ISSN 1990-519X, Cell and Tissue Biology, 2016, Vol. 10, No. 4, pp. 284–289. © Pleiades Publishing, Ltd., 2016.
Original Russian Text © Yu.S. Gavrilova, N.P. Bgatova, A.O. Solov'eva, K.E. Trifonova, A.P. Lykov, Yu.I. Borodin, V.I. Konenkov, 00000, published in Tsitologiya, 00000, Vol. 00000, No. 00000, pp. 00000–00000.

Target Cells for Lithium in Different Forms within a Heterogeneous Hepatocarcinoma-29 Population

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Received November 2, 2015

Abstract—Liver cancer is an aggressive and heterogeneous human tumor. Lithium compounds block proliferation and induce apoptosis of hepatocarcinoma cells, but cannot cause the death of an entire population of tumor cells. The aim of this study was to reveal morphological types of target cells for different lithium preparations on the basis of their action on hepatocarcinoma-29 cells. The viability of hepatocarcinoma-29 cells was assessed by the MTT test. A dose-dependent decrease in viability was revealed upon addition of native and nanosized lithium carbonate and citrate. Target cells for lithium salts were revealed based on the morphological criteria for five differentiation stages of hepatocarcinoma-29 cells. It was shown that hepatocarcinoma-29 proliferating cells of differentiation stages I and II are the target cells for native and nanosized lithium citrate, while differentiated cells of differentiation stages III and IV are the target cells for nanosized lithium carbonate. It was revealed that hepatocarcinoma-29 cells are more sensitive to nanosized lithium salts rather than to their native forms. This makes it possible to affect tumor growth more effectively.

Keywords: lithium salts, nanosized lithium forms, hepatocarcinoma, target cells

DOI: 10.1134/S1990519X16040076

INTRODUCTION

Hepatocarcinoma is one of the most aggressive tumors in human beings. Despite advances that have been made in diagnostics and treatment, it is in fifth place in prevalence rate and third place by death rate among the pathologies in the world due to its resistance to chemotherapy (Zhou et al., 2015). Heterogeneity of a population of tumor cells is an important factor in the development of resistance to therapy (Cassidy et al., 2015). Tumor heterogeneity may be the result of different causes related to the action of cellular (specifically, genetic or epigenetic) and noncellular factors, for example, stromal factors (Alizadeh et al., 2015; Cassidy et al., 2015). The diversity of tumor cells may also be related to genomic instability (Li, Wang, 2015) and the influence of the microenvironment (Cassidy et al., 2015; Li, Wang, 2015). One theory concerning cancer stem cells (Tsynkalovskii et al., 2008) suggests that the tumor develops as two distinct populations of cells. The first one, which is smaller in size, contains malignant stems calls that are long-living, capable of migration, self-renewal, and differentiation malignant (Ma et al., 2008; Khan et al., 2015). The second is larger; it makes up the majority of the tumor and is represented by partially differentiated tumor cells (Ma et al., 2008). Investigation of the possibility to selectively affect different types of tumor cells is currently important for the development of tumor growth suppression methods.

Methods with different mechanisms of action, including ones affecting the cell cycle, are used in developing targeted therapy methods. Application of lithium salts for apoptosis induction and cell cycle arrest of tumor cells in G2 + M phase is one area of research in this field (Hossein et al., 2012). Lithium structures are considered potential agents of targeted therapy that are able to slow tumor growth. Specifically, it has been shown that application of nanosized lithium carbonate particles blocks hepatocarcinoma growth (Bgatova et al., 2014). However, the authors of that work could not inhibit tumor progression completely. This may be related to hepatocarcinoma heterogeneity (Ma, Chan, 2008).

Accordingly, the aim of this study was to reveal morphological types of target cells for different forms of lithium salts during their action on hepatocarcinoma-29.

MATERIALS AND METHODS

Hepatocarcinoma-29 was created and checked by employees of the Institute for Cytology and Genetics, Siberian Branch, Russian Academy of Sciences (Kaledin et al., 2009). HC-29 cells were incubated in RPMI medium supplemented with 10% of fetal bovine serum in a CO_2 incubator at 37°C. The cells were seeded at a density of 2.0×10^6 cells/1 mL.

[1] *Abbreviations:* HC-29—hepatocarcinoma-29, GSK 3β—glycogen synthetase kinase 3β 3β.

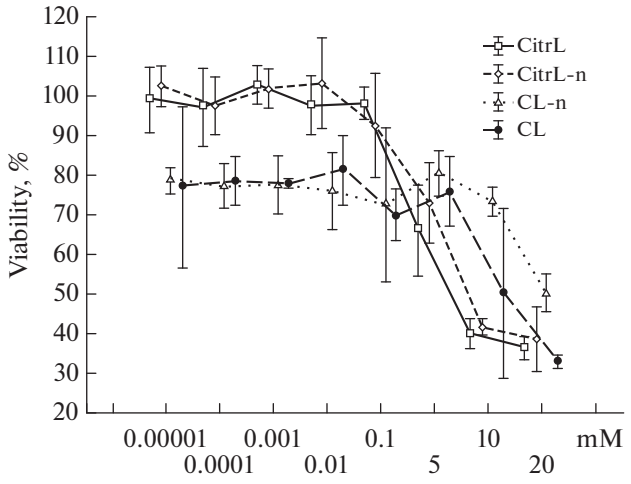

Fig. 1. Viability of hepatocarcinoma-29 upon the action of lithium salts in different forms assessed by MTT-test. CitrL—lithium citrate in native form, CitrL-n—lithium citrate in nanosized form, CL—lithium carbonate in native form, and CL-n—lithium carbonate in nanosized form.

Lithium carbonate (Chemetall, Germany) and lithium citrate (OOO Komponent-Reaktiv, Russia) in native or nanosized forms were used as tumor growth inhibitors. Nanosized forms of lithium salts were made from native forms by mechanoactivation of specimens thereof in an AGO-2C planetary activator mill (Isupov et al., 2013). The size of lithium salts particles was measured by a JEM 1400 electron microscope (Japan) to be 10 nm.

The cytotoxicity of the entities was assessed by MTT-test, which allows one to reveal the number of viable cells by the changes in the solution optic density (Sylvester, 2011). This method is based on the ability of cellular mitochondria dehydrogenases to reduce MTT reagent 3-(4,5-dimethylthiasol-2-il)-2,5-diphenyl-2H-tetrazolium bromide (Sigma, United States) to violet crystal formazan, which is soluble in dimethyl sulfoxide. Consequently, the amount of generated formazan is directly proportional to the number of metabolically active cells, with the intensity of staining directly reflecting their viability.

HC-29 cells were seeded into 96-well culture plates at a concentration of 20–30 × 10^3 cells per well and incubated 24 h at 37°C in a humidified environment with 5% carbon dioxide. These conditions were standard. Lithium compounds were added then to the plates in wide concentration range from 0.00001 mM to 20 mM, and the cells were cultivated with lithium salts for 24 h at standard conditions. Wells with HC-29 cells in culture medium but without lithium compounds were used as control. MTT reagent was added afterward to each well in a final concentration of 250 μg/mL, the cells were incubated for 4 h, and 100 μL of dimethyl sulfoxide (DMSO) was added further to each well. After formazan crystals had dissolved in 60 min, the optical density of the substrate was measured in the wells by a Multiskan FC microplate photometer (ThermoScientific, United States) at 620 nm. The percentage of viable tumor cells was calculated using the formula (optical density of experimental solution/optical density of control solution) × 100%.

We chose two concentrations of the compounds—specifically, 5 and 10 mM—for revealing the morphology of the target cells for lithium salts. No fewer than 40% of cells remained viable at these concentrations. Intact HC-29 cells (control) were used as a control. For light-optical investigation, HC-29 cell suspension was fixed in 4% *para*-formaldehyde solution prepared on Hanks' medium, additionally fixed 1 h in 1% OsO_4 (Sigma, United States) prepared on phosphate buffer, pH 7.4; dehydrated in ethanol solutions of increasing percentage; and embedded in Epon (Serva, Germany). Semifine sections 1-μm thin were made in a Leica EM UC7 ultramicrotome (Germany/Switzerland), stained with toluidine blue, and assessed by a Leica DMEM light microscope (Germany).

Morphometric analysis was done in the ImageJ computer software (Wayne Rasband, United States). Nuclear and cytoplasmic diameters, nuclear and cytoplasmic volumes, and nuclear–cytoplasmic ratio were determined in HC-29 tumor cells. All in all, 756 cells were measured. According to previously obtained data (Bgatova et al., 2015), it is possible to specify five differentiation stages relying on the cell nuclear–cytoplasmic ratio. Mean values (M) and standard deviations for the nuclear–cytoplasmic ratio were calculated for the cells of each differentiation stage. The significance of the differences among designated differentiation stages of HT-29 cells was assessed in the Statistica 6.0 software using the Mann–Whitney U-criterion at a significance level of 95%, meaning $p < 0.05$.

Reagents used: 3-(4,5-dimethylthiasol-2-il)-2,5-diphenyl-2H-tetrazolium bromide and osmium tetroxide (Sigma, United States), as well as Epon (Serva, Germany).

RESULTS

The viability of HC-29 cells was assessed by the MTT-test. The viability decreased in a dose-dependent manner upon the action of different forms of lithium carbonate and citrate (Fig. 1). Given lithium salt concentrations of less than 5 mM, a smaller ratio of viable HC-29 cells was observed if lithium carbonate in native and nanosized forms had been added. A more profound effect on the cells' viability was observed at lithium salt concentrations of 5 and 10 mM, when native and nanosized forms of lithium citrate, rather than lithium carbonate, were used. Specifically, cultivating HC-29 cells with lithium citrate in native form in concentrations of 5 and 10 mM resulted in a content of viable cells of 66 and 40%, respectively (Fig. 1). To reveal morphological types of HC-29 cells underlying the decrease in the ratio of viable cells, lithium salt concentrations of 5 and 10 mM were chosen.

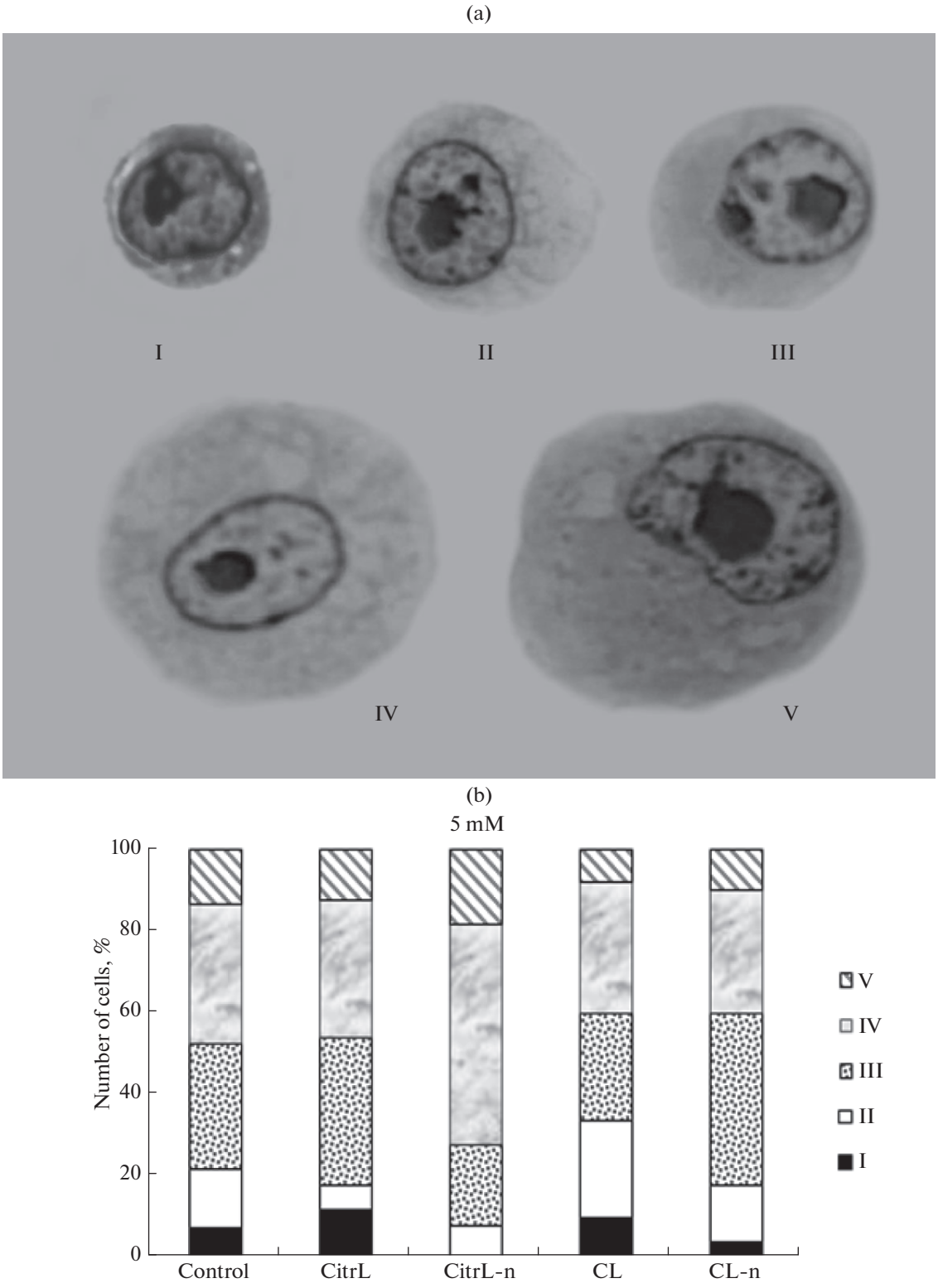

Fig. 2. The distribution of hepatocarcinoma-29 cells by differentiation stages I–V. (A) Morphological types of HC-29 cells in different differentiation stages. (B, C) The ratio of HC-29 cells of different differentiation stages upon addition lithium salts at concentrations of (B) 5 and (C) 10 mM. CitrL—lithium citrate in native form, CitrL-n—lithium citrate in nanosized form, CL—lithium carbonate in native form, and CL-n—lithium carbonate in nanosized form.

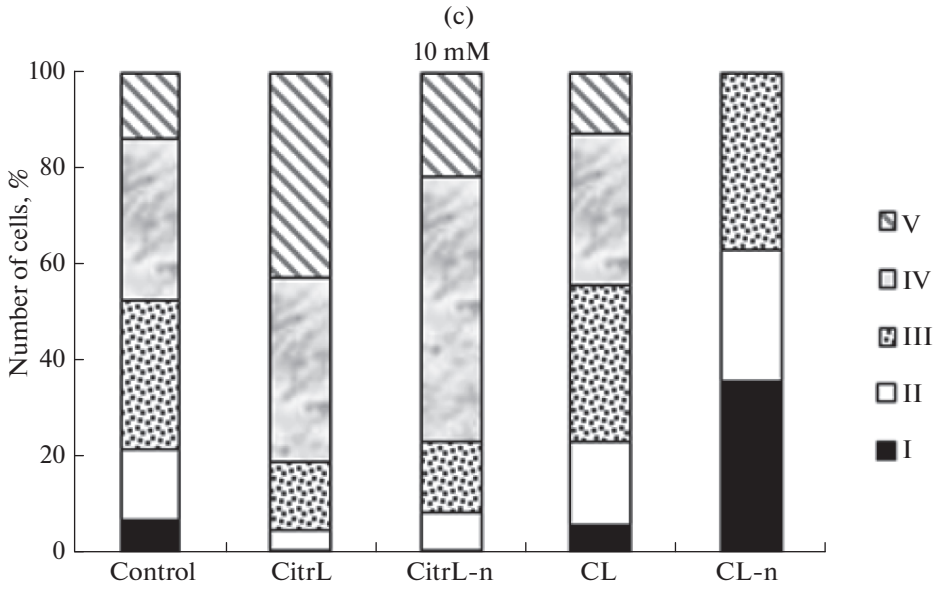

Fig. 2. Contd.

According to previous data on the distribution of hepatocarcinoma-29 cells by differentiation stages and based on defined morphological criteria for five differentiation stages (Bgatova et al., 2015), HC-29 cells of all studied groups were divided into five groups depending on the nucleocytoplasmic index (NCI) value (Fig. 2a). These values were 0.49 ± 0.08 for group I, 0.34 ± 0.03 for group II, 0.25 ± 0.03 for group III, 0.15 ± 0.03 for group IV, and 0.07 ± 0.02 for group V. Analysis of tumor cells distribution by differentiation stages in control group showed that the cells of stage I came to 7.5% of the total cell number; stage II, 14.5%; stage II, 30.8%; stage IV 33.9%; and stage V, 13.2%. Addition of lithium salts changed this ratio (Figs. 2b and 2c).

During cultivation of HC-29 cells with lithium salts at a concentration of 5 mM, there was observed a decrease in the proportion of differentiation stage II cells if the native form of lithium citrate was used and a decrease in the proportion of differentiation stages I, II, and III if the nanosized form of lithium citrate was used (Fig. 3a). The proportion of different HC-29 cell types did not change upon the addition of lithium carbonate in native form at the same concentration. The effect of lithium carbonate in nanosized form consisted in a decrease in the proportion of differentiation stage I cells (Fig. 3b).

A decrease in the proportion of differentiation stage I, II, and III cells took place during cultivation of HC-29 cells with lithium citrate at 10 mM concentration in native and nanosized forms (Fig. 3c). Acting by lithium carbonate in native form did not lead to changes in the proportion of different cell types as compared to control group (Fig. 3d). In view of the data on 50% cell viability upon cultivation with 10 mM lithium carbonate in native form (Fig. 1), cell death did not depend on the differentiation stage. No cells of differentiation stages IV and V were detected upon addition of 10 mM lithium carbonate in nanosized form (Fig. 3d).

DISCUSSION

The results obtained in this study indicate a dose-dependent decrease in viability of HC-29 cells upon their cultivation with lithium salts in different forms.

Utilizing morphological criteria for differentiation stages of HC-29 cells (Bgatova et al., 2015) facilitated revealing the target cells, which were the most sensitive to the action of lithium in different forms. Those were cells of differentiation stages I and II. The percentage of those cells decreased predominantly after addition of lithium citrate in nanosized form at concentrations of 5 and 10 mM. The addition of 10 mM lithium carbonate in nanosized form resulted in elimination of cells of differentiation stages IV and V. It was shown earlier (Bgatova et al., 2015) that HC-29 predominantly consists of diploid proliferating I–III differentiation stages cells, which have maximum NCI values. Cells of differentiation stages IV and V were classified as differentiated cells with limited proliferation potential. Consequently, lithium citrate target cells are predominantly poorly differentiated proliferating cells, while lithium carbonate target cells are HT-29 differentiated cells. The revealed differences between lithium citrate and carbonate in nanosized form and their native forms may be accounted for by

the fact that nanoparticles of a substance have higher reactive capacity than does the initial form of the substance (Tee et al., 2015).

Different mechanisms of tumor cell death upon the action of lithium salts are discussed in the literature. There are data on the effect of lithium on tumor cell proliferation and apoptosis induction (Hossein et al., 2012; Li et al., 2014). The ability of lithium to inhibit glycogen synthetase kinase 3β (GSK 3β) intracellular ferment (Greenblatt et al., 2010; Freland, Beaulieu, 2012; Hossein et al., 2012; Li et al., 2014), which plays an important role in signal pathways involved in carcinogenesis process. Specifically, studying prostate cancer cells revealed that inhibiting GSK 3β increases the sensitivity to TNF-related apoptosis-inducing ligand (TRAIL), which mediates apoptosis in tumor cells (Hossein et al., 2012). It is known that TRAIL protein presented in different human tissues binds to specific DR-receptors—decoy receptors 1–5—and, thus, transmits an apoptosis signal into the cell through activation of caspases 8 and 10 (Lim et al., 2015). It is known from the literature that lithium can block proliferation in tumor cells by arresting the cell cycle in G2 + M phase (Hossein et al., 2012). The ability has been revealed of lithium to affect autophagy processes in tumor cells through inhibition of inositol monophosphatase, a ferment that participates in carcinogenesis (O'Donovan et al., 2015). The revealed morphological types of target cells for different forms of lithium salts may be related to the initiation of different mechanisms of cell death.

Thus, using morphological criteria for five differentiation stages of hepatocarcinoma-29 allowed to reveal the target cells for different forms of lithium salts. Poorly differentiated cells of differentiation stages I and II may predominantly be the target cells of lithium citrate. A decrease in these cells ratio was noted to be more pronounced if lithium citrate in nanosized form was applied. Using native form of lithium carbonate did not reveal prevailing elimination of certain HC-29 cell type. The target cells for nanosized form of lithium carbonate are predominantly the cells of differentiation stages IV and V. Consequently, hepatocarcinoma-29 cells are more sensitive to the damaging action of nanosized lithium salts, which give an opportunity of more effective impact on tumor

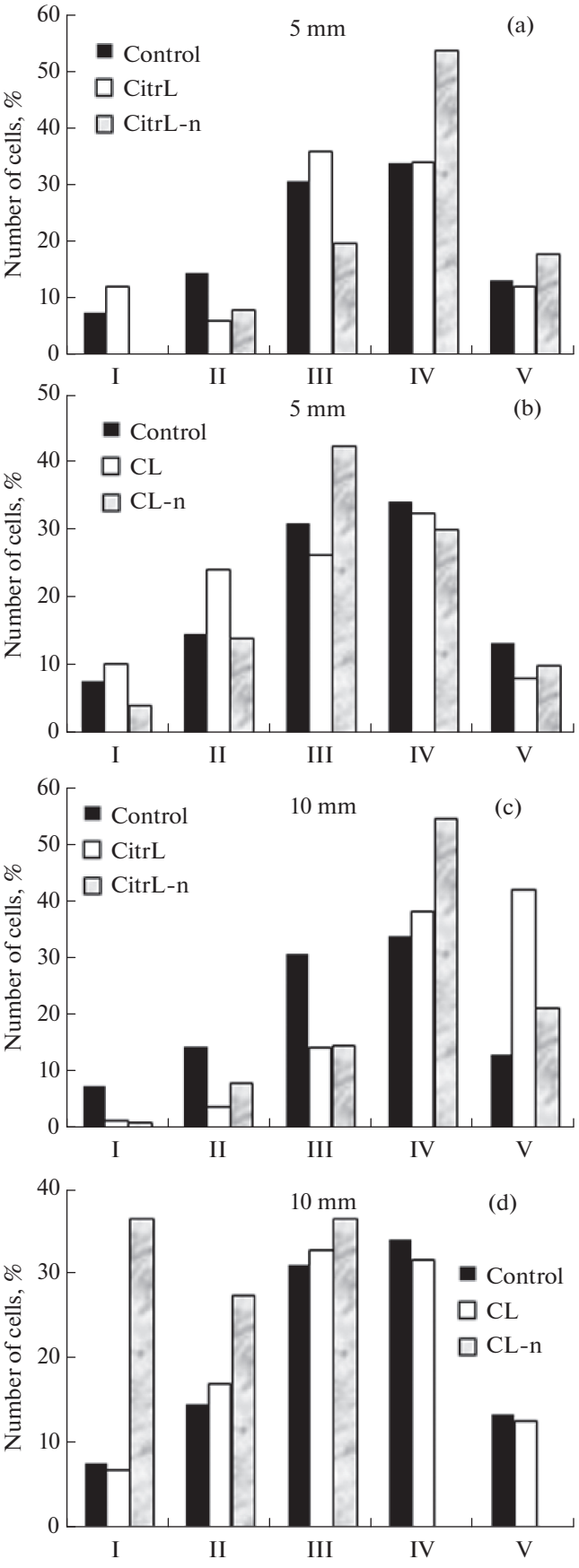

Fig. 3. The effect of lithium salts in different concentrations on the number of HC-29 cells of different (I–V) differentiation stages. (a, c) The addition of native and nanosized lithium citrate at (a) 5 and (c) 10 mM. (B, d) The addition of native and nanosized lithium carbonate at (b) 5 and (d) 10 mM. CitrL—lithium citrate in native form, CitrL-n—lithium citrate in nanosized form, CL—lithium carbonate in native form, and CL-n—lithium carbonate in nanosized form.

growth compared to the action of the same substances in native forms.

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Translated by A. Bigildeev

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