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Research

Antiproliferative Potential of Lithium Salts Against Hepatocellular Carcinoma Cell Line

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ABSTRACT

To investigate the antiproliferative activity of nanosized particles of lithium salts against hepatocellular carcinoma cells line-29 (HCC-29) with special attention on apoptosis. HCC-29 cell line was inoculated by the intraperitoneal injection in mice. The different officinal and nanosized particles of the lithium salts effect on cell viability, and proliferation, and cell cycle distribution, and apoptosis, and membrane expression of GSK3β, was studied *In vitro*. The nanosized particle of lithium citrate and lithium carbonate treatment caused a reduction of survival rate, proliferation activity of HCC-29 cell line. Immunophenotype analysis revealed increased apoptosis by nanosized particles of lithium carbonate, and increased a number cell in subG0-G1 (apoptotic cells), and decreased the number of cells in G2-M phase. Nano sized particles of lithium citrate and lithium carbonate treatment also increased membrane expression of GSK-3β. The nano sized particle of lithium citrate and lithium carbonate suppresses cell viability and proliferation, induced apoptosis, especial lithium carbonate, and increased membrane expression of GSK-3β *In vitro*.

Keywords: Lithium salts, Hepatocellular carcinoma cell-29 cell line, Apoptosis, Cell cycle, GSK-3β.

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INTRODUCTION

Hepatocellular carcinoma may be a result of hepatitis caused by hepatitis virus (B and C), alcohol consumption, and metabolic diseases [1-3]. Human HCC characterized by tumor heterogeneity and this fact are the reason un-sufficient therapy, and cancer-related death [4].

It is known that tumour cell mediated growth and production of growth factors via signalling pathways, including such as Notch1-HES-ASCL1, Ras-Raf-MEK-MAPK, PI3K-Akt-MAPK and glycogen sinzu-3β (GSK-3β) [5-6]. GSK-3β play a key role in Wnt/b-catenin signaling pathway, which is one of the most activated signaling pathway in tumors, including HCC [7].

Lithium salts previously were used as agent for therapy of bipolar disorders [5]. Recently were observed that lithium salts can inhibit inositol monophosphatase and glycogen synthase kinase-3β (GSK-3β) [8]. It is shown that on the background therapy of patients with pheochromocytomas and medullar thyroid cancer lithium chloride, showed a significant growth suppression of these tumours [6]. In addition, it is shown that lithium salts are able to arrest tumour cells in G2 phase of the cell cycle [9].

However, there is no information about the cytotoxic potential of nanosized particles of lithium salts. Based on our earlier data on anti-proliferative effect of different dosage of lithium salts *In vitro*, we hypothesized that nanosized particle of lithium salts would inhibit of proliferative activity of HCC-29 cell line via inhibition of GSK-3β.

In present paper, using different lithium salts, we have investigated whether nano sized particles of lithium citrate and lithium carbonate diminished HCC-29 cell line function. In this study we demonstrate that treatment of HCC-29 cell line with nano sized decreased cell viability and proliferation. Important, treatment also leads to changes in cell cycle distribution and apoptosis. Finally, we show that treatment HCC-29 cell line with nano sized particle of lithium salts leads to increasing of membrane expression of GSK-3β by cells. These results suggest that nano sized particle of lithium salts has potential as anti-proliferative drug for hepatocellular carcinoma.

MATERIALS AND METHODS

Animals

The study was carried out on 8-10-weeks-oldfemale CBA/Lac mice (20 to 25 g) from vivarium of Institute of Physiology and Basic Medicine. Mice were housed in group of 10 in stainless-steel wire-bottom cages before use during the 2-week acclimation period, and received standard mouse chow and water ad libitum, and were maintained at room temperature (21 to 24°C), relative humidity (25 to 50%), and lighting (12 hours/day) were carefully controlled. The experimental proceedings on mice and the facilities used to hold the experimental animals were in compliance with the rules of the Institute of Clinical and Experimental Lymphology Animal Care and Use Committee. The present protocols were approved by Institutional Animal Care and Use Committee of the Institute of Clinical and Experimental Lymphology, Russia.

Hepatocellular carcinoma cell line

Hepatocellular carcinoma cell-29 (HCC-29) cell line was obtained by intraperitoneal injection of 5 × 10^6 cells/ mice, 10-14 days after injection, the peritoneal cavity was lavaged with cold phosphate-buffer saline, after determination of viability by trypan blue (cell viability was > 95%), HCC-29 cell line were re-suspended in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine and 50 µg/mL gentamycin (Sigma).

Preparation of nano sized particles of lithium salts

Mechanical crushing of lithium citrate and lithium carbonate was done in high-intensive ball mills for increasing a mass fraction small-sized (less than 0.5 µm) fraction of particles [10].

Cell viability

To determine the cell viability, cells were plated onto 96-well plates (1 × 10^4 cells/well). After 24 hours incubation with different lithium salts in dosage 5 mM, 5g/L 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to the cell suspension for further 4 hours, followed by addition of dimethylsulfoxide (DMSO, Sigma) at 100 L/well for cell lysis. Then, absorbance was measured at 570 nm. Each assay was carried out in triplicate. Cell survival was expressed as percentage (A value of treated to control cells ratio × 100).

Proliferation assay

Cell proliferation of HCC-29 cell line was measured by the MTT rapid colorimetric assay. In brief, cells were seeded into 96-well plate at a density of 10^4 cells/well with a different lithium salts in dosage 5 mM, and alone in RPMI-1640 medium supplemented by heat-inactivated 10% fetal calf serum (FCS), 0.3 mg/mL L-glutamine, 5 mM HEPES buffer, and 80 µg/mL of gentamycin and incubated during 72 hours. The MTT assay was performed by replacing the standard medium with 100 µL of serum-free medium containing 5 g/L 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide and incubated for 4 hours at 37°C. The formazan in viable cells was dissolved with 100 µL of DMSO and determined by reading optical densities (OD) in microplate reader (Stat Fax 2100, USA) at an absorption wave length of 570 nm.

Cell cycle

Cell cycle distribution and measurement were performed by flow cytometry. To this order, 1 × 10^6 cell/mL before and after incubation 5 mM of different lithium salts during 24 hours, were washed with cold PBS and fixed in 70% ethanol (-20°C) at 4°C. After 2 hours, fixed cells were pelleted and stained with propidium iodide (20 µg/mL) in the presence of RNAse A (100 µg/mL) for 30 minutes at 37°C, and about 3 × 10^4 cells were analyzed using a fluorescence-activated cell sorter Canto II and data were analyzed by using Diva software (Becton-Dickinson, USA).

Apoptosis

Apoptosis in the HCC-29 cell line was measured by using the Annexin V-FITC/PI Apoptosis Detection kit (Becton-Dickinson, USA). Before and after 24 hours incubation with different lithium salts in dosage 5 mM in 24-well plates at density of 1 × 10^6 cell/well, fluorescent intensities were determined by flow cytometry. Alive cells grouped in the lower left part of the panel, early apoptotic cells grouped in the lower right part of the panel, and late apoptotic cells grouped in the higher right part of the panel. The experiment was repeated at least three times.

GSK-3β by FACS analysis

The number of HCC-29 cell line expressed on membrane GSK-3β was measured by using a fluorescence-activated cell sorter Canto II and data were analyzed by using Diva software. Briefly, the 1 × 10^6 cell/mL before and after incubation with 5 mM of different lithium salts during 24 hours, was stained with primary antibody anti-GSK3 beta antibody during 30 minutes in dark at room temperature, then washed with 0.5 mL 1% bovine serum albumin in PBS, then stained with secondary antibody goat anti-rabbit IgG (H+L) for 30 minutes at room temperature in dark, then cells was washed with 0.5 mL 1% bovine serum albumin in PBS, and then HCC-29 cell line was re-suspended in 0.5 mL FACS buffer (0.05% NaN_3+5% bovine serum albumin in PBS), antibody was purchased from Abcam (USA). Dead cells were excluded by electronic gating and 30000 gating events were analyzed for each sample.

Statistical analysis

Data was processed with the software Statistica 10.0 (Stat Soft Inc., USA). Data was obtained from 3 experiments. The Kolmogorov-Smirnov test was used to test for normal distribution of all variables. To reveal significant differences between the parameters compared, the distribution-free Mann-Whitney U-criterion was employed. Difference were considered significant at the level of $p<0.05$. To analyze correlation relationships between characteristics, the Spearman rank correlation coefficient was employed.

RESULTS AND DISCUSION

Lithium salts decreased survival of HCC-29 cell line

To investigate the role of lithium in the survival of HCC-29 cell line, the cell viability was analyzed by MTT assay. **Table 1** shows that both forms of the lithium citrate caused a gradual reduction in the percentage of viable cells in dose 5 mM.

Table 1: Survival ratio of HCC-29 cell line (Me; Q25-Q75).

Parameters	Survival ratio (%)
Basal	100
Lithium chloride	81.89; 74.69-88.73
Lithium citrate	72.17; 54.84-85.43
	$P_{u\ 2\text{-}3}$ = 0.003
	$P_{u\ 2\text{-}5}$ = 0.003
Lithium citrate nanosized particle	57.64; 54.79-57.92
	$P_{u\ 4\text{-}5}$ = 0.003
	$P_{u\ 4\text{-}6}$ = 0.003
Lithium carbonate	89.42; 82.66-99.85
Lithium carbonate nano sized particle	80.97; 77,73-84.60

Whereas, the lithium chloride and both forms of the lithium carbonate caused a slow reduction in the percentage of viable cells compared to control, and lithium citrate. Also, the lithium citrate nanosized particles caused a significant ($p<0.05$) reduction in percentage of viable cells compared to lithium carbonate both forms. These results suggested that lithium salts contributed to the reduced survival of HCC-29 cell line.

Lithium salts suppressed proliferation of HCC-29 cell line

In order to further characterize the effect of lithium salts on the proliferation of HCC-29 cell line, MTT proliferation assay was also performed.

Table 2: Antiproliferative effect of officinal and nanosized particles of lithium salts (Me; Q25-Q75).

Parameters	Proliferation (OD at λ=570 nm)
Basal	0.82; 0.68-0.94
Lithium chloride	0.68; 0.61-0.70
	$P_{u\ 1\text{-}2}$ = 0.013
Lithium citrate	0.66; 0.52-0.81
Lithium citrate nanosized particle	0.53; 0.52-0.55
	$P_{u\ 1\text{-}4}$ = 0.0007
	$P_{u\ 2\text{-}4}$ = 0.003
Lithium carbonate	0.70; 0.68-0.78
Lithium carbonate nanosized particle	0.55; 0.54-0.56
	$P_{u\ 1\text{-}6}$ = 0.000
	$P_{u\ 2\text{-}6}$ = 0.03

	$P_{u\ 5\text{-}6}$ = 0.001

Table 2 shows that after exposure to different lithium salts for 24 hours, the proliferation capacity of HCC-29 cell line significantly decreased. So, officinal form of the lithium citrate and lithium carbonate *in vitro* in dosage 5 mM caused a tendency to inhibit proliferation of HCC-29 cell line, but not a significant ($p>0.05$). Whereas nanosized particles of the lithium citrate and lithium carbonate in the same dosage cause a significant ($p<0.05$) decrease proliferation of HCC-29 cell line.

Also, nanosized particles of the lithium citrate and lithium carbonate a significantly ($p<0.05$) decreased HCC-29 cell line proliferative activity compared to lithium chloride. The lithium carbonate nanosized particles more efficiently decreased HCC-29 cell line proliferation ($p<0.05$) compared to officinal form of lithium carbonate. These results suggested that lithium salts contributed to the reduced proliferation of HCC-29 cell line.

Lithium salts induced apoptosis in HCC-29 cell line

Since GSK-3β play a crucial role in tumor cells survival pathway and lithium salts, a well-known general GSK-3β inhibitor, we detected early apoptotic cells (Annexin V+/Pi), and apoptotic cells (Annexin V+/Pi+ and Annexin V-/Pi+, early apoptosis and late apoptosis respectively), and necrotic cells (Annexin V-/Pi+) by flow cytometry (**Table 3**).

Parameters	Necrosis (%)	Apoptosis (%)
Basal	0.00; 0.00-0.03	9.40; 7.30-11.40
Lithium chloride	0.40; 0.40-0.40	55.45; 20.40-91.40
		$P_{u\ 1\text{-}2}$ = 0.004
Lithium citrate	0.20; 0.00-0.30	78.60; 27.40-88.10
		$P_{u\ 1\text{-}3}$ = 0.002
Lithium citrate nanosized particle	0.10; 0.00-0.40	5.50; 5.50-5.50
		$P_{u\ 2\text{-}4}$ = 0.02
		$P_{u\ 3\text{-}4}$ = 0.02
Lithium carbonate	0.10; 0.00-0.20	69.25; 40.70-96.80
		$P_{u\ 1\text{-}5}$ = 0.003
Lithium carbonate nanosized particle	0.20; 0.10-0.60	79.00; 35.30-95.30
		$P_{u\ 1\text{-}6}$ = 0.003

Table 3. Effect of Lithium salts on HCC-29 apoptosis and necrosis (Me; Q25-Q75).

We have no estimate any changes in percent of necrotic HCC-29 cell line after exposure with different lithium salts in dosage 5 mM significant ($p>0.05$). Whereas, lithium salts in same dosage cause a significant ($p<0.05$) of apoptosis of HCC-29 cell line, exclude the nano sized particles lithium citrate. Also, officinal form of the lithium citrate and lithium carbonate, and nano sized lithium carbonate increased apoptosis of HCC-29 cell line, compared to lithium chloride ($p<0.05$). These results confirmed that lithium salts treatment led to HCC-29 cell line apoptosis.

Lithium salts on HCC-29 cell line affect cell cycle distribution

To determine whether lithium salts-induced inhibition of HCC-29 cell line proliferation was due to altered cell cycle regulation, HCC-29 cell line was treated with lithium salts in dosage 5 mM for 24 hours. Cell cycle distribution was monitored by flow cytometry of DNA content (**Table 4**). So, lithium salts cause a significant ($p<0.05$) increase number of HCC-29 in subG0-G1 cell cycle (apoptotic cells).

The lithium chloride, and lithium citrate nano sized particles a significant decreased a number of HCC-29 cell line in G0G1 phase of cell cycle. Whereas, the lithium carbonate nanosized particles a significant ($p<0.05$) increased a number of HCC-29 cell line in G0-G1 phase of cell cycle. Also, the lithium citrate officinal form, and both forms of the lithium carbonate a significant ($p<0.05$) increased a number of HCC-29 cell line in G0-G1 phase of cell cycle compared to effect of the lithium chloride. Also, we found that the lithium citrate nano sized particles a significant ($p<0.05$) decreased in number of HCC-29 cell line in G0-G1 compared to officinal form of the lithium citrate. Whereas, the nano sized particles of the lithium carbonate a significant ($p<0.05$) increased in number of HCC-29 cell line in G0-G1 compared to officinal form.

The lithium chloride, and the nano sized particles of the lithium citrate a significant ($p<0.05$) increased a number of HCC-29 cell line in S phase of cell cycle. Whereas, the both forms of the lithium carbonate a significant ($p<0.05$) decreased a number of HCC-29 cell line in S phase of cell cycle.

We found that only nano sized particles of the lithium citrate, and the lithium carbonate a significant ($p<0.05$) decreased a number of HCC-29 cell line in G2-M phase of cell cycle. Whereas, both form of the lithium citrate, and the nanosized particle of the lithium carbonate a significant ($p<0.05$) decreased a number of HCC-29 cell line in G2-M.

Table 4. Distribution cell cycle HCC-29 under Lithium salts (Me; Q25-Q75).

Parameters	Phase of cell cycle			
	subG0-G1	G0-G1	S	G2-M
Basal	0.20; 0.20-0.20	82.70; 82.60-83.33	11.30; 10.87-11.40	5.80; 5.37-5.80
Lithium chloride	0.90; 0.90-0.90	73.80; 73.80-75,87	14.12; 11.40-18.80	6.34; 6.00-6.50
	$P_{u\ 1-2}$ =0.004	$P_{u\ 1-2}$ =0.004	$P_{u\ 1-2}$ =0.016	--
Lithium citrate	0.50; 0.50-0.50	77.00; 77.00-77.40	12.64; 11.40-17.40	4.30; 4.00-4.50
	$P_{u\ 1-3}$ = 0.006	$P_{u\ 2-3}$ = 0.037		$P_{u\ 2-3}$ = 0.02
	$P_{u\ 2-3}$ = 0.045			
Lithium citrate nano sized particle	1.00; 1.00-1.10	75.00; 75.00-76.30	16.27; 11.40-18.60	4.22; 3.10-5.00
	$P_{u\ 1-4}$ = 0.004	$P_{u\ 1-4}$ = 0.004	$P_{u\ 1-4}$ = 0.016	$P_{u\ 1-4}$ = 0.016
	$P_{u\ 3-4}$ = 0.013	$P_{u\ 3-4}$ = 0.037	--	$P_{u\ 2-4}$ = 0.004
Lithium carbonate	0.80; 0.80-0.80	86.00; 85.00-86.00	9.59; 9.00-11.40	4.20; 4.20-4.87
	$P_{u\ 1-5}$ = 0.006	$P_{u\ 2-5}$ = 0.006	$P_{u\ 2-5}$ = 0.01	
Lithium carbonate nanosized particle	0.70; 0.70-0.70	87.67; 87.00-88.40	9.50; 8.50-11.40	3.90; 3.20-4.30
	$P_{u\ 1-6}$ = 0.006	$P_{u\ 1-6}$ = 0.004	$P_{u\ 2-6}$ = 0.01	$P_{u\ 1-6}$ = 0.02
	--	$P_{u\ 2-6}$ = 0.004	--	$P_{u\ 2-3}$ = 0.004
	--	$P_{u\ 5-6}$ = 0.016	--	--
P_u - Mann-Whitney U-criterion				

Effect of Lithium salts on HCC-29 cell line expressed on membrane GSK-3β

So, GSK-3β are a survival factor for cancer, and lithium inhibit GSK-3β activity, we determined effect of lithium salts on a number of HCC-29 cell line expressed on membrane GSK-3β by flow cytometry (**Table 5**).

Table 5: Effect of Lithium salts on membrane GSK-3βin HCC-29 cell line (Me; Q25-Q75).

Parameters	Level of expression of GSK3β (%)
Basal	0.70; 0.70-0.90
Lithium chloride	0.40; 0.40-0.50
	$P_{u\ 1-2}$ = 0.049
Lithium citrate	0.50; 0.30-0.50 $P_{u\ 1-3}$ = 0.049
Lithium citrate nanosized particle	1.10; 0.90-1.10
	$P_{u\ 2-4}$ = 0.049
	$P_{u\ 3-4}$ = 0.049
Lithium carbonate	0.50; 0.30-0.50

	$P_{u\ 1\text{-}5}$ = 0.049
Lithium carbonate nanosized particle	20.30; 18.00-20.30
	$P_{u\ 1\text{-}6}$ = 0.049
	$P_{u\ 2\text{-}6}$ = 0.049
	$P_{u\ 5\text{-}6}$ = 0.049
P_u - Mann-Whitney U-criterion	

The lithium chloride, and the lithium citrate officinal form, and the lithium carbonate officinal form a significant (p<0.05) decreased a number of HCC-29 cell line expressed on membrane GSK-3β. Whereas, the nanosized particle of the lithium citrate, and the lithium carbonate a significant (p<0.05) increased a number of HCC-29 cell line expressed on membrane GSK-3β compare to the basal, and the lithium chloride, and officinal form of the lithium citrate, and the lithium carbonate.

The present study showed that lithium salts played a role in the survival rate, proliferation capacity, apoptosis, cell cycle distribution and membrane expression of GSK-3β on hepatocellular carcinoma-29 cell line via GSK-3β pathway. The main findings in the present work include the following new data: nanosized particle of the lithium citrate, and the lithium carbonate *In vitro* significantly decreased the proliferation of HCC-29 cell line, had a controversial effect of the apoptosis, and distribution cell cycle of HCC-29 cell line, and increased the expression of GSK-3β on HCC-29 cell line surface.

In vitro results in the present study demonstrated that nano sized particle of the lithium citrate, and the lithium carbonate at dosage 5 mM caused inhibition of proliferative capacity of HCC-29 cell line. In contrast, officinal forms of the lithium salts exhibited absence of antiproliferative activity on HCC-29 cell line.

The lithium chloride in dosage 20 mM induced arrest of tumor cells in S and G2 phase of cell cycle [9]. In the present study, we have studied the effect of lithium salts in dosage 5 mM on HCC-29 cell line distribution cell cycle, with special attention to effect of nano sized particles of the lithium salts. It has been shown that all studied lithium salts in this dosage increased percent in G0-G1, and decreased percent in G0-G1, and S, and in G2-M by nano sized particles of the lithium citrate. However, we have not been shown that nano sized particles of the lithium citrate increased percent of apoptotic cells when used the Annexin V-FITC/PI Apoptosis Detection kit, but nano sized particles of the lithium carbonate increased percent of apoptotic HCC-29 cell line.

In our study, lithium salts, a well-known general GSK-3β inhibitor [9], was shown to increase a number of HCC-29 cell line expressed on membrane GSK-3β. Glycogen Synthase Kinase 3 involved in a variety of cellular processes including glycogen metabolism, gene transcription, apoptosis [5,7,11,12]. GSK-3β is important survival factor for cancer, including hepatocellular carcinoma [13,14]. It is known that lithium chloride in dosage 20 mM induced apoptosis, cell arrest various tumour's by targeting GSK-3β [9]. Earlier we found that nanosized particles of lithium salts inhibit HCC-29 cell line proliferation in lowest dosage compare to officinal form [15]. Also, we obtained that different form of lithium salts target HCC-29 cell line at the different stage of cell differentiation [16]. So, lithium citrate decreased survival of HCC-29 cell line on the I-II stage of cell differentiation. Whereas, lithium carbonate target HCC-29 cell line on IV-V stage of cell differentiation. Recently, we on basis of light-microscopy, electronic microscopy established heterogeneity of HCC-29 cell line, and divided it at 5 stage of cell differentiation [17]. Moreover, we observed that lithium carbonate nano sized particles more efficiently induced autophagy in HCC-29 [18].

Here, we found that nano sized particle of lithium citrate and lithium carbonate in dosage 5 mM more efficiently inhibit proliferation of HCC-29 cell line compare to officinal form of lithium citrate, and lithium carbonate, and lithium chloride (as control for anti-proliferative activity of lithium).

There is a limitation in our work. First, we did not examine the cytoplasmic and nucleus localization of GSK-3β in HCC-29 cell line.

CONCLUSION

In summary, the present study extends our understanding of the role of GSK-3β signaling in hepatocellular carcinoma and the efficiency of nano sized particles of lithium salts as anti-proliferative drug. We have demonstrated that nano sized particle lithium salts treatment HCC-29 cell line leads to suppression of survival rate, and inhibition of cell proliferation *In vitro*.

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AUTHORS' CONTRIBUTIONS

Alexander Lykov designed the study, contributed to the *In vitro* study, data entry and analyzed data, wrote the paper. Natalia Bgatova designed the study, contributed to the microscopy and approved the final manuscript. Yulia Taskaeva contributed to *In vivo* study, GSK-3β FACS analysis, read and approved the final manuscript. Sabina Nogovitsina contributed to *In vivo* study, read and approved the final manuscript. Olga Poveshchenko designed the study, read and approved the final manuscript.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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