ISSN 1990-519X, Cell and Tissue Biology, 2017, Vol. 11, No. 4, pp. 261–267. © Pleiades Publishing, Ltd., 2017.
Original Russian Text © N.P. Bgatova, Yu.S. Gavrilova, A.P. Lykov, A.O. Solovieva, V.V. Makarova, Yu.I. Borodin, V.I. Konenkov, 2017, published in Tsitologiya, 2017, Vol. 59, No. 3, pp. 178–184.

Apoptosis and Autophagy in Hepatocarcinoma Cells Induced by Different Forms of Lithium Salts

N. P. Bgatova*, Yu. S. Gavrilova, A. P. Lykov, A. O. Solovieva, V. V. Makarova, Yu. I. Borodin, and V. I. Konenkov

Scientific Institute of Clinical and Experimental Lymphology, Novosibirsk, 630060, Russia

**e-mail: n_bgatova@ngs.ru*

Received October 12, 2016

Abstract—Hepatocellular carcinoma (HCC) is an aggressive cancer that is resistant to drug therapy. It is believed that the development of HCC is correlated with misregulation of programmed cell death. Discovery of effective inducers of HCC cell death is very important for HCC therapy. The aim of this work was to identify structural changes leading to the death of HCC cells exposed to nanosized and original forms of lithium salts. Structural features of autophagy and apoptosis were revealed in HCC cells after their incubation with various forms of lithium salts by light, electron microscopy, and flow cytometry. It was shown that nanosized forms of lithium carbonate and lithium citrate had a pronounced effect on HCC-29 cells. Of these forms, the nanosized lithium citrate induced mainly apoptosis, while the nanosized form of lithium carbonate, along with apoptosis, induced autophagic death of HCC cells.

Keywords: lithium carbonate, lithium citrate, nanosized forms of lithium carbonate and lithium citrate, hepatocarcinoma-29, apoptosis, autophagy

DOI: 10.1134/S1990519X17040022

INTRODUCTION

Hepatocarcinoma (HCC) is a widespread aggressive tumor that is resistant to drug therapy (Shen and Cao, 2012). The development of HCC is accompanied by impaired regulation of programmed cell death (Degterev and Yuan, 2008). The programmed cell death is divided into three general types: apoptosis, autophagy, and programmed necrosis (Edinger and Thompson, 2004). It has been proposed that hepatocarcinoma cells can undergo apoptosis, autophagy, and necrosis (Cui et al., 2013), Unlike necrosis apoptosis and autophagy do not provoke local and systemic inflammation and are considered as targets for anticancer therapy (Zhang et al., 2016).

The same extracellular signaling molecules and intracellular messengers frequently stimulate opposite cell responses, from proliferation to apoptosis (Gomperts et al., 2013). Different signaling pathways are involved in the regulation of apoptosis. Apoptosis is associated with Wnt signaling and glycogen-synthase kinase-3β (GSK-3β) as a key negative regulator (McCubrey et al., 2016). GSK-3β inhibition affects the cell proliferation and apoptosis development in colorectal, pancreas, and ovarian cancer cells (Wang et al., 2008; de Araujo et al., 2015).

Lithium compounds make up one of five classes of GSK-3β inhibitors (Quiroz et al., 2004). Lithium carbonate (LiC) suppresses GSK-3β activity and cyclin E expression and inhibits the cell proliferation due to the cycle arrest of cancer cells in G_2/M (Erdal et al., 2005, Tsui et al., 2012). It also induces apoptosis in tumor cells (Li et al., 2015). New findings show that lithium modulates autophagy in cancer cells (O'Donovan et al., 2015).

Lithium's toxicity limits its application for cancer therapy (Chiu et al., 2013). We found that nanosized forms of lithium salts in doses less than the initial forms were more effective for HCC cells (Gavrilova et al., 2016). Morphological division of HCC cells for differentiation stages (Bgatova et al., 2016) reveals that the targets for initial (LC, LiC) and nanosized lithium citrate (nLC) and carbonate (nLiC) forms are different cell types. We suggested that these agents triggered different mechanisms of the cell death (Gavrilova et al., 2016). The aim of this study was to disclose structural changes resulting in death of HCC cells after cell exposure to lithium salts.

Abbreviations: HCC-29-hepatocarcinoma-29, LCl—lithium chloride, LiC—lithium carbonate, nLiC—lithium carbonate nanosized form, LC—lithium citrate, nLC—lithium citrate nanosized form.

MATERIALS AND METHODS

Cells

Experiments were performed using the cell line HCC-29 (hepatocarcinoma) obtained from the Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences (Kaledin et al., 2009). HCC-29 cells were expanded via injecting (10^6 cells/mouse) and growing them in CBA females for 2–3 weeks.

nLC and nLiC were obtained by sample mechanoactivation in a planetary activator mill by the method described in (Isupov et al., 2015). The size of lithium salt particles determined with the Image software (United States) was 10 nm. Lithium chloride, the most frequently used lithium salt (Li et al., 2014), was used as control.

A dose of 5 mM was used to determine early structural modifications triggered by lithium salts in HCC-29 cells. The cell viability with this dose was about 50% (Gavrilova et al., 2016).

Cell morphology

Intact (control) cells and cells exposed to 5 mM lithium salts for 24 or 48 h were fixed with 2.5% glutaraldehyde (Sigma, United States) in phosphate buffer, pH 7.4, treated with 1% OsO_4 (Sigma, United States) in phosphate buffer, pH 7.4, dehydrated with increasing concentrations of ethanol and imbedded into Epon (Serva, Germany). Semithin sections (1 μM) were cut with an EM UC7 Leica ultratome (Germany/Switzerland), stained with toluidine blue, and visualized under a LEICA DME light microscope (Germany). Ultrathin sections 70–100 nm in thickness were contrasted with a saturated water solution of sodium uranyl acetate and lead citrate and analyzed with a JEM 1400 electron microscope. Morphometric analysis was performed with the Image J software (Wayne Rasband, United States). The cell number with apoptotic nuclei, nuclear invaginations, and binucleated cells was calculated in HCC-29 cultures exposed to lithium salts for 48 h.

Cell-cycle assay

HCC-29 cells treated with lithium salts were washed twice and fixed with 96% ethanol for 16–18 h at 4°C. The cells washed from ethanol were stained with 100 mg/mL propidium iodide (BD, United States) with 0.2% Triton X-100 (Sigma, United States) and 0.15 mg/mL RNAase (Vector, Russia) added to it for 20 min at 37°C and analyzed with a FACS Canto II flow cytometer (BD, United States).

Apoptosis

Apoptosis in HCC-29 cells cultivated with lithium salts for 24 h was assayed by annexin V binding using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingem™, United States). The data are presented as the percentage of apoptotic cells in the total cell number.

Autophagy

HCC-29 cells cultivated with lithium salts for 24 h were washed and incubated with antibodies to the LC3 autophagic marker (Abcam, United Kingdom), dilution 1 : 400, for 1 h at 37°C. The cells were washed and incubated with Alexa Fluor 647 secondary antibodies (Abcam, United Kingdom), dilution 1 : 400, for 30 min at 37°C. The cells were washed and fixed. Expression of the LC3 autophagic marker was assayed with an FACS Canto II flow cytometer (BD, United States).

Statistics

Statistical treatment was performed with the Statistica 6.0 Mean value (M), standard deviation (SD), median (Me), low (LQ), and upper (UQ) quartiles were calculated. Significant difference was estimated with the Mann–Whitney U-test. $P < 0.05$ was considered statistically significant.

Reagents

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide glutaraldehyde (Sigma, United States), osmium tetroxide (Sigma, United States), Epon (Serva, Germany), Anenxin V-FITV Apoptosis Detection Kit (BD Pharmingem™, United States), antibodies to LC3 and Alexa Fluor 647 antibodies (Abcam, United Kingdom).

RESULTS AND DISCUSSION

The most pronounced structural modifications were observed in HCC-29 cells exposed to nLC. Endocytic vesicles on the cell periphery (Fig. 1a) and invaginations of various sizes and locations on the nuclear periphery (Fig. 1b), as well crossing the full nuclear diameter (Fig. 1c), were observed after treatment for 24 h. Cells manifesting apoptotic features (large accumulation of condensed chromatin on the nuclear periphery) appeared at 24 and 48 h after treatment (Fig. 1d).

The highest number of apoptotic cells assessed with flow cytometry was registered after HCC-29 cell exposed to nLC (Fig. 2a). Compared to lithium salts, nLiC was an effective apoptosis inducer in HCC-29 cells treated for 48 h (Figs. 2b, 2c). It was demonstrated that 1 mM nLC induced apoptosis in HCC-29 cells more strongly than nLiC and lithium chloride at the same dose (Lykov et al., 2015).

HCC-29 cells treated with LiC displayed structural features of autophagy. The most effective was nLiC.

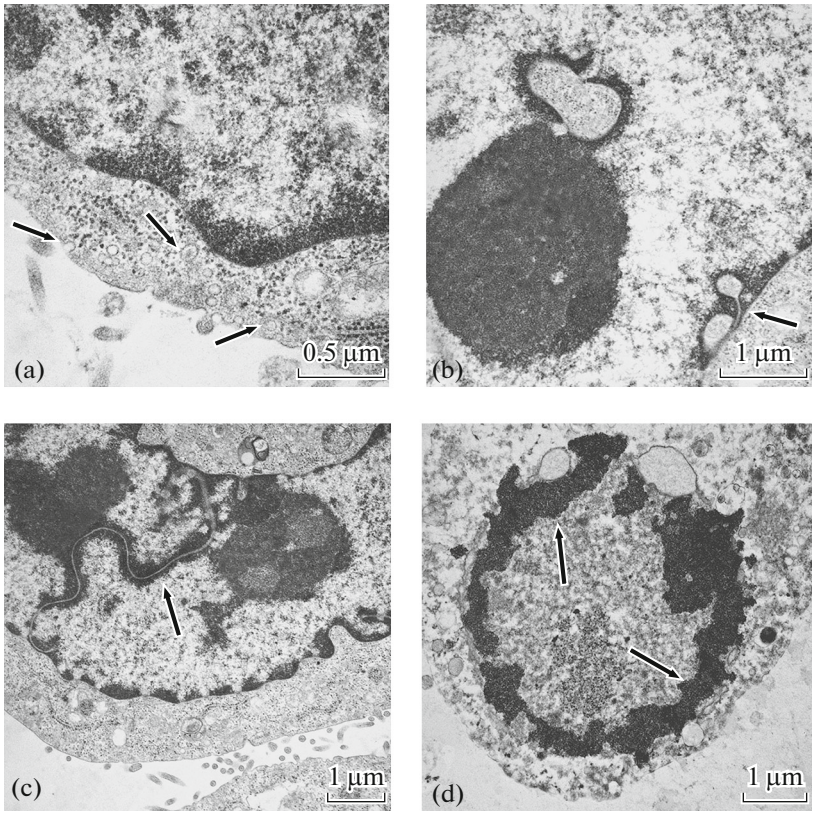

Fig. 1. Structural changes in hepatocarcinoma-29 (HCC-29) cells treated with nanosized lithium citrate. Arrows show (a) endocytic vesicles, (b), the intranuclear channel, (c), formation of an intranuclear channel crossing the nucleus, and (d) chromatin condensation during apoptosis.

Twenty-four hours after nLiC administration, phagosomes with lithium particles (Fig. 3a), as well as autophagosomes with mitochondria, cytoplasmic fragments, extended cisterns of the granular endoplasmic reticulum (Fig. 3b), and autophagosome accumulation with the membranes of the endoplasmic reticulum and lysosomes, were observed (Fig. 3c). After 24 and 48 h, the cells exhibited structural characteristics of autophagic death. Cells that do not swell and are without condensed chromatin contain a significant amount of autophagosomes and autophagolysosomes (Figs. 3d, 3e).

The development of autophagy in HCC-29 cells treated with nLiC was confirmed with flow-cytometry assay. nLiC produced a higher number of cells expressing the autophagy marker LC3 than other lithium salts and forms ($p < 0.05$) (Fig. 3f).

The cell-death program is determined by the cell-differentiation level and their position in the cell cycle. Unlike apoptosis triggered in various stages of the cell cycle, autophagic cell death develops mainly in nondividing cells (G_0-phase) (Chen et al., 2016). The cell number of HCC-29 in the G_0/G_1 stage after administration of nLiC increased, whereas their number in the S-phase declined compared to that of chloride-treated and control cells ($p < 0.05$) (Fig. 4a). HCC-29 cell accumulation in the G_0/G_1 phase after nLiC administration may be a structural basis for autophagy development in differentiated cells.

It was found that nLC and nLiC reduced HCC-29 number in the G_2/M-phase. It has also been reported that lithium inhibits cell proliferation and cell-cycle arrest in the G_2/M-phase have been reported (Tsui et al., 2012). nLC induced the highest frequency of apoptosis in HCC-29 cells (Fig. 2a). It mostly affected proliferating cells, because the number of binuclear cells in the HCC-29 population significantly diminished (Fig. 4b). The decrease has been accompanied with increased number of cells with nuclear invaginations (Fig. 4c) that is an evidence of a higher frequency of pathological forms of division (Vargas et al., 2012) after HCC treatment with nLC.

Lithium is a common drug for of bipolar disorder treatment (Malhi et al., 2012). Lithium compounds applied in different diseases exert various results (Mota de Freitas et al., 2016). It has been widely documented that lithium salts (mostly studies is lithium chloride) trigger apoptosis in cancer cells (Li et al., 2014). The main mechanism of the apoptosis triggered with lithium salts is inhibition of GSK-3β and Akt signaling (Li et al., 2015).

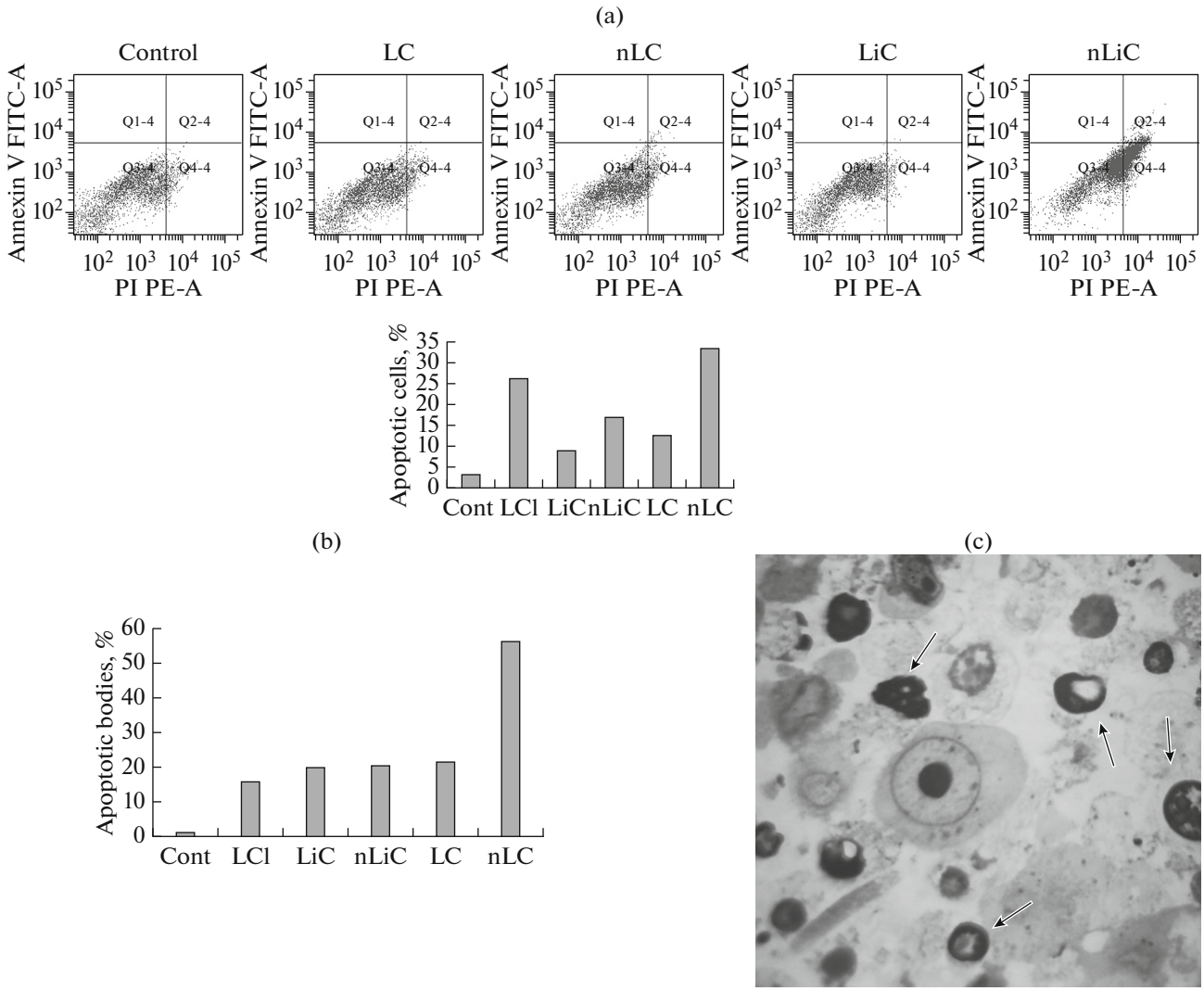

Fig. 2. Apoptosis in HCC-29 cells cultured treated with lithium salts. (a) Apoptotic cell number at 24 h after lithium-salt administration, flow-cytometry assay; (b) number of apoptotic bodies in cells cultivated with lithium salts for 48 h; (c) and apoptosis in cells (arrows) cultivated with nanosized lithium citrate for 48 h. Toluidine blue staining. Magnification: 400×. Here and in Figs. 3 and 4: HCC-29—hepatocarcinoma-29; LCl—lithium chloride; LiC—lithium carbonate, initial form; nLiC—nanosized lithium carbonate; LC—lithium citrate, initial form; nLC—nanosized lithium citrate.

On the other hand, there exists some evidence that lithium induces autophagy (Motoi et al., 2014). Structural features of autophagy were shown to exist in motor neurons of chicken embryos exposed to lithium salts: accumulation of large dense vesicles, autophagic vacuoles, neurofilaments, degradation of rough endoplasmic reticulum, and diminished number of free ribosomes (Calderó et al., 2010). Lithium affects autophagosome production in neurons in neurodegenerative disorders (Chang et al., 2011). Esophageal and colorectal cancer cells exposed in culture to lithium have experienced autophagy (O'Donovan et al., 2015).

The mechanism underlying the lithium-induced autophagy is considered to be inhibited with inositol monophosphatase (IMP) followed by reduced level of inositol 1,4,5-triphosphate (Sarkar et al., 2005). It has been proposed that autophagy is a double-edge sword for cancer cells. On one hand, it is a mechanism for their survival, but, on the other hand, it promotes their death (Peng et al., 2013; Zhang et al., 2016). It has been suggested that enhanced autophagic death of tumor cells resistant to apoptosis facilitates the therapeutic efficiency of anticancer drugs (O'Donovan et al., 2015).

Lithium's inhibition of the key targets GSK-3β and IMP results in opposite effects on cell-death development (induction of apoptosis or autophagy). The choice is determined by the drug dose. Low lithium concentrations inhibit IMP and induce autophagy. Higher lithium doses inhibit GSK-3β activity and

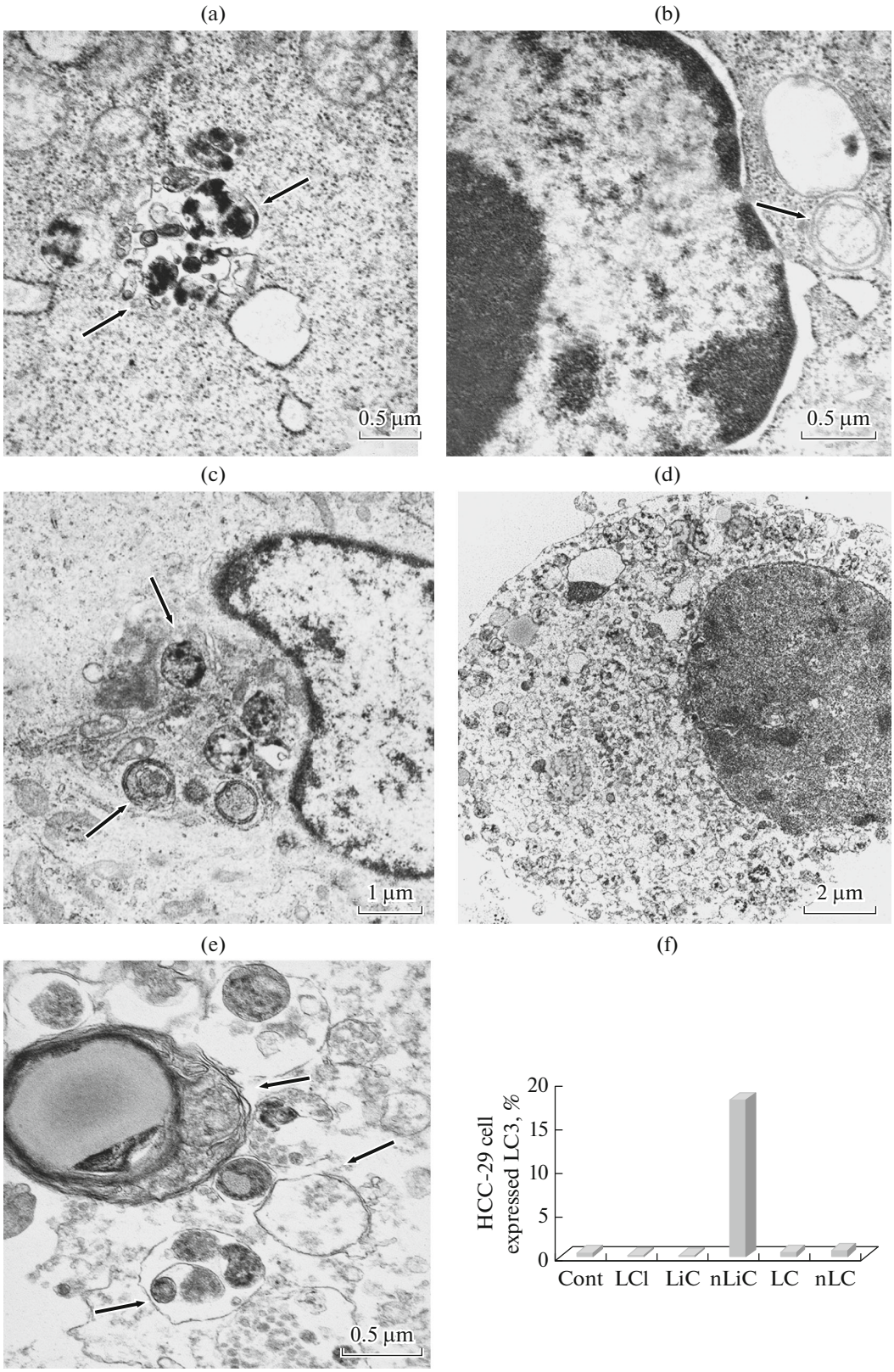

Fig. 3. Structural changes in HCC-29 cells exposed to nanosized lithium carbonate for 24 h. (a) Lithium carbonate particles in phagosomes (arrow), (b) autophagosome with mitochondria (arrow) and cistern extension in granular endoplasmic reticulum membrane, (c) autophagosome with endoplasmic membrane and lysosomes (arrows), (d) autophagosomes and autophagolysosomes in the cytoplasm with nuclear structural integrity, (e) autophagosomes and autophagolysosomes in the cytoplasm (arrows), and (f) expression of LC3-marker in HCC-29 cells.

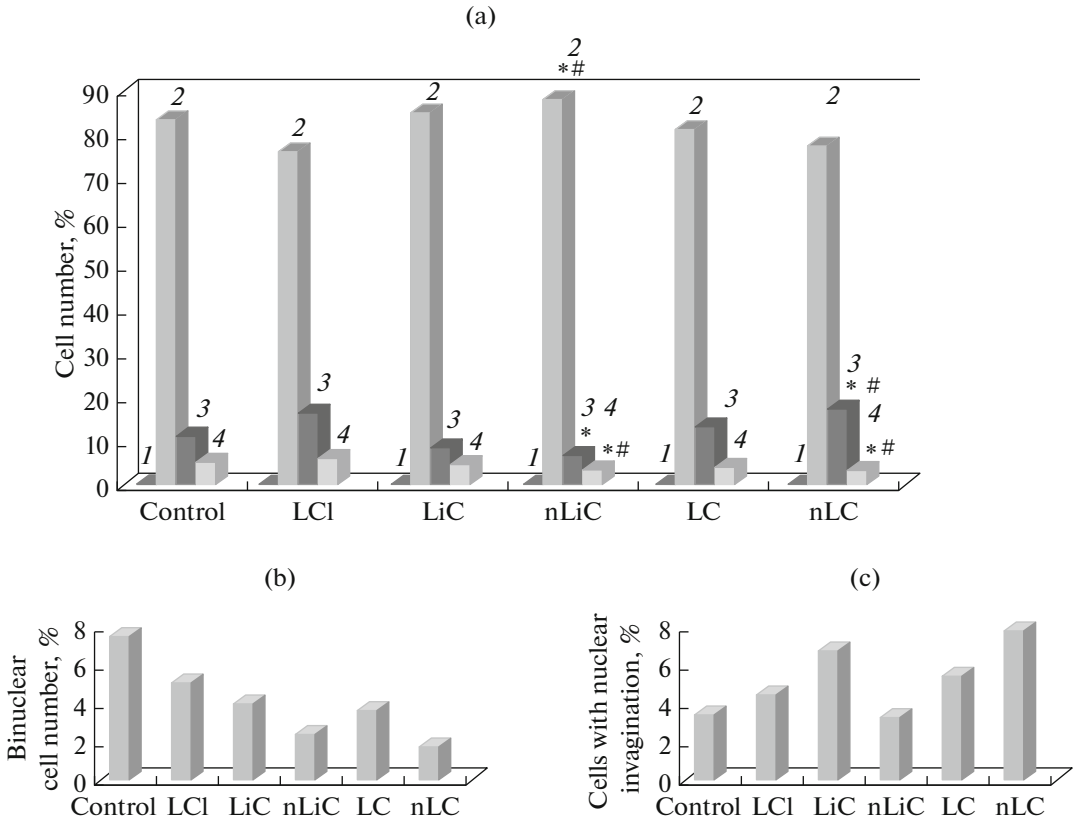

Fig. 4. (a) Cell-cycle distribution, (b) number of binuclear cells, and (c) number of cells with nuclear invaginations in HCC-29 culture treated with 5 mM lithium salts. (*1*) Sub-G_0/G_1 phase, (*2*) G_0/G_1 phase, (*3*) S phase, and (*4*) M-phase. * Statistically significant compared to control; # statistically significant compared to nanosized lithium salt; difference with nanosized lithium salt, $p < 0.05$.

trigger apoptosis (Dell'Osso et al., 2016). Lithium may, via different mechanisms, activate or suppress autophagy depending on the cell type and administration routes (Fabrizi et al., 2012). Further studies are needed to understand the mechanisms underlying the induction of apoptosis or autophagy in HCC-29 cells exposed to nLC or nLiC.

In conclusion, the results of this study show that lithium salts produce structural modification causing the death of HCC-29 cells. The most active of these cells are nLiC and nLC. nLC mainly induces apoptosis. nLiC induces both apoptotic and autophagic death of HCC-29 cells.

REFERENCES

Bgatova, N.P., Omel'yanchuk, L.V., Pozhidaeva, A.A., Semeshin, V.V., Lykov, A.P., Poveschenko, O.V., Makarova, O.P., Pachkovskaya, L.N., Borodin, Yu.I., and Konenkov, V.I., Morphological criteria of cell differentiation stages in experimental hepatocarcinoma and evaluation of antitumor drug efficiency, *Bull. Exp. Biol. Med.*, 2015, vol. 160, no. 1, pp. 109–114.

Calderó, J., Brunet, N., Tarabal, O., Piedrafita, L., Hereu, M., Ayala, V., and Esquerda, J.E., Lithium prevents excitotoxic cell death of motoneurons in organotypic slice cultures of spinal cord, *Neuroscience*, 2010, vol. 165, pp. 1353–1369.

Chang, J.W., Choi, H., Cotman, S.L., and Jung, Y.K., Lithium rescues the impaired autophagy process in CbCln3(Δex7/8/Δex7/8) cerebellar cells and reduces neuronal vulnerability to cell death via IMPase inhibition, *J. Neurochem.*, 2011, vol. 116, pp. 659–668.

Chen, Y.J., Fang, L.W., Su, W.C., Hsu, W.Y., Yang, K.C., and Huang, H.L., Lapatinib induces autophagic cell death and differentiation in acute myeloblastic leukemia, *Onco Targets Ther.*, 2016, vol. 9, pp. 4453–4464.

Chiu, C.T., Wang, Z., Hunsberger, J.G., and Chuang, D.M., Therapeutic potential of mood stabilizers lithium and valproic acid: beyond bipolar disorder, *Pharmacol. Rev.*, 2013, vol. 65, pp. 105–142.

Cui, J., Gong, Z., and Shen, H.M., The role of autophagy in liver cancer: molecular mechanisms and potential therapeutic targets, *Biochim. Biophys. Acta*, 2013, vol. 1836, pp. 15–26.

de Araujo, W.M., Robbs, B.K., Bastos, L., de Souza, W.F., Vidal-Cabral, F., Viola, J.P., and Morgado-Diaz, J.A., PTEN overexpression cooperates with lithium to reduce the malignancy and to increase cell death by apoptosis via PI3K/Akt suppression in colorectal cancer cells, *J. Cell Biochem.*, 2015. doi 10.1002/jcb.25294

Degterev, A. and Yuan, J., Expansion and evolution of cell death programs, *Nature Rev. Mol. Cell Biol.*, 2008, vol. 9, pp. 378–390.

Dell'Osso, L., Del, Grande, C., Gesi, C., Carmassi, C., and Musetti, L., A new look at an old drug: neuroprotective effects and therapeutic potentials of lithium salts, *Neuropsychiatr. Dis. Treat.*, 2016, vol. 12, pp. 1687–1703.

Edinger, A.L., and Thompson, C.B., Death by design: apoptosis, necrosis and autophagy, *Curr. Opin. Cell Biol.*, 2004, vol. 16, pp. 663–669.

Erdal, E., Ozturk, N., Cagatay, T., Eksioglu-Demiralp, E., and Ozturk, M., Lithium-mediated downregulation of PKB/Akt and cyclin E with growth inhibition in hepatocellular carcinoma cells, *Int. J. Cancer*, 2005, vol. 115, pp. 903–910.

Fabrizi, C., Somma, F., Pompili, E., Biagioni, F., Lenzi, P., Fornai, F., and Fumagalli, L., Role of autophagy inhibitors and inducers in modulating the toxicity of trimethyltin in neuronal cell cultures, *J. Neural. Transm. (Vienna)*, 2012, vol. 119, pp. 1295–1305.

Gavrilova, Y.S., Bgatova, N.P., Solovieva, A.O., Trifonova, K.E., Lykov, A.P., Borodin, Yu.I., and Konenkov, V.I., Target cells for lithium in different forms within a heterogeneous hepatocarcinoma-29 population, *Cell Tissue Biol.*, 2016, vol. 10, no. 4, pp. 284–289.

Gomperts, B., Walser, T.C., Spira, A., and Dubinett, S.M., Enriching the molecular definition of the airway "field of cancerization:" establishing new paradigms for the patient at risk for lung cancer, *Cancer Prev. Res. (Phila)*, 2013, vol. 6, pp. 4–7.

Isupov, V.P., Bulina, N.V., and Eremin, N.V., Structural and disperse characteristics of lithium carbonate after mechanical activation and thermal processing, *Inorg. Mater.*, 2015, vol. 51, no. 4, pp. 1–7.

Kaledin, V.I., Zhukova, N.A., Nikolin, V.P., Popova, N.A., Belyaev, M.D., Baginskaya, N.B., Litvinova, E.A., Tolstikova, T.G., Lushnikova, E.L., and Semenov, D.E., Hepatocarcinoma-29, a metastasizing transplantable mouse tumor inducing cachexia, *Bull. Exp. Biol. Med.*, 2009, vol. 148, no. 6, pp. 903–908.

Li, H., Huang, K., Liu, X., Liu, J., Lu, X., Tao, K., Wang, G., and Wang, J., Lithium chloride suppresses colorectal cancer cell survival and proliferation through ROS/GSK-3β/NF-κB signaling pathway, *Oxid. Med. Cell Longev.*, 2014. doi 10.1155/2014/241864

Li, L., Song, H., Zhong, L., Yang, R., Yang, X.Q., Jiang, K.L., and Liu, B.Z., Lithium chloride promotes apoptosis in human leukemia NB4 cells by inhibiting glycogen synthase kinase-3 beta, *Int. J. Med. Sci.*, 2015, vol. 12, pp. 805–810.

Lykov, A.P., Poveshenko, O.V., Bondarenko, N.A., Bgatova, N.P., Makarova, O.P., and Konenkov, V.I., Antiproliferative potential of officinal forms and nanopaparticles of lithium salts, *Bull. Exp. Biol. Med.*, 2016, vol. 160, no. 6, pp. 827–830.

Malhi, G.S., Tanious, M., Das, P., and Berk, M., The science and practice of lithium therapy, *Aust. N. Z. J. Psychiatry*, 2012, vol. 46, pp. 192–211.

McCubrey, J.A., Rakus, D., Gizak, A., Steelman, L.S., Abrams, S.L., Lertpiriyapong, K., Fitzgerald, T.L., Yang, L.V., Montalto, G., Cervello, M., Libra, M., Nicoletti, F., Scalisi, A., Torino, F., Fenga, C., Neri, L.M., Marmiroli, S., Cocco, L., and Martelli, A.M., Effects of mutations in Wnt/β-catenin, hedgehog, Notch and PI3K pathways on GSK-3 activity—diverse effects on cell growth, metabolism and cancer, *Biochim. Biophys. Acta*, 2016, vol. 1863, pp. 2942–2976.

Mota de Freitas, D., Leverson, B.D., and Goossens, J.L., Lithium in medicine: mechanisms of action, *Met. Ions Life Sci.*, 2016, vol. 16, pp. 557–584.

Motoi, Y., Shimada, K., Ishiguro, K., and Hattori, N., Lithium and autophagy, *ACS Chem. Neurosci.*, 2014, vol. 5, pp. 434–442.

O'Donovan, T.R., Rajendran, S., O'Reilly, S., O'Sullivan, G.C., and McKenna, S.L., Lithium modulates autophagy in esophageal and colorectal cancer cells and enhances the efficacy of therapeutic agents in vitro and in vivo, *PLoS One*, 2015, vol. 10, p. e0134676. doi 10.1371/journal.pone.0134676

Peng, Y.F., Shi, Y.H., Shen, Y.H., Ding, Z.B., Ke, A.W., Zhou, J., Qiu, S.J., and Fan, J., Promoting colonization in metastatic HCC cells by modulation of autophagy, *PLoS One*, 2013, vol. 8, p. e74407. doi 10.1371/journal.pone.0074407

Quiroz, J.A., Gould, T.D., and Manji, H.K., Molecular effects of lithium, *Mol. Interv.*, 2004, vol. 4, pp. 259–272.

Sarkar, S., Floto, R.A., Berger, Z., Imarisio, S., Cordenier, A., Pasco, M., Cook, L.J., and Rubinsztein, D.C., Lithium induces autophagy by inhibiting inositol monophosphatase, *J. Cell Biol.*, 2005, vol. 170, pp. 1101–1111.

Shen, Y. and Cao, D., Hepatocellular carcinoma stem cells: origins and roles in hepatocarcinogenesis and disease progression, *Front. Biosci.*, 2012, vol. 1, pp. 1157–1169.

Tsui, M.M., Tai, W.C., Wong, W.Y., and Hsiao, W.L., Selective G2/M Arrest in a p53 (Val135)-transformed cell line induced by lithium is mediated through an intricate network of MAPK and β-catenin signaling pathways, *Life Sci.*, 2012, vol. 24, pp. 312–321.

Vargas, J.D., Hatch, E.M., Anderson, D.J., and Hetzer, M.W., Transient nuclear envelope rupturing during interphase in human cancer cells, *Nucleus*, 2012, vol. 3, pp. 88–100.

Wang, J.S., Wang, C.L., Wen, J.F., Wang, Y.J., Hu, Y.B., and Ren, H.Z., Lithium inhibits proliferation of human esophageal cancer cell line Eca-109 by inducing a G2/M cell cycle arrest, *World J. Gastroenterol.*, 2008, vol. 7, pp. 3982–3989.

Zhang, C., Jia, X., Wang, K., Bao, J., Li, P., Chen, M., Wan, J.B., Su, H., Mei, Z., and He, C., Polyphyllin VII induces an autophagic cell death by activation of the JNK pathway and inhibition of PI3K/AKT/mTOR pathway in HepG2 cells, *PLoS One*, 2016, vol. 11, p. e0147405. doi 10.1371/journal.pone.0147405

Translated by I. Fridlyanskaya