumor cells was demonstrated using various cell cultures of hepatic cancer [16], glioma C6 [17], breast cancer MCF-7 cells [18]. In the case of prolonged suppression of tumor cell growth (from 12 h to 4 days) the biopolymer system exhibited higher effectiveness than aqueous solution of the antitumor drug substance: the traditional drug formulation of the antitumor drug substance suppressed cell growth for up to 12 h, while biopolymer microspheres with the encapsulated antitumor drug substance suppressed tumor growth up to 4 days. Due to certain problems related to time limits of cell cultivation [17] it is hard to investigate correctly longer periods of suppression of cell tumor growth. Effectiveness of polymer microspheres with encapsulated norcantaridine [16], temozolomide [17], and

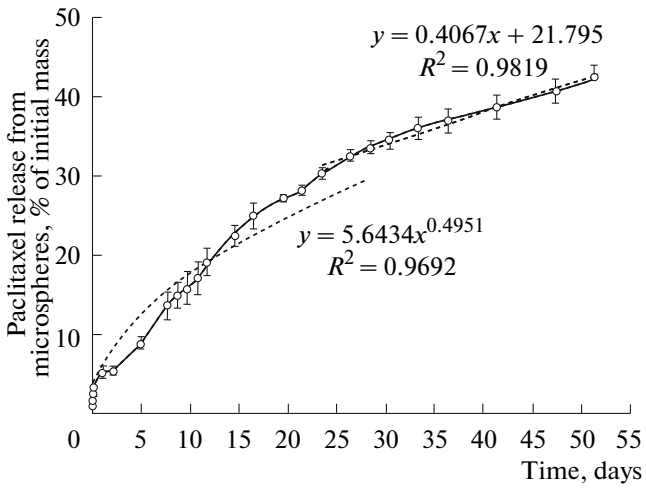

Fig. 2. Kinetics of paclitaxel release from PHB-based biopolymer microspheres.

tamoxifen [18]. However, kinetics of release of these antitumor agents is not ideal in terms of both duration and the kinetic profile. Duration of drug substance release from these microspheres did not exceed 1 month and in all the cases early steps of drug substance release were characterized by the burst effect; the latter may represent serious limitations for the development of an antitumor drug formulation system. Our biopolymer antitumor systems are characterized by effective prolonged antitumor effect due to sustained unimodal drug substance release from microspheres and also due to high biocompatibility of the polymer drug form (microspheres without drug substance).

CONCLUSIONS

In the present study we have investigated the biopolymer drug formulation for sustained release of the antitumor drug substance paclitaxel and kinetics of its release from microspheres. We have demonstrated here biocompatibility and biological activity in vitro of the drug formulation towards human breast cancer cells. Results of this study suggest that sustained release of paclitaxel from PHB-based microspheres due to drug substance release from the polymer matrix and hydrolytic destruction of the polymer causes suppression of proliferation of breast cancer MCF-7 cells. In contrast to the traditional drug formulation of paclitaxel this effect was directly depended on concentration of microspheres and duration of their incubation with cells. Thus, the developed polymer systems

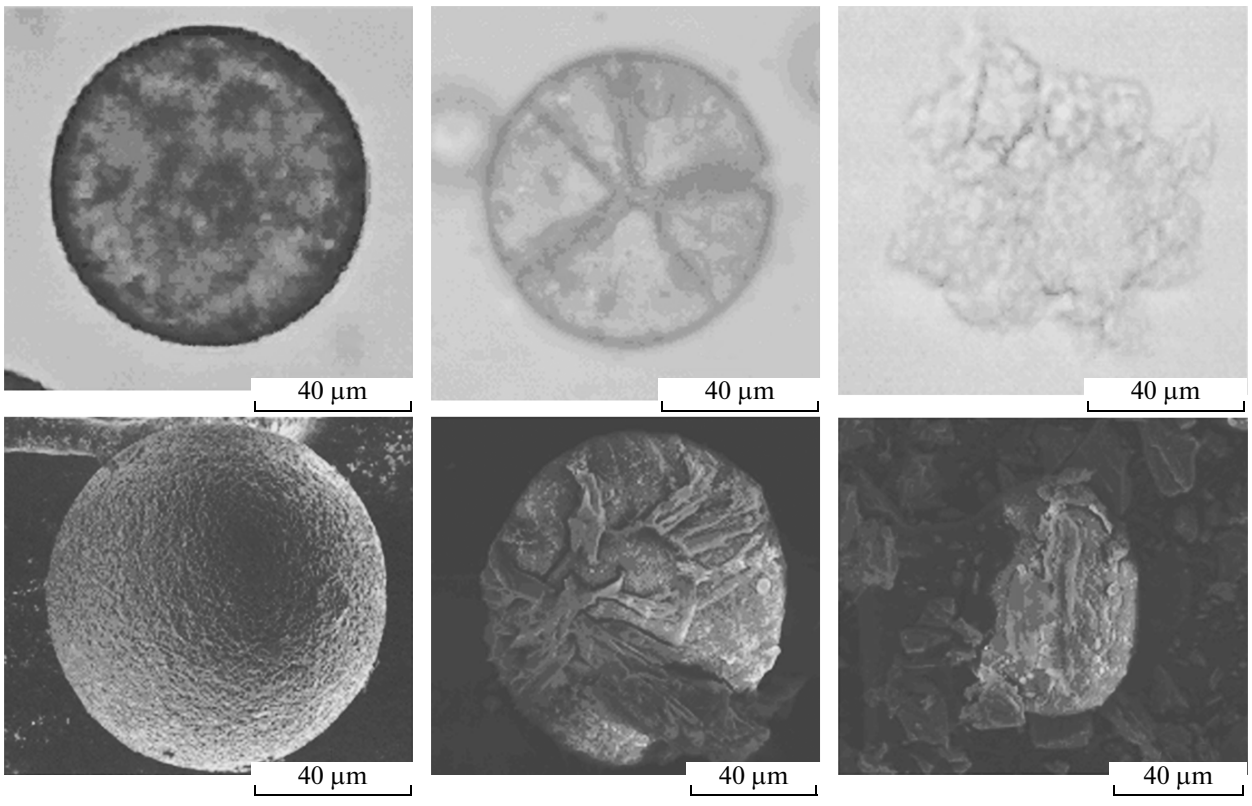

Fig. 3. Hydrolytic degradation of PHB-based biopolymer microspheres with the encapsulated antitumor drug paclitaxel: photographs from left to right 1 day, 30 days, 90 days; on the top—light microscopy, on the bottom—scanning electron microscopy, ion emission.

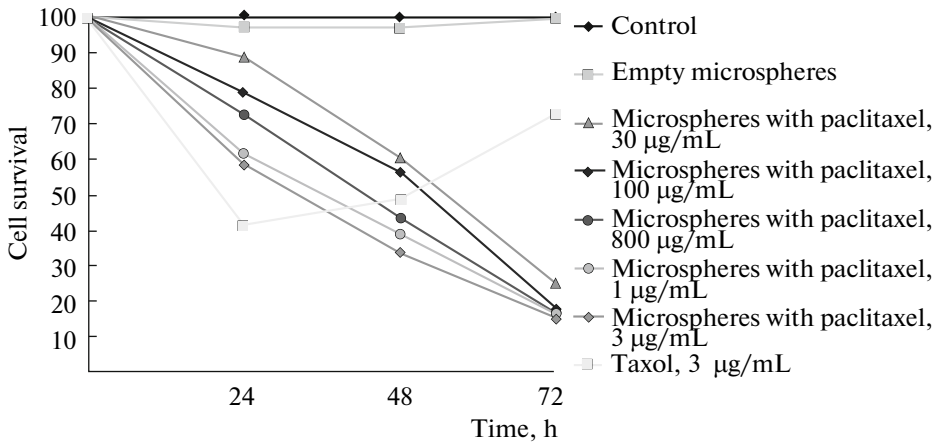

Fig. 4. The dependence of cell survival on duration of their incubation with various concentrations of microspheres. Group designations are shown on the right.

may represent a base for the development of novel antitumor drug preparations of prolonged action.

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