

Lithium chloride has a biphasic effect on prostate cancer stem cells and a proportional effect on midkine levels

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Abstract. Prostate cancer (PCa) is the second most frequent type of cancer in men worldwide and the levels of differentiation growth factor midkine (MK) are increased in PCa. Cancer and/or the treatment process itself may lead to psychiatric disorders. Lithium chloride (LiCl) has anti-manic properties and has been used in cancer therapy; however, it has a queried safety profile. In addition, cancer stem cells are responsible for the heterogeneous phenotype of tumor cells; they are involved in progression, metastasis, recurrence and therapy resistance in various cancer types. The aims of the present study were to investigate the effect of different concentrations of LiCl on PCa stem cells (whether a shift from tumorigenic to non-tumorigenic cells occurs) and to determine if these results can be explained through changes in MK levels. Monolayer and spheroid cultures of human prostate stem cells and non-stem cells were incubated with low (1, 10 μ M) and high (100, 500 μ M) concentrations of LiCl for 72 h. Cell proliferation, apoptotic indices, MK levels and ultrastructure were evaluated. Cells stimulated with low concentrations showed high proliferation, low apoptotic indices, high MK levels and more healthy ultrastructure. Opposite

results were obtained at high concentrations. Furthermore, stem cells were more sensitive to stimulation and more resistant to inhibition than non-stem cells. LiCl exhibited concentration-dependent effects on stem cell and non-stem cell groups. MK levels were not involved in the biphasic effect of LiCl; however, they were proportionally affected. To the best of our knowledge, the present study was the first to show the effect of LiCl on PCa stem cells through MK.

Introduction

Prostate cancer (PCa) is second only to lung cancer as the most frequent type of cancer in men worldwide. In terms of mortality, PCa ranks sixth internationally as a cause of cancer-related mortality in men. Each year, PCa is responsible for ~250,000 mortalities in men worldwide (1,2). PCa is a heterogeneous disease that exhibits a range of clinical behaviors, from indolent, slow-growing tumors to aggressive, fast-growing tumors with lethal progression. Accurate discrimination between cases of aggressive and non-aggressive PCa, using parameters such as clinical stage, prostate-specific antigen level and Gleason score, are not adequate to diagnose the risk category (3). In addition, chemotherapy resistance is a major problem in the treatment of PCa, particularly at advanced stages (3,4).

Cancer stem cells (CSCs) have specific functional features, including self-renewal capacity, long-term repopulation potential and differentiation ability in order to give rise to a heterogeneous phenotype of tumor cells. Therefore, CSCs differ from the bulk tumor cells, and are known to be involved in metastasis, recurrence and therapy resistance in various cancer types. The genetic evolution and epigenetic plasticity (i.e., genetic heterogeneity) of CSCs promote the emergence of tumor adaptations, consequently leading to therapeutic failure (5-7).

Midkine (MK) is a heparin binding small protein (13 kDa) with growth factor and cytokine actions. Together with pleiotrophin/heparin-binding growth-associated molecule, MK comprises a family of heparin binding growth factors. MK is highly expressed at the mid-gestation period, however, its expression is decreased and/or lost in adults. MK exhibits

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Abbreviations: PCa, prostate cancer; MK, midkine; EMT, epithelial-mesenchymal transition; LiCl, lithium chloride; CSCs, cancer stem cells; GSK-3 β , glycogen synthase kinase-3 β ; DMEM-F12, Dulbecco's modified Eagle's medium-F12; LRP, low density lipoprotein receptor-related protein; PE, phycoerythrin; FITC, fluorescein isothiocyanate; TEM, transmission electron microscopy; FACS, fluorescence-activated cell sorting; PI, propidium iodide; ELISA, enzyme-linked immunosorbent assay; Dox, doxorubicin; Eto, etoposide; Vin, vinblastine

Key words: lithium chloride, midkine, prostate cancer, cancer stem cell, biphasic effect

its activity through several receptors, including anaplastic lymphoma kinase, protein tyrosine phosphatase ζ , Notch 2, low density lipoprotein receptor-related protein (LRP), proteoglycans and integrins. MK has significant roles in inflammation immunity, blood pressure, development, tissue protection/renewal/repair, cancer [including tumor growth, chemoresistance, transformation, anti-apoptosis, epithelial-mesenchymal transition (EMT)] and determination of cell fate (from autophagy to cell resistance/survival or autophagic cell death/apoptosis) (8-12). High MK expression has been observed in several cancer types, including PCa, in preclinical and clinical studies (9,13,14).

Lithium chloride (LiCl) is a Food and Drug Administration-approved drug for bipolar psychiatric disorder. LiCl has been a well-known drug with anti-manic properties for 60 years. It has cancerogenic and anti-cancerogenic properties, meaning that it can inhibit or stimulate the growth of normal and cancer cells [i.e., it has a biphasic effect]. LiCl acts through inhibition of the glycogen synthase kinase-3 β (GSK-3 β) pathway (an agonist known to activate the Wnt/ β -catenin signaling), the phosphoinositide signaling pathway and adenylate cyclase (15-19). Cancer and/or the cancer treatment itself may lead to psychiatric disorders causing a reduction in life quality and a desire to overcome the illness (20). Therefore, the safety profile of the anti-manic drug LiCl needs to be re-evaluated.

CSCs have also been identified in PCa (21). Advances in research have shown that, similar to normal tissue, certain types of cancer have a hierarchical organization where tumorigenic CSCs differentiate into non-tumorigenic progenies (22). CSCs, LiCl with biphasic effects and the differentiation growth factor MK appear to be part of the Russian Roulette of cancer therapy. Therefore, the aims of the present study were to investigate the effect of different concentrations of LiCl on PCa stem cells (whether a shift from tumorigenic to non-tumorigenic situation occurs or not) and to determine if these results can be explained through MK levels.

Materials and methods

Cell culture and reagents. The DU145 human PCa cell line (HTB-81) was supplied by the American Type Culture Collection (Manassas, VA, USA) and was grown in a monolayer culture in Dulbecco's modified Eagle's medium-F12 (DMEM-F12; Biological Industries Israel Beit-Haemek Ltd., Kibbutz Beit-Haemek, Israel) supplemented with 10% heat-inactivated fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO₂. Cells in semi-confluent flasks (70% of confluency) were harvested using 0.05% trypsin (Sigma-Aldrich). Harvested cells were added to DMEM-F12 for trypsin inactivation and centrifuged (NF200; Nüve Laboratory and Sterilization Technology, Ankara, Turkey) at 500 x g for 3 min at 22°C and then resuspended in culture medium (23).

Stem cell sorting. For fluorescence-activated cell sorting (FACS), cells were detached using non-enzymatic cell dissociation solution (Sigma-Aldrich) and $\sim 5 \times 10^4$ cells were

incubated with cluster of differentiation CD(133) and CD44 antibodies diluted to 1:100 in FACS wash (0.5% bovine serum albumin, 2 mM Na₂N₃ and 5 mM ethylenediaminetetraacetic acid; Miltenyi Biotec, Bisley, UK) for 15 min at 4°C: An isotype and concentration-matched phycoerythrin (PE) (monoclonal mouse IgG1; clone, IS5-21F5; dilution, 1:100; catalog no., 130-092-212; Miltenyi Biotec) and fluorescein isothiocyanate (FITC)-labeled (monoclonal mouse IgG1; 1:100; clone, IS5-21F5; catalog no., 130-092-213; Miltenyi Biotec) control antibody were used, and the tested samples were PE-labeled CD133/1 (monoclonal mouse; clone, AC133; dilution, 1:100; catalog no., 130-080-801; Miltenyi Biotec) and FITC-labeled CD44 (monoclonal mouse; clone, G44-26; dilution, 1:100; catalog no., 555478; BD Pharmingen, San Diego, CA, USA). After 3-5 min, the cells were washed with FACS wash and resuspended with FACS wash. The cells were sorted into CD133^{high}/CD44^{high} (CSC) and non-CSC subpopulations using a FACS Aria cell sorter (BD Biosciences), and termed DU145(+) and DU145(-), respectively. The two subpopulations were cultured in two different settings: Monolayer culture or multicellular tumor spheroids (23,24).

Experimental design. Stem cells and non-stem cells were incubated with low (1 and 10 μ M) and high (100, 500 μ M) concentrations of LiCl for 72 h at 37°C in a humidified atmosphere of 5% CO₂. The four drug groups and an untreated control group were evaluated for total cell numbers, apoptotic index [flow cytometric Annexin V-FITC/propidium iodide (PI) staining], MK levels (enzyme-linked immunosorbent assay; ELISA) of monolayer cultures and ultrastructure of spheroid cultures [transmission electron microscopy (TEM)].

Cell proliferation index analysis. A starter kit (catalog no., M1293-0020; ChemoMetec A/S, Allerød, Denmark) and software (NucleoView Software, version 1.0; ChemoMetec A/S) compatible with an automated cell counter (NucleoCounter; ChemoMetec A/S) were used to determine the total cell number. The kit included lysis buffer, stabilization buffer and nucleocassettes. Briefly, cells were harvested every 24 h for 72 h. Cells were pre-treated with lysis and stabilization buffers to dissolve cell aggregates and lyse cell membranes for 5-10 min. Pre-treated cells were loaded to nucleocassettes coated with PI dye and their nuclei were stained with PI. Nucleocassettes were placed into the cell counter for 30-35 sec to measure PI fluorescence. Subsequently, cell counts were analyzed using the software and recorded (24).

MK concentration level analysis. MK protein concentration levels were detected using an ELISA kit (catalog no., CDYE-LISA; Cellmid Limited, Sydney, Australia), according to the manufacturers' instructions with minor modifications. Briefly, 100 μ l/well of test samples (the supernatants of lysed stem cells and non-stem cells), positive controls (one supplied in the ELISA kit and one a high concentrated MK from T98 glioblastoma cells) and standards (concentrations of 0, 25, 50, 100, 250, 500, 750 and 1,000 pg/ml were obtained by diluting MK master standard in a concentration of 10⁷ pg/ml) were all incubated at room temperature for 2 h with continuous shaking. Standards, controls and samples were run in triplicate. After every step except for the application of stop reagent,

four washing applications with washing buffer supplied by the kit were performed for stem cells and non-stem cells. Detector antibody (100 μ l/well) was added and incubated at room temperature for 1 h with continuous shaking. Then, samples were incubated with 100 μ l/well streptavidin-peroxidase solution for 20 min and 100 μ l/well Substrate Solution for 15 min in the dark at room temperature with continuous shaking. After 15 min, 100 μ l/well stop solution was added in order to inactivate the enzyme and detect blue color formation. Then, results were measured at a wavelength of 450 nm using an ELISA microplate reader within 3 min of stopping (24).

Apoptotic index analysis. One of the manifestations of apoptosis is the translocation of phosphatidylserine (PS) from the inner membrane to the outer side of the plasma membrane. Externalization of PS was studied by the Annexin V binding assay. The apoptotic index was evaluated using flow cytometric Annexin-V-FITC/PI staining (BD Biosciences), according to the manufacturer's instructions. Briefly, cells were washed twice with phosphate-buffered saline, then resuspended in binding buffer containing 0.01 M HEPES, 0.14 mM NaCl and 2.5 mM CaCl_2 . A cell suspension (1×10^5 cells in 100 μ l binding buffer) was incubated with 5 μ l FITC-labeled Annexin V dye and PI (BD Biosciences) for 15 min in the dark at room temperature. Following incubation, PI fluorescence and Annexin V were measured simultaneously in a FACSCalibur machine and analyzed with the instrument's operating software (CellQuest; BD Biosciences). Data acquisition and analysis were undertaken with CellQuest version 5.1 and Windows Multiple Document Interface for Flow Cytometry version 2.8 programs (24). The programs calculated 4 types of situations (viability, early apoptosis, late apoptosis and death) and presented the data through panels with 4 quadrants: The lower left quadrant shows density plots for the number of viable cells (Annexin V⁻, PI⁻); the lower right quadrant shows the number of earlier stages of apoptotic cells (Annexin V⁺, PI⁻); the upper right quadrant shows the number of late stages of apoptotic cells (Annexin V⁺, PI⁺); and the upper left quadrant shows the number of dead cells. The program provided the percentage using the formula: Cells in quadrant/total cell number. In the graph of results, only the percentage of apoptotic cells (the sum of lower right and upper right) is presented.

Multicellular spheroid model. For spheroid cultures, the tumor cells grown as a monolayer were resuspended with trypsin and the clonogenic potential of various phenotypic populations was analyzed in a 3D non-adherent culture condition (plates coated with 3% Noble Agar; Gibco; Thermo Fisher Scientific, Inc.). The cells were counted, resuspended and plated at 1×10^6 cells per well in a 6-well plate. The plates were inspected for colony (sphere) growth 1 week after initiation. The number of colonies within each well was counted under a microscope (Olympus BX-51; Olympus, Hamburg, Germany) and images of representative fields were captured (24).

Ultrastructure analysis. Harvested spheroids were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h at 4°C and post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 20-30 min at 4°C. Spheroids were incubated in 1% uranyl acetate for 1 h at 4°C, dehydrated in a

graded ethanol series and embedded in in Epon 812 substitute as Epoxy Embedding Medium (Sigma-Aldrich). Samples were cut using a rotating blade microtome (Leica Biosystems, Heerbrugg, Switzerland) to a thickness of 70 nm and sections were mounted on copper grids. Sections were subsequently stained with 5% uranyl acetate for 30 min and counterstained with Reynold's lead citrate for 3-5 min at 22°C. Sections were examined with a JEM-1011 TEM (JEOL, Inc., Peabody, MA, USA). Photographs of stem cell and non-stem cell spheroids applied with the lowest (1 μ M) and highest (500 μ M) concentrations of LiCl were captured at several different magnifications (24).

Statistical analysis. SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2010 (Microsoft, Inc., Redmond, WA, USA) were used for the statistical analysis. Results were statistically analyzed using one-way analysis of variance and relevant differences were analyzed by post-hoc comparisons using the Bonferroni method. Data are represented as mean \pm standard error of the mean ($n=6$). Experiments were repeated three times, each experiment was triplicated. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell proliferation index analysis. Figure 1 clearly shows that low concentrations of LiCl significantly increased cell number, while high concentrations of LiCl significantly decreased cell number in both stem ($P_{500}=0.0001$; $P_{100}=0.001$; $P_{10}=0.001$; $P_1=0.0001$) and non-stem cells ($P_{500}=0.0001$; $P_{100}=0.001$; $P_{10}=0.0001$; $P_1=0.0001$) in comparison with the control group ($P<0.001$). The inhibition rate observed with high concentrations of LiCl in the non-stem cell group was higher than that observed for the stem cell group ($P_{500}=0.02$; $P_{100}=0.04$); however, the increase in proliferation observed with low concentrations of LiCl was much lower than in the stem cell group ($P_{10}=0.02$; $P_1=0.03$). Furthermore, treatment with 500 μ M LiCl decreased cell numbers significantly more than in the 100 μ M group and 1 μ M LiCl increased cell numbers significantly more than in the than 10 μ M group for both stem ($P_{500}=0.001$; $P_1=0.001$) and non-stem cell groups ($P_{500}=0.001$; $P_1=0.0001$).

Apoptotic index analysis. It was determined that high concentrations of LiCl significantly increased the apoptotic index, while low concentrations significantly decreased the apoptotic index in both stem cells ($P_{500}=0.00001$; $P_{100}=0.00001$; $P_{10}=0.04$; $P_1=0.02$) and non-stem cells ($P_{500}=0.00001$; $P_{100}=0.00001$; $P_{10}=0.03$; $P_1=0.01$) in comparison with the control group. High concentrations of LiCl increased the apoptotic index in non-stem cells significantly more than in stem cells ($P_{500}=0.002$; $P_{100}=0.004$) and low concentrations of LiCl decreased the apoptotic index in non-stem cells significantly more than in stem cells ($P_{10}=0.04$; $P_1=0.02$). In addition, the apoptotic index was lowest at 1 μ M LiCl and highest at 500 μ M LiCl for both stem cell group ($P_{500 \text{ vs. } 100}=0.0001$; $P_{1 \text{ vs. } 10}=0.03$) and non-stem cell group ($P_{500 \text{ vs. } 100}=0.002$; $P_{1 \text{ vs. } 10}=0.01$) (Fig. 2).

MK concentration level analysis. As shown in Fig. 3, low concentrations of LiCl significantly increased MK levels, while high concentrations significantly decreased MK levels in both

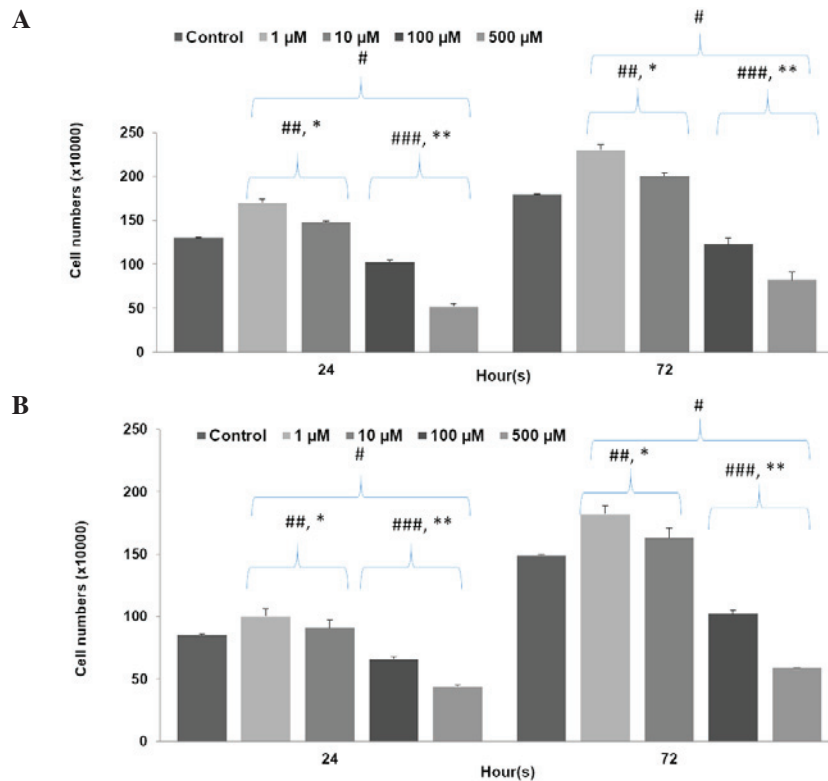


Figure 1. Cell proliferation index analysis. Number of (A) DU145(+) and (B) DU145(-) cells following treatment with LiCl (0, 1, 10, 100, 500 μ M) for 24 or 72 h. DU145(+) and DU145(-) represent stem cells and non-stem cells, respectively. Cell numbers were highest in the stem cell group at 1 μ M LiCl. Data are presented as the mean \pm standard error of the mean (n=6). Experiments were repeated three times, each experiment was triplicated. *P<0.05 vs. control group; **P<0.05 for 100 μ M LiCl vs. 500 μ M LiCl; ***P<0.05 for 1 μ M LiCl vs. 10 μ M LiCl; #P<0.05 for stem cells vs. non-stem cells at high concentrations of LiCl; **P<0.05 for stem cells vs. non-stem cells at low concentrations of LiCl.

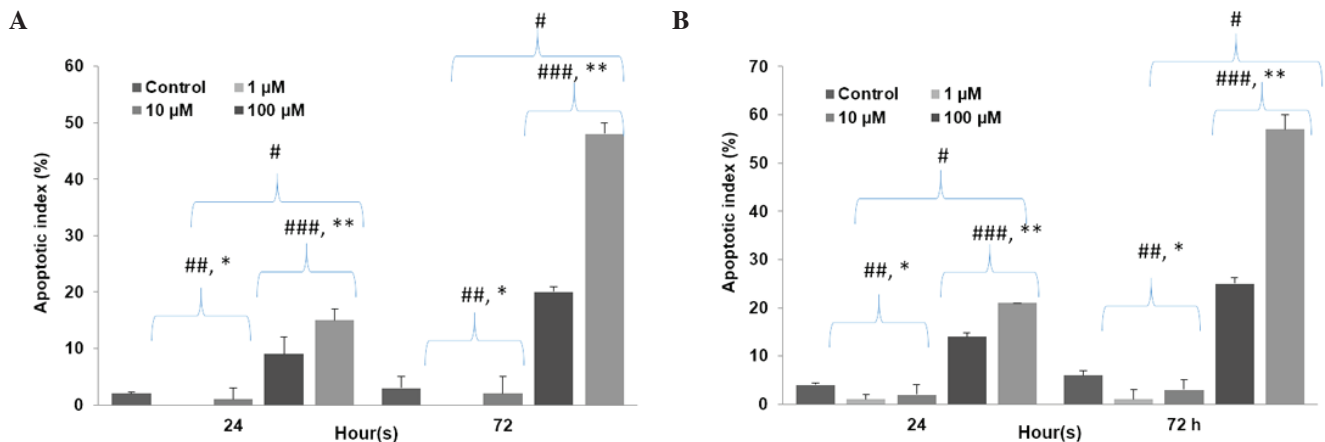


Figure 2. Apoptotic index analysis. (A) DU145(+) and (B) DU145(-) cells following treatment with LiCl for 24 or 72 h. The apoptotic index was lowest in stem cell group at 1 μ M LiCl group. DU145(+) and DU145(-) cells represent stem cells and non-stem cells, respectively. Data were represented as mean \pm standard error of the mean (n=6). Experiments were repeated three times, each experiment was triplicated. *P<0.05 vs. control group; **P<0.05 for 100 μ M LiCl vs. 500 μ M LiCl; ***P<0.05 for 1 μ M LiCl vs. 10 μ M LiCl; #P<0.05 for stem cells vs. non-stem cells at high concentrations of LiCl; **P<0.05 for stem cells vs. non-stem cells at low concentrations of LiCl.

stem cells ($P_{500}=0.0001$; $P_{100}=0.0001$; $P_{10}=0.01$; $P_1=0.001$) and non-stem cells ($P_{500}=0.0001$; $P_{100}=0.001$; $P_{10}=0.03$; $P_1=0.001$) in comparison with the control group. The inhibition rates caused by high concentrations of LiCl in the non-stem cell group were significantly higher than in the stem cell group ($P_{500}=0.01$; $P_{100}=0.01$); however, the proliferation stimulation rates of cells MK levels treated with low concentrations of LiCl was significantly lower in the non-stem cell group than

the stem cell group ($P_{10}=0.0001$; $P_1=0.001$). Application of 500 μ M LiCl decreased MK levels significantly more than 100 μ M and 1 μ M LiCl increased MK levels significantly more than 10 μ M for both stem group ($P_{500 \text{ vs. } 100}=0.01$; $P_{1 \text{ vs. } 10}=0.01$) and non-stem cell group ($P_{500 \text{ vs. } 100}=0.01$; $P_{1 \text{ vs. } 10}=0.02$).

Ultrastructure analysis. In the stem cell group, spheroids treated with 1 μ M LiCl had the same structure as the control

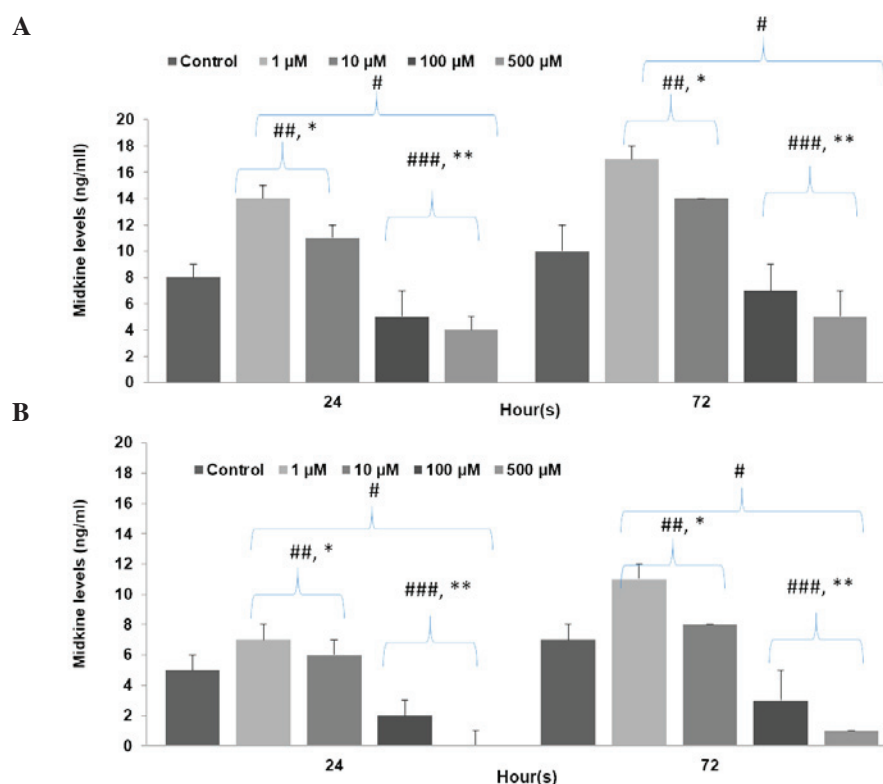


Figure 3. Midkine (MK) concentration level analysis. MK levels in (A) DU145(+) and (B) DU145(-) cells following treatment with LiCl (0, 1, 10, 100, 500 μ M) for 24 or 72 h. DU145(+) and DU145(-) represent stem cells and non-stem cells, respectively. MK levels were highest in stem cell group at 1 μ M LiCl. Data are presented as the mean \pm standard error of the mean (n=6). Experiments were repeated three times, each experiment was triplicated. *P<0.05 vs. control group; **P<0.05 for 100 μ M LiCl vs. 500 μ M LiCl; ***P<0.05 for 1 μ M LiCl vs. 10 μ M LiCl; #P<0.05 for stem cells vs. non-stem cells at high concentrations of LiCl; ##P<0.05 for stem cells vs. non-stem cells at low concentrations of LiCl.

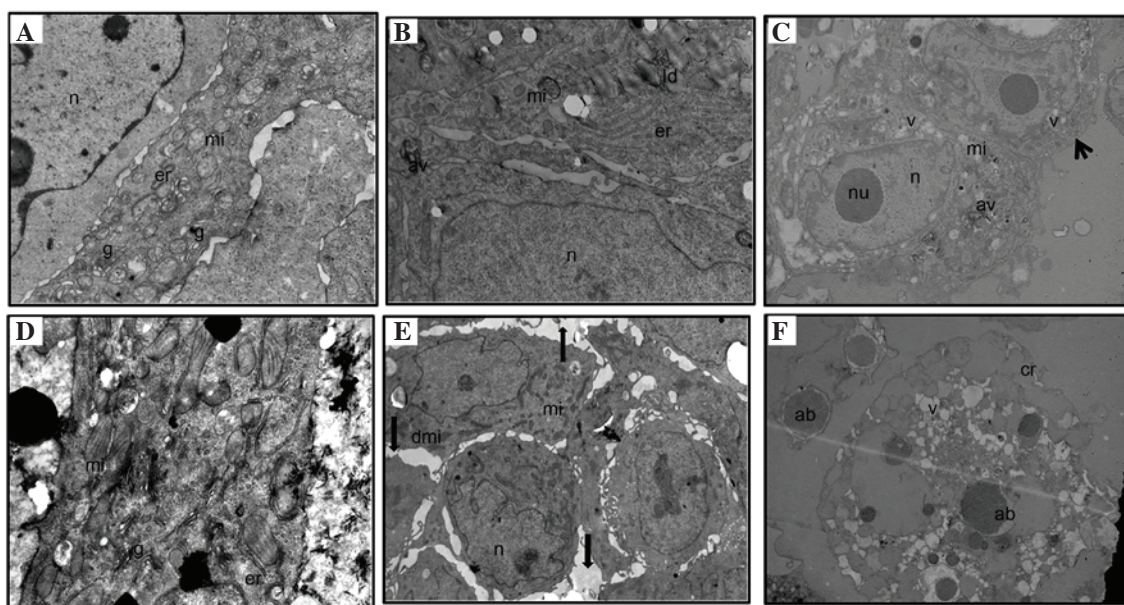


Figure 4. Ultrastructure analysis of spheroids of (A-C) stem cells and (D-F) non-stem cells. (A) Control group (magnification, x12,000), (B) 1 μ M LiCl group (magnification, x10,000), (C) 500 μ M LiCl group (magnification, x7,500; arrow indicates a healthy cell with some autophagic vacuoles), (D) control group (magnification, x12,000), (E) 1 μ M LiCl group (magnification, x6,000; arrows indicate gaps between cells in the spheroid), (F) 500 μ M LiCl group (magnification, x7,500). n, nucleus; mi, mitochondria; er, endoplasmic reticulum; g, golgi apparatus; ld, lipid droplets; nu, nucleolus; av, autophagic vacuoles; dmi, damaged mitochondria; cr, cell remnants (lytic cells); ab, apoptotic bodies.

group, displaying intact spheroid integrity (i.e., strong cell-to-cell adhesions), heterochromatic nucleus, healthy

mitochondria and prominent endoplasmic reticulum. One or two autophagic vacuole-like structures and numerous lipid

droplets were observed in the stem cell group, however, no membrane breakdown in the plasma and nucleus membranes, and no vacuoles were observed (Fig. 4A and B). Treatment of stem cells with the highest concentration of LiCl (500 μ M) resulted in 3 various cell ultrastructures: i) Apoptotic cells; ii) necrotic cells; and most frequently, iii) healthy cells, some of which had 3-5 autophagic vacuoles (Fig. 4C). The control group of non-stem cells showed a healthy structure, as did the control group of stem cells. The non-stem cell group treated with 1 μ M LiCl had a similar structure to the control group with certain exceptions, such as the loss of spheroid integrity (i.e. weak and/or lost cell-to-cell adhesions) in certain spheroids and a few damaged mitochondria in certain cells (Fig. 4D and E). By contrast, destructive images were frequently observed in non-stem cells treated with 500 μ M LiCl, including no spheroid structure only apoptotic bodies, the presence of single cells with apoptotic appearance, and cell remnants (lytic cells). No healthy cells were observed (Fig. 4F).

Discussion

To the best of our knowledge, the present study showed for the first time that the effects of LiCl on PCa stem cells are also concentration-dependent/biphasic, as in normal prostate cells, and MK levels are changed directly proportional to this effect in PCa stem cells *in vitro*. Hossein *et al* (25) previously evaluated LiCl with various concentrations (2.5, 10 and 25 mM) in the androgen-independent human prostate cell line, DU145. In the study, the viability of DU145 cells in the presence or absence of LiCl (2.5-25 mM) was assessed as a percentage of viable cells compared with the control (absence of LiCl). After 48 h, DU145 cells showed a 32 and 53% reduced cell viability with 10 and 25 mM LiCl, respectively, and a significantly decreased cell viability of 13% was observed with low and high doses of LiCl after 72 h (25). In addition, LiCl [half maximal inhibitory concentration (IC_{50}), 20 nM] was combined with IC_{50} concentrations and low concentrations of other well-known anti-neoplastic drugs, such as doxorubicin (Dox), etoposide (Eto) or vinblastine (Vin) (25). The study determined the synergistic effect of LiCl with these drugs and concluded that the IC_{50} concentrations of all three drugs combined with LiCl demonstrated a decreased cell percentage in the G1 phase and increased p53 levels compared with the control or LiCl alone (25).

A different study performed by Azimian-Zavareh *et al* (26) used androgen-dependent PCa LNCap cells and the same drug treatments (Dox, Eto and Vin). The results showed that LiCl increases apoptosis of these cells in the presence of Eto, which is S and G2 phase-specific drug. Suganthi *et al* (15) treated human breast cancer cells (MCF-7) with low (1, 5 and 10 mM) and high (50 and 100 mM) concentrations of LiCl. The results were similar to those of Hossein *et al* (25) and Azimian-Zavareh *et al* (26), and indicated that LiCl induces cell survival by inhibiting apoptosis through regulation of GSK-3 β , caspase-2, Bcl2-associated X protein and cleaved caspase-7, and by activation anti-apoptotic proteins (Akt, β -catenin, B cell lymphoma-2, and cyclinD1). However high concentrations induced apoptosis by reversing these effects. Considering the results of the aforementioned studies, it can be

concluded that LiCl exhibits a cytotoxic effect in a dose- and time-dependent manner.

The etiology of cancer is complex and appears to include several mechanisms: i) Normal cells with mutations or epigenetic changes can become cancer cells; ii) normal stem cells can transform into CSCs via specific mechanisms; iii) CSCs can originate from cancer cells that are hierarchically downstream of CSCs but have not differentiated and have acquired the capacity for self-renewal; and iv) cancer cells can be derived from progenitor cells or from more differentiated cells via a dedifferentiation process (EMT) (6,27-32). EMT appears to have an important role by endowing cells with some of the characteristics and behaviors of CSCs (33). It is unclear which characteristic is responsible for cancer progression or metastasis. However, it is clear that only one injured cell or one cell that manages to escape from therapy, regardless of whether it is a stem cell or non-stem cell, can lead to the progression and recurrence of cancer, consequently causing therapy to collapse (6,29). Furthermore, it is well-known that CSCs in the bulk of the tumor are responsible for poor prognosis and therapy resistance, particularly in chemotherapy. Preclinical and clinical trials targeted to eradicate this population have improved prognosis (29,34). Wang *et al* (32) used afatinib, a small-molecule inhibitor of the epidermal growth factor receptor and erb-b2 receptor tyrosine kinase 2 and 4 tyrosine kinases, in order to preferentially eliminate side population cells with CSC characters in cell lines and patient-derived leukemia cells, by decreasing ATP binding cassette subfamily G member 2 expression. In these cells, afatinib also acted in parallel to suppress self-renewal capacity and tumorigenicity. CSC self-renewal is one approach to cancer therapy, amongst others.

The Wnt/ β -catenin signaling pathway, which is one of the major targets of LiCl, has a significant role in several developmental processes and the maintenance of adult tissue homeostasis by regulating cell proliferation, differentiation, migration, genetic stability and apoptosis, and maintaining adult stem cells in a pluripotent state (35). Silva *et al* showed that the application of 20 and 40 mM LiCl increased the number of stem-like cells in retinoblastoma through the Wnt/ β -catenin pathway, maintaining stem cell renewal and leading to tumor formation (36). Concomitant with the study by Silva *et al* (36), Cai and Zhu (37) showed that LiCl improved the self-renewal of human gastric cancer stem-like cells by using 10 mM LiCl. Another study by Teng *et al* (38) stimulated the Wnt/ β -catenin signaling pathway of lung cancer cells via treatment with 10 mM LiCl. This resulted in enhanced proliferation, clone formation and drug resistance abilities of the cells through an increase in the number of stem cells, as determined by upregulation of the stem cell marker, OCT-4.

MK is highly correlated with cancer resistance and EMT (9,10,14). Wnt ligands bind to Frizzled-LRP5/6 receptor complexes and activate the cytoplasmic scaffold protein, Dishevelled, resulting in inhibition of β -catenin phosphorylation and degradation (38). One of the candidate receptors of MK is LRP, therefore, MK and Wnt signaling pathways have a common receptor (14).

In contrast to the studies by Silva *et al* (36), Cai and Zhu (37), and Teng *et al* (38), the present study used micromolar concentrations of LiCl following initial optimization

experiments. Similar to studies by Hossein *et al* (25) and Azimian-Zavareh *et al* (26), which used the same cell lines as the present study, low concentrations of LiCl were found to increase stem cell proliferation as well as non-stem cell proliferation in the current study; however, opposite results were obtained in the high LiCl concentration groups. In addition, in the low LiCl concentration groups, the apoptotic index was very low and MK levels were very high. Opposite results were obtained in the high LiCl concentration group. Furthermore, the stem cell group with the lowest apoptotic index and highest MK levels increased their cell numbers significantly more than non-stem cells during low LiCl concentration stimulation. The stem cell group was only marginally effected by the application of high LiCl concentrations, exhibiting a lower apoptotic index and higher MK levels than non-stem cells.

Ultrastructure evaluation of stem cell and non-stem cell spheroids treated with the lowest (1 μ M) and highest (500 μ M) concentrations of LiCl showed the biphasic effect more prominently. Ultrastructure evaluation showed that stem cells treated with the lowest concentration of LiCl (1 μ M) had similar ultrastructure to the control group. By contrast, stem cells treated with 500 μ M LiCl showed apoptotic and necrotic cells, as well as numerous healthy cells. That means healthy spheroids are common scheme. The ultrastructure of non-stem cells at 1 μ M LiCl was also similar to the control group; a low degree of damage was rarely observed, including loss of cell-to-cell interactions and spheroid integrity. LiCl (500 μ M) damaged non-stem cells severely, as indicated by their apoptotic appearance, cell remnants (lytic cells), apoptotic bodies and the loss of spheroid integrity (numerous single damaged cells). Comparison of the electron micrographs of the stem and non-stem cell control groups (prior to treatment) revealed no differences.

EMT is characterized by the loss of polarity of epithelial cells and decreased adhesion with surrounding cells, resulting in single, motile cells (i.e., metastatic ability). Therefore, the inhibition of cellular adhesive ability is associated with EMT (33,39). According to this data, no loss of spheroid integrity and no single cell motility was observed in the group treated with the lowest LiCl concentration, where high levels of MK were determined. This indicates that MK may not exhibit its mechanism of action through EMT. MK levels in the stem cell groups treated with high concentrations of LiCl were higher than the non-stem cell group, however, MK levels did not appear to be involved in the PCa stem cell fate resistance pathway. A previous study revealed the co-existence of multiple genetically diverse clones within the same tumor (40). Upon treatment, this intratumoral diversity, which is associated with distinct treatment sensitivity due to genetic heterogeneity, caused different clones to exhibit distinct mechanisms of resistance within the same tumor. Similar to normal tissue, certain types of cancer have hierarchical organization where tumorigenic CSCs differentiate into non-tumorigenic progenies (29,40). We propose that this hierarchy may explain why MK levels in PCa stem cells were lower than expected. Hierarchy may have occurred spontaneously or after LiCl application. Thus, MK levels are not involved in the biphasic effect of LiCl, however, they are affected proportionally.

In conclusion, the present study showed that LiCl acted through cancer suppression or promotion through stem cells in

an *in vitro* prostate cancer model in a concentration dependent manner. In addition, the underlying mechanism was evaluated through MK, which is a neglected biomarker in numerous studies. The present study concluded that MK had no effect on the prostate cancer stem cell resistance mechanism during LiCl administration. The current findings elucidate the important pathophysiological process that drives PCa progression toward resistance/lethality during chemotherapy with LiCl.

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