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Lithium Has Neuroprotective Effect On Neuroblastoma Cell Line In Low Dosages

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Research Article

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ABSTRACT

Lithium (Li) was presented as a protective agent in neuron degeneration, which is an important process in neurodegenerative diseases. This study aimed to determine the effect of specific amounts of Li on neuroblastoma cells by considering several specific genes which act on neuroprotection. Li solutions were prepared as 1 µM, 15 µM (low dosage), 30 µM and 45 µM (high dosage) concentrations and then applied to the neuroblastoma cell line. XTT and trypan blue assays were performed to determine cell proliferation and viability. mRNA expression levels of NES, BDNF, GRIN2A, LRRK2, PRKN, and SNCA were detected by quantitative real-time polymerase chain reaction (qRT-PCR). Cell viability was detected as significantly increased in cells treated with low dosage Li. However, it was significantly decreased in high-dosage applied cells compared to untreated control. In addition, cell proliferation ratios were significantly decreased in high-dosage applied cells compared to the control. It was demonstrated that mRNA expression levels of several genes (NES, LRRK2, PRKN) were significantly upregulated.

Regarding BDNF, gene expression was significantly upregulated in the cells only treated with very low amount of Li. However, no significant data could be obtained for GRIN2A. Furthermore, the mRNA expression level of SNCA was determined as significantly downregulated compared to the control. Statistically significant expression of NES, LRRK2, PRKN, BDNF, and SNCA genes due to the variable Li concentrations applied to cells suggests that Li acts on transcriptional regulation of certain genes associated with neuronal survival. These findings support that dose-dependent Li treatment might have a protective effect on neurodegenerative diseases.

Keywords: Neurodegeneration, lithium, neuroprotection, gene expression

Lityumun Düşük Dozajlarda Nöroblastoma Hücre Hattı Üzerinde Nöroprotektif Etkisi

ÖZ

Lityum (Li), nörodejeneratif hastalıklarda önemli bir süreç olan nöron dejenerasyonunda koruyucu bir ajan olarak sunulmuştur. Bu çalışmanın amacı, belirli miktarlarda Li'nin nöroblastoma hücreleri üzerindeki etkisini, nöroproteksiyon üzerinde etki eden birkaç spesifik geni dikkate alarak belirlemekti. Li solüsyonları 1 µM, 15 µM (düşük doz), 30 µM ve 45 µM (yüksek doz) konsantrasyonlarında hazırlandı ve ardından nöroblastoma hücre hattına uygulandı. Hücre proliferasyonunu ve canlılığını belirlemek için sırasıyla XTT ve tripan mavisi deneyleri yapıldı. NES, BDNF, GRIN2A, LRRK2, PRKN ve SNCA'nın mRNA ekspresyon seviyeleri, kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) ile tespit edildi. Hücre canlılığı, düşük dozda Li ile tedavi edilen hücrelerde önemli ölçüde arttığı tespit edildi, ancak yüksek doz uygulanan hücrelerde, tedavi edilmeyen kontrole kıyasla önemli ölçüde azaldı. Ayrıca yüksek doz uygulanan hücrelerde hücre çoğalma oranları kontrole göre önemli ölçüde azaldı. Birkaç genin (NES, LRRK2, PRKN) mRNA ekspresyon seviyelerinin önemli ölçüde yukarı regüle edildiği gösterildi. BDNF ile ilgili olarak, genin ekspresyonu, yalnızca çok düşük miktarda Li ile tedavi edilen hücrelerde önemli ölçüde yukarı doğru düzenlenmiştir. Ancak GRIN2A için anlamlı bir veri elde edilememiştir. Ayrıca, SNCA'nın mRNA ekspresyon seviyesinin kontrole kıyasla önemli ölçüde aşağı regüle edildiği belirlendi. Hücrelere uygulanan değişken Li konsantrasyonları nedeniyle NES, LRRK2, PRKN, BDNF ve SNCA genlerinin istatistiksel olarak anlamlı ifadesi, Li'nin nöronal sağkalım ile ilişkili bazı genlerin transkripsiyonel düzenlemesi üzerinde etkili olduğunu düşündürür. Bu bulgular, doza bağlı Li tedavisinin nörodejeneratif hastalıklar için koruyucu bir etkiye sahip olabileceğini desteklemektedir.

Anahtar sözcükler: Nörodejenerasyon, lityum, gen ekspresyonu

Süreç

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Introduction

Neurodegeneration is the main process of neurodegenerative disease. It is caused by the progressive loss of function and structure of a neuron. There are various neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), multiple sclerosis (MS), Parkinson's disease (PD), and Alzheimer's disease (AD). Today, there is no therapeutic application in reversing the progression of neuron degeneration[1, 2].

Li, which is used to treat mood disorders such as mania, hypomania, depression, and bipolar disorder, has a neuroprotective effect in the brain when it is daily used[3, 4, 5]. Li provides neuroprotection by inactivating N-methyl-D-aspartate (NMDA) receptor. It decreases gene expressions of pro-apoptotic proteins such as p53 and Bax while increasing the expression of cytoprotective proteins such as Bcl-2. In addition, Li activates Akt (Protein kinase B), known as 'cell survival kinase,' which plays an active role in cell survival[3, 6]. Moreover, it has been demonstrated that Li changes the expression of the *NES* gene, which is an intermediate filament and highly expressed in neural progenitor cells[4, 7, 8]. Despite these findings, the role of Li on neuron cells is still debated.

Neuroblastoma (NB) cell lines are derived from the neural crest and can be easily used for detecting the ability of neuronal differentiation with the application of various agents[9]. Many genes, predominantly expressed in neurons, play an active role in neuronal differentiation, survival, and regeneration. Of these, the Nestin protein, which NES encodes, is a biological marker for neuroblastoma cell lines. In addition, Brain-Derived Neurotrophic Factor (*BDNF*) encodes a nerve growth factor protein. The binding of this protein to its cognate receptor promotes neuronal survival in the adult brain. The expression of this gene is reduced in AD and PD patients[10]. Glutamate Ionotropic Receptor NMDA Type Subunit 2A (*GRIN2A*) encodes the NMDA receptor subunit, an ion channel protein. This structure plays crucial roles in certain kinds of memory and learning[11].

NES (OMIM 600915), *BDNF* (OMIM 113505), and *GRIN2A* (OMIM 138253) genes are all required for survival, regeneration, and proliferation of neural progenitor cells by mitogen stimulus[12-14]. Moreover, Leucine Rich Repeat Kinase (*LRRK2*; OMIM 609007), Parkin RBR E3 Ubiquitin Protein Ligase (*PRKN*; OMIM 602544) and alpha-synuclein (*SNCA*; OMIM: 163890) gene with a single-nucleotide polymorphism (rs356219) are also associated with the etiology of neurodegenerative diseases such as PD and AD[15].

Here, the effect of lithium concentrations on neuroblastoma cells was evaluated by considering neuron viability, proliferation, and transcriptional regulation of certain genes which play a role in neuronal survival. Interestingly, differential mRNA expression of several neuron-specific genes (*NES, LRRK2, PRKN*, *BDNF*, *GRIN2A,* and *SNCA*) in neuroblastoma cells treated with variable concentrations of Li suggests that certain amount of Li acts on neuronal survival via activating several pathways.

Material Method

This study was designed with the Gülhane Military Medical Academy, GATA Ethics Committee decision [23/10/2015- GATA/AR-2015-22/Ethics Committee Decision 2015- Session (240) 05].

Preparation Of Li Solutions: Li (499811-Sigma-Aldrich) as an element was used in our experiments. Li was diluted in isotonic saline solution as 1 µM, 15 µM, 30 µM, 45 µM, 60 µM, 75 µM, and 90 µM concentrations.

Cell Culture: Neuroblast cell line (NE-4C, ATCC, Cat. No: CRL-2925) was cultured and grown in 6 well-plates with RPMI-8226 1640 (Sigma-Aldrich-R8758) including 10 % (v/v) fetal bovine serum (Biochrom AG, Germany), 1% (v/v) l-glutamine and 1% (v/v) gentamicin (Biological Industries, Israel) at 37°C in 5% CO_2.

Application Of Lithium: LD 50 dosage was found as 75 µM Li concentration. For study groups, 1 ml of Li solution for each concentration [Low dosage: (1µM, 15µM)], High dosage: (30µM, 45µM)]) was used. Control group cells were grown in culture medium and not treated with Li solutions. The cells were examined immediately after the Li application and 24 hours later.

XTT Cell Proliferation Assay: The cytotoxic effects of Li solutions were analyzed by using the protocol of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)- 5-[(phenylamino)carbonyl)]-2H-tetrazolium hydroxide) assay kit on neuroblastoma cells. In addition, the "XTT assay" kit (Trevigen XTT Cell proliferation assay kit-Cat No: 4891-025-K) was used to measure cell proliferation ratios in accordance with the manufacturer's instructions. In spectrophotometric viability tests such as XTT

assay, the viability of the untreated cells is accepted as 100%, and the viability of the treated cells is determined as a percentage (%) according to these cells[16]. In our experiments, neuroblastoma cells were seeded in a 96-well plate at a density of 10^3 - 10^5 cells/well in 100 µl of culture medium with or without lithium. Li was used in 1 µM, 15 µM, 30 µM, 45 µM, 60 µM, 75 µM, and 90 µM concentrations. We cultured the cells in a CO_2 incubator at 37°C for 24 hours. 10 µl XTT mixture was added to each well and incubated for 2 hours in a CO_2 incubator at 37°C. The results were obtained by using an "ELISA reader" with absorbance at 450 nm[17]. This study was repeated three times for each condition. LD 50 dosage was found as 75 µM Li concentration.

Cell Viability Assay: Trypan blue (Sigma Aldrich Co. 302643) was used as a stain to determine the cell viability. It was diluted at 0.8 mM in PBS and mixed with the cells in a 1:1 ratio. In total, a hundred cells were counted in each condition. Viable and non-viable cells were evaluated by using a hemocytometer (6+9)[18]. Three different studies were conducted for each condition.

RNA Isolation and Cdna Synthesis: All culture flasks were harvested by Trypsin-EDTA solution (Sigma Aldrich Cat No: T4049) 24 hours after Li applications. Total RNA was extracted via NucleoSpin RNA mini kit (NucleoSpin RNA, Mini kit for RNA purification/ Machenerey-Nagel) according to the manufacturer's protocol. cDNAs were synthesized by using RevertAid First Strand cDNA synthesis kit (ThermoFisher). The quality of cDNAs was checked with 2% agarose gel.

Qrt-PCR: The mRNA expression levels of *NES*, *BDNF*, *GRIN2A*, *LRRK2*, *PRKN*, and *SNCA* were analyzed. The primer sequences can be accessed in PrimerBank database (http://pga.mgh.harvard.edu/cgi-bin/primerbank). qRT-PCR conditions were 45 cycles of 95˚Cº 10'', 56˚C 15'', 72˚C 15'' (Roche Light Cycler 1.5). β-Actin [beta-actin housekeeping gene (5'-GTC CCT CAC CCT CCC AAA AG-3' (forward) and 5'-GCT GCC TCA ACA CCT CAA CCC-3' (reverse)] was used as an internal control. Each qRT-PCR reaction is performed in 20 µl. This study was repeated three times and results were analyzed by "Roche Light Cycler1.5 software" by $2^{-\Delta\Delta CT}$ method.

Statistical Analysis: Mean values and standard deviations were obtained for cell viability, proliferation assays as well as for qRT-PCR results. Student's *t* test was used to determine the statistical significance. $p<0.05$ was considered significant.

Results

NE-4C cells were treated with variable concentrations of Li solutions in low and high-dosage groups. It was detected that although cell viability was significantly increased in low-dosage Li-applied cells, it was significantly decreased in cells treated with high-dosage Li compared to untreated control neuroblastoma cells (Figure 1, Table 1). According to the XTT assay, all cell proliferation ratios were statistically insignificant in cells treated with low Li solutions. However, it was significantly decreased in the high amount of Li solution applied cells (Figure 2).

In this study, mRNA expression levels of *NES, BDNF, GRIN2A, LRRK2, PRKN*, and *SNCA* genes were determined by qRT-PCR both in the control and study group. Accordingly, it was demonstrated that mRNA expression of *NES* was significantly upregulated with the increased level of Li in the neuroblastoma cell line compared to untreated control (Figure 3A). the mRNA expression level of *LRRK2* was significantly upregulated by the treatment of 15 µM and 45 µM Li solutions to neuroblastoma cell line compared to control (Figure 3B). In addition, it was shown that *PRKN* gene expression was significantly increased only in 45 µM Li treated cells compared to untreated control (Figure 3C). No statistically significant data could be obtained for other applications. Furthermore, mRNA expression level of *BDNF* was significantly but slightly increased only in 1 µM Li treated cells compared to untreated control (Figure 3D). However, it was detected that mRNA expression of *SNCA* was significantly downregulated both in low and high dosage Li applied cells (Figure 3E). On the other hand, no significant data could be obtained for *GRIN2A* (Figure 3F). It should also be noted that there are some blank columns because of the clogged capillary tubes.

Table 1. Cell viability ratios of neuroblastoma cells in Li treated groups and untreated control.

		Low Dosage Group				High Dosage Group			
	Untreated	1µM	P value	15µM	P value	30µM	P value	45µM	P value
Neuroblastoma cells	93±4%	97±2%	p<0.05	95±4%	p<0.05	92±2%	p<0.05	90±3%	p<0.05

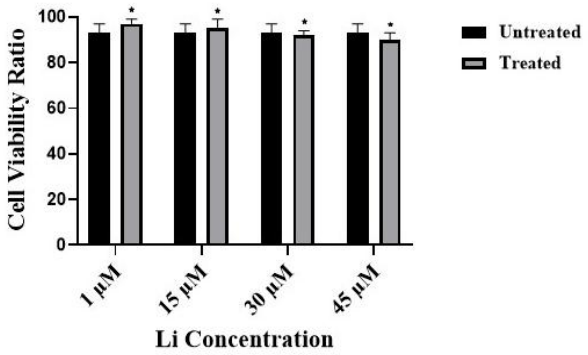

Figure 1. The effect of variable Li concentrations on viability of neuroblastoma cells.
X axis represents Li concentrations and y axis indicates cell viability ratios. P values represents the comparisons between untreated and treated cells. *: p<0.05

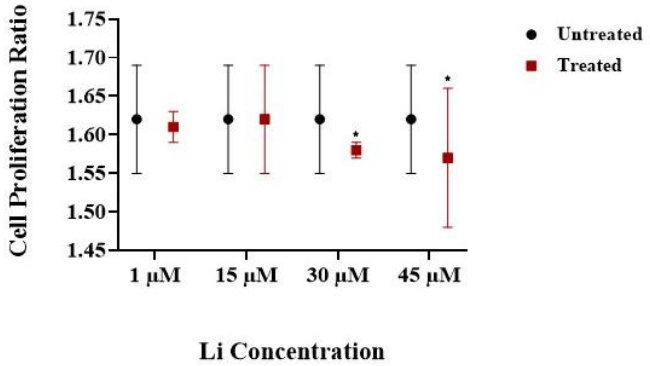

Figure 2. The result of XTT assay to estimate the proliferation ratio of neuroblastoma cells after Li treatments.
P values were calculated by the comparisons between untreated and treated cells. *: p<0.05
Proliferation ratios for 1 µM and 15 µM Li treated cells are not statistically significant (p≥0.05).

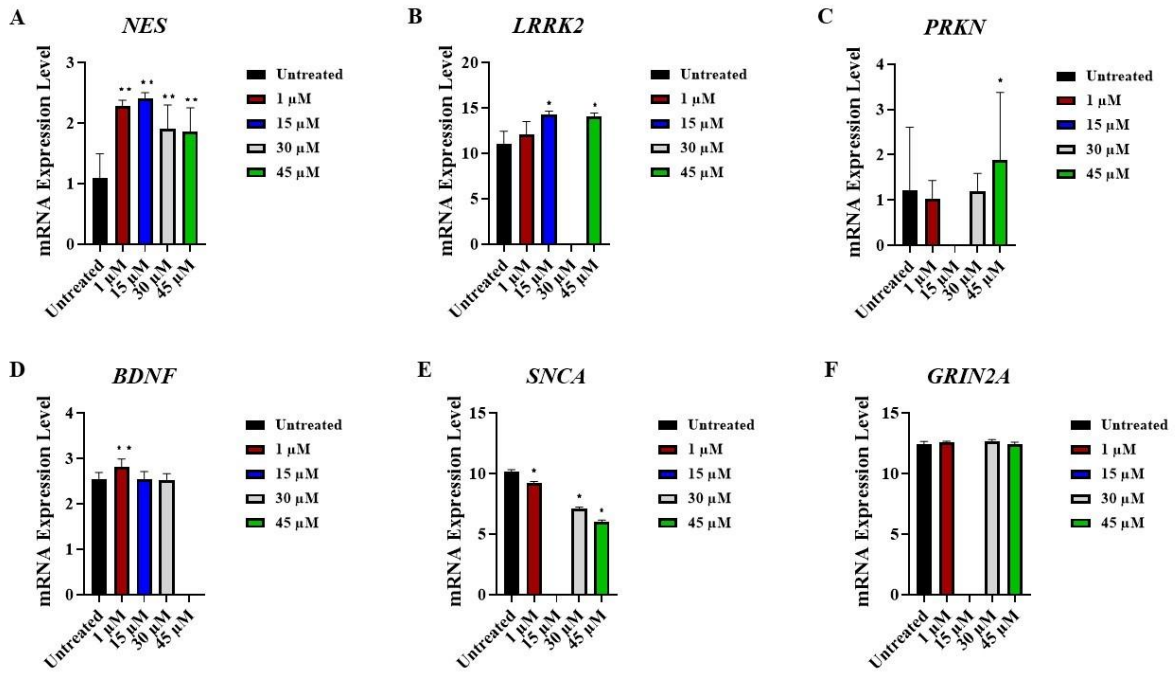

Figure 3. mRNA expression analysis of selected genes. Untreated represents control cells without Li application. *: p<0.05 **: p<0.005. P values were calculated by the comparisons between untreated and treated neuroblastoma cells. Blank columns represent not obtained data for related genes.

Discussion

The study results indicated that low-level Li increased neuron cell viability. However, cell viability and proliferation were decreased in high-level Li-applied neuron cells. Moreover, it was detected that Li was responsible for the transcriptional regulation of several neuron-specific genes.

Nerve cell degeneration can be identified as decreased functional activity and degeneration in nerve axons and their terminal branches. The destruction of the nerve cells occurs in the last step. These pathological conditions are pathognomonic in neurodegenerative diseases. The process of nerve degeneration is studied in the field of the neuroanatomy of the brain and neurophysiology of neural pathways[19]. Although specific protein accumulations and anatomic vulnerability typically define neurodegenerative diseases, they share many fundamental processes associated with progressive neuronal dysfunction and death caused by such as oxidative stress, programmed cell death, and neuroinflammation[20].

Li is an alkali metal which is naturally present in all vertebrate tissues and body fluids at low concentrations. It is used in manic-depressive illness as a drug. In addition, it was recommended to treat acute brain injury and chronic neurodegenerative diseases for its potentially beneficial effects[21, 22].

Interestingly, it was detected that neuron cell viability was decreased with the application of the increased amount of Li solution in this study. By the way, high dosage Li also decreased the cell proliferation. Li has been shown to increase cell proliferation in cerebellar granule and cerebral cortical cell cultures. Studies with bromodeoxy Uridine (BrdU) have demonstrated that this effect is in the S phase of replication[23]. This finding revealed that Li changed cell behavior (especially proliferation and apoptosis) by regulating gene expressions[23]. Furthermore, as high dosage Li decreased neuron cell proliferation in this study, it might be speculated that Li affected the mechanisms of necrosis or apoptosis. Reduced cell proliferation rate is correlated with decreased viability in cells treated with higher amount of Li. The results are consistent with the literature findings[24, 25]. This data also suggest that high level Li may be detrimental to neuron cells and dose adjustments should be done before use.

On the other hand, effects of Li were evaluated on the transcriptional regulation of several neuron specific genes which play an active role in neuron differentiation, survival, and regeneration. Accordingly, Li changed the mRNA expression level

of *NES* (Figure 3A). It is required for the survival, regeneration, and proliferation of neural progenitor cells[26]. Upregulation of *NES* suggests that it might stimulate neural progenitor cells and attempt to proliferate them upon application of Li. However, NES's mRNA expression level changed between low and high-dosage Li-applied cells. Although data are not statistically significant to make a comment on cell proliferation in cells treated with low dosage Li, it can be concluded that the proliferation ratio is decreased in high dosage Li applied neuron cells. This is also correlated with decreased mRNA expression of *NES* in neuroblastoma cells treated with high dosage Li. In addition, another neuron-specific gene, *LRRK2* encodes a kinase protein, and it is present in the mitochondrial outer membrane. Mutations (particularly G2019S) in this gene increase the kinase activity and have been associated with PD[27]. Several studies reported that LRRK2 protein involved in neurite outgrowth, autophagy, and immune cell functions[28,29]. Moreover, *PRKN* encodes parkin protein which functions as an E3 ubiquitin ligase and enhances cell survival by suppressing both mitochondria-dependent and -independent apoptosis[30]. Mutations in this gene are also known to cause PD [31]. Both *LRRK2* and *PRKN* genes were upregulated only in 15 µM and 45 µM Li applied cells, and data were not sufficient to make a precise comment about the effect of Li on these genes (Figure 3B-C). However, statistically significant upregulation of the gene m RNA levels in a certain amount of Li-treated neuroblastoma cells at least indicates the stimulation of neuroprotection-related pathways. In this situation, toxic effects of high amount of Li should be considered.

On the other hand, *BDNF* encodes a member of the nerve growth factor family proteins[32]. Expression of this gene is reduced in AD and PD patients. In addition, this gene plays a role in the regulation of the stress response and in the biology of mood disorders[33]. *BDNF* mRNA expression level was very slightly but significantly upregulated in neuroblastoma cells treated with a very low amount of Li, demonstrating the neuroprotection effect of low dose Li in neurons (Figure 3D). However, *GRIN2A* gene is associated with *NMDA* receptors, memory, and learning[33]. mRNA expression of *GRIN2A* was either not obtained or not statistically significant. For this reason, no precise comment could be declared in terms of the effect of Li on *GRIN2A* expression. Furthermore, alpha-synuclein is a neuronal protein that regulates synaptic vesicle trafficking and subsequent neurotransmitter release. It is abundant in the brain and is located mainly in the axon terminal of the presynaptic neurons. The release of neurotransmitters relay signals between neurons and are critical for normal brain function. *SNCA* has also been implicated in the pathogenesis of PD and SNCA peptides are a major component of amyloid plaques in the brains of patients with AD[34, 35].

Moreover, it is accumulated after spinal cord injury and causes damaged nerve fibers and neurons[36]. It was reported that downregulated alpha-synuclein triggered functional recovery, reduced neuroinflammation, and microglial activation in rats with spinal cord injury[36]. In addition, Zhao et al. reported that Li was involved in epigenetic regulation by decreasing DNA methylation, which eventually reduced the expression of alpha-synuclein[37]. Our study also showed that mRNA expression of *SNCA* was downregulated depending on the application of the increased amount of Li. It can be concluded that Li regulates the expression of *SNCA* via novel pathways or mechanisms to protect nerve cells.

To sum up, our data suggest that dosage-dependent Li treatment of neurodegenerative diseases such as AD and PD may reduce symptoms and disease progression via regulating the expression of several gene mRNA levels. This implies Li is a protective agent for neurodegenerative diseases. However, further clinical and molecular biology-based experimental studies should be done to understand how Li affects the expression levels of mRNAs of these genes and to reveal the exact mechanism of neuroprotection in more detail.

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Conflict Of Interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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