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Ultrastructural Changes in Hepatocellular Carcinoma-29 Cells after Treatment with Lithium Carbonate

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We studied the effect of lithium carbonate on hepatocellular carcinoma-29 cells in CBA male mice after injection in a dose of 20 mM along the tumor periphery. Transmission electron microscopy revealed a decrease in the volume density of the granular endoplasmic reticulum in the cell cytoplasm and an increase in the total numerical and volume density of autophagosomes and autolysosomes and zones of destruction of intracellular organelles. The ability of lithium carbonate to activate intracellular degradation processes in tumor cells and to stimulate cell death can be used to develop new combined strategies in the chemotherapy for hepatocellular carcinoma.

Key Words: *hepatocellular carcinoma; lithium carbonate; ultrastructural organization; autophagy*

Hepatocellular carcinoma (HCC) is one of the most malignant tumors characterized by high heterogeneity and refractory to systemic chemotherapy [4,9]. Lithium salts are traditionally used for the treatment of bipolar disorders; however, more and more studies of lithium in different fields of experimental oncology appear recently. It was shown that lithium salts could affect different signaling pathways used by tumor cells for the growth and development: PI, PI3K/Akt/mTOR, MAPK/ERK, Wnt/β-catenin, *etc*. This effect of lithium salts is mainly realized via inhibition of inositol monophosphatase and isoform of glycogen synthase kinase 3 (GSK3α and GSK3β), as well as some other enzymes [6,8,12]. By modulating intracellular signaling, lithium can affect the cell cycle and proliferation and can stimulate apoptosis and autophagy in tumor cells.

Autophagy is the process of degradation of intracellular proteins and organelles that is necessary for the maintenance of intracellular homeostasis. Autophagy plays a dual role in cancer [10]: it can contribute to survival of cancer cells under stress or nutritional deficiencies, but at the same time, it can suppress tumor growth by stimulation of autophagy of tumor cells. According to published data, HCC is characterized by deficient autophagy [5]; however, the role of autophagy in the development and progression of HCC is still poorly understood [7]. Lithium stimulates autophagy mainly via modulation of the PI signaling pathway [12]. Little is known about the effects of lithium salts on tumor cell autophagy and the development of HCC.

We assessed ultrastructural changes occurring in HCC cells under the influence of lithium carbonate *in vivo*.

MATERIALS AND METHODS

The experiments were conducted on 3-month-old CBA mice weighing 18-20 g. The animals were given standard water and food *ad libitum*. Tumor cell line HCC-29 was kindly provided by the Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences [3]. For modeling tumor process, HCC-29 cells were transplanted intraperitoneally to CBA male mice; in 10 days, the ascitic fluid was collected, 2×10^6 cells (in 100 μl PBS) were injected intramuscularly into the right thigh. The animals were divided into 3 groups (5 mice per group): mice with untreated tu-

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mor (control); mice treated with 100 μl 0.9% physiological saline (PS); and mice receiving injections of 20 mM lithium carbonate (Li_2CO_3) in 100 μl PS. The drugs were administered intramuscularly along the periphery of the tumor. Material for analysis (intramuscular tumor) was isolated on day 23 of the experiment in the morning. The animals were sacrificed by cervical dislocation under ether anesthesia.

For transmission electron microscopy, the autopsy material was fixed in 4% paraformaldehyde in Hanks medium, fixed for 1 h in 1% OsO_4 in phosphate buffer (pH 7.4), dehydrated in increasing concentration of ethanol, and embedded in epon. Semi-thin sections (1 μ) were prepared on a Leica EM UC7 ultramicrotome (Leica Microsystems), stained with toluidine blue, and areas for electron microscopy were selected under a Leica DME light microscope (Leica Microsystems). Ultrathin sections (70-100 nm) were prepared from the selected material on a Leica EM UC7 ultratome, contrasted with saturated aqueous solution of uranyl acetate and lead citrate, and photographed under a JEM 1400 electron microscope (Jeol).

For each study group, 100 HCC cells were independently selected containing the nucleus and continuous clear cell membrane. Morphometry was performed on photographs at ×8000 using ImageJ software and an open test system. For each cell, the volume (Vv) and numerical (N_A) densities of mitochondria, granular endoplasmic reticulum, autophagosomes, autolysosomes, lysosomes, and destruction zones of intracellular organelles were estimated.

Statistical processing of the results was performed using Statistica 6.0 and Microsoft Excel. Significance of differences between the studied parameters was determined using the Mann—Whitney *U* test at $p<0.05$. The mean (*M*) and standard deviation (*SD*) were calculated.

RESULTS

HCC-29 was formed by large and predominantly round cells arranged in structures resembling hepatic cords. Cells nuclei had irregular shape and contained large irregular lumps of condensed chromatin. Signs of cell degeneration (swollen homogenized mitochondria with smoothed cristae, few cisterns of the granular endoplasmic reticulum, and numerous free ribosomes) were noted (Fig. 1, *a*). Structures typical of autophagy were seen: numerous autolysosomes (vesicles with single membrane and contents at different stages of degradation) and occasionally autophagosomes (round structures with two membrane layers and narrow electron-transparent gap between them and intact intracellular material) (Fig. 1, *b*). Lysosomes with relatively homogeneous content were rare, had small sizes and moderate electron density. The zones of destruction of intracellular organelles were visualized as sites of clear cytoplasm containing destroyed cell components (Fig. 1, *b*). The absence of glycogen accumulation in HCC-29 cells was noted.

Morphometry of subcellular components in cells of heterogeneous population HCC-29 showed that the numerical density of mitochondria after lithium carbonate treatment significantly increased by 1.74 times ($p<0.0001$) in comparison with the control group and by 1.4 times ($p<0.005$) in comparison with PS group (Fig 2). The differences in the volume density of mitochondria between the studied groups were in-

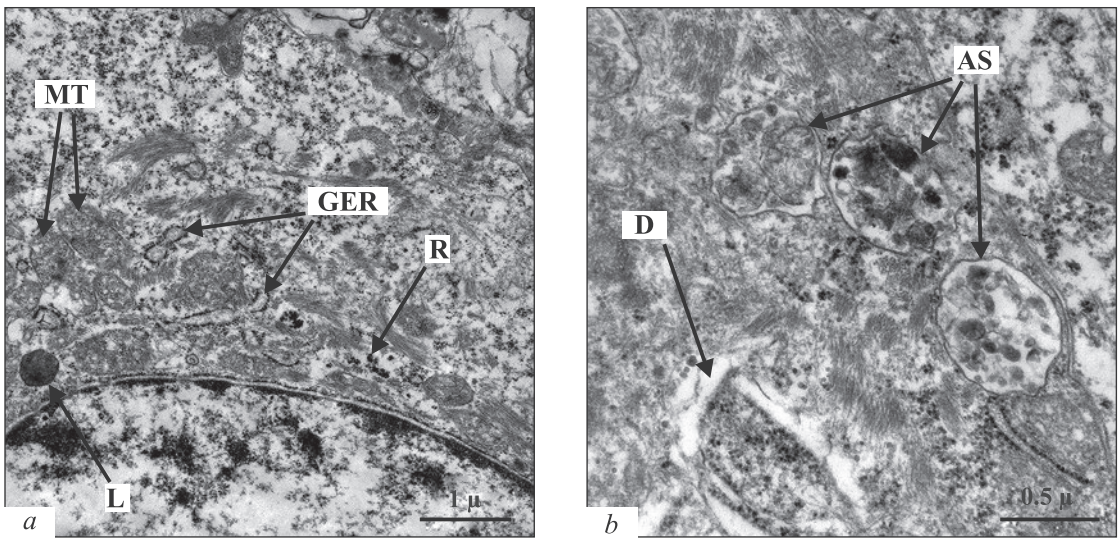

Fig. 1. Ultrastructural changes in HCC-29 cells after injection of 20 mM lithium carbonate along the tumor periphery: cell ultrastructure (*a*), autolysosomes and destruction zone (*b*). MT: mitochondria, GER: granular endoplasmic reticulum, R: ribosomes, L: lysosome, D: zones of destruction of intracellular organelles, AS: autophagy structures (autolysosomes).

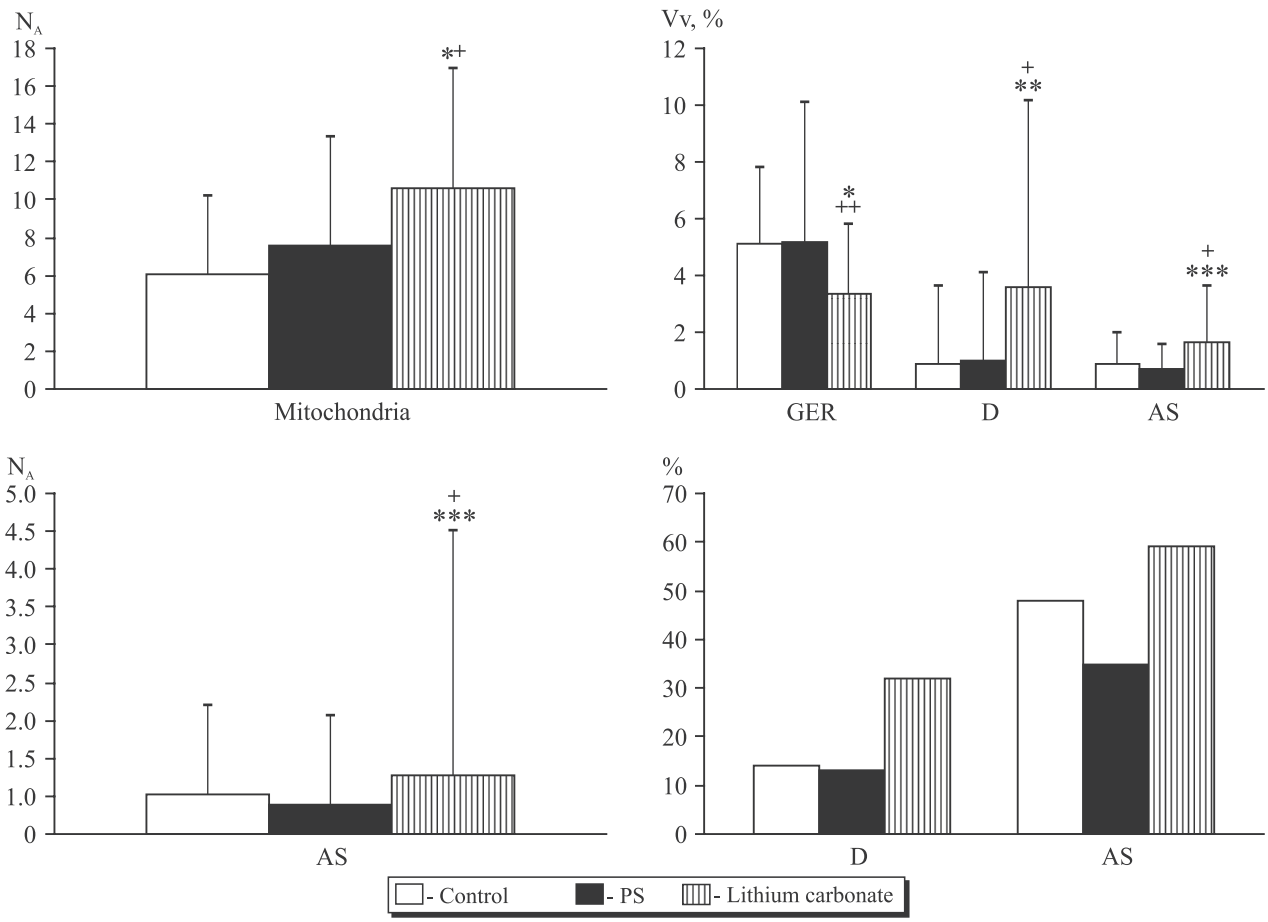

Fig. 2. Numerical (N_A) and volume (V_v) density of subcellular structures, relative number of cells (%) with destructions (D) and autophagy structures (AS; autophagosomes and autolysosomes). GER: granular endoplasmic reticulum. *p<0.0001, **p<0.005, ***p<0.05 in comparison with the control; ^{+}p<0.005, ^{++}p<0.0001 in comparison with the PS group.

significant, but there was a tendency to its increase (control — 5.7±3.2; PS — 5.5±3.05; lithium carbonate — 5.94±3.48). The volume density of the granular endoplasmic reticulum after injection of lithium carbonate was by 1.5 times lower (p<0.0001) than in the control and PS group (Fig. 2). The number of cells with zones of destruction of intracellular organelles after injection of lithium carbonate was higher by 2.3 and 2.5 times in comparison with the control and PS groups, respectively (Fig. 2). The volume density of destruction zones was significantly higher (p<0.005) after injection of lithium carbonate: by 4 and 3.5 times in comparison with the control and PS groups, respectively (Fig. 2). Injection of lithium carbonate along the tumor periphery also led to an increase in the number of cells with autophagosomes and/or autolysosomes to 59% (in comparison with 48 and 35% in the control and PS groups, respectively; Fig. 2). The total numerical density of autophagosomes and autolysosomes in the lithium carbonate group increased by 1.4 times in comparison with the control (p<0.05) and by 1.9 times in comparison with the PS group (p<0.005) (Fig. 2). The total volume density of autophagosomes and autolysosomes after the introduction of lithium carbonate increased by 1.8 times in comparison with the control (p<0.05) and by 2.4 times in comparison with the PS group (p<0.005) (Fig. 2). Volumetric and numerical densities of lysosomes did not significantly differ.

In our previous *in vitro* studies, a dose-dependent decrease in the viability of HCC-29 cells after treatment with lithium carbonate [2] and induction of apoptosis and autophagy in HCC-29 cells [1] under the effect of lithium salts were demonstrated in the MTT test.

In this *in vivo* study, lithium carbonate induced destructive changes in the cytoplasm of HCC-29 cells and increased the total numerical and volume densities of autophagosomes and autolysosomes, which attested to initiation of autophagy and activation of intracellular degradation processes. The increase in the numerical density of mitochondria could be associated with increased energy requirements for autophagy and the decrease in the volume density of granular endoplasmic reticulum could be a result of reticulophagy.

Lithium induces autophagy mainly via inhibition of inositol monophosphatase [12] presumably leading to accumulation of myoinositol-1,4,5-triphosphate (IP3), desensitization and reduction of IP3R (IP3 receptors) [11], and subsequent release of Beclin-1 from a specific complex consisting of IP3R, Beclin-1, and Bcl-2, which ultimately stimulates autophagy [13]. As experimental studies of lithium in oncology and, in particular, in HCC are limited, further studies of the molecular and morphological bases for the development of ultrastructural changes and the induction of autophagy after treatment with lithium carbonate are required.

Thus, injection of lithium carbonate in a dose of 20 mM along the periphery of experimental hepatocarcinoma-29 in the thigh region of CBA mice induced ultrastructural changes in the cytoplasm of tumor cells: a decrease in the volume density of the granular endoplasmic reticulum and an increase in the numerical density of mitochondria, total numerical and volume densities of autophagosomes and autolysosomes, and zones of destruction of intracellular organelles. The ability of lithium carbonate to activate intracellular degradation processes in tumor cells and induce cell death can be used for the development of new combined strategies in the chemotherapy for hepatocellular carcinoma. Combined application of lithium carbonate and common chemotherapeutic drugs allows simultaneous modulation of different cellular signaling pathways for integration of different types of cell death.

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