Research Article

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Bahareh Sadat Yousefsani, Romina Askian and Jalal Pourahmad*

A new approach on lithium-induced neurotoxicity using rat neuronal cortical culture: Involvement of oxidative stress and lysosomal/mitochondrial toxic Cross-Talk

<https://doi.org/10.1515/mgmc-2020-0003>

Received November 24, 2019; accepted March 04, 2020.

Abstract: Lithium (Li) is a widely-used medication for the treatment of patients with bipolar disorder. Li causes different complications. One of the most important adverse effects of Li is neurotoxicity. Neurotoxicity is usually irreversible which may lead to very important complications. The symptoms of Li-induced neurotoxicity include tremor, delirium, seizures, coma, and death. In this study, we wanted to evaluate the exact sub-cellular mechanisms of Li-induced neurotoxicity.

For this purpose, we used primary neuronal cortical culture for investigating lithium-induced neurotoxicity. We applied the postnatal rat pups for isolating the cortical neurons. After that, we evaluated neural viability, neural reactive oxygen species (ROS), lipid peroxidation, mitochondrial membrane potential (MMP), lysosomal membrane integrity (LMI), and reduced (GSH) and oxidized (GSSG) glutathione.

Our results demonstrated that the cytotoxic effect of Li has mediated through lysosomal membrane leakage associated with ROS formation and reduction of MMP. Furthermore, the incubation of isolated neurons with Li caused rapid GSH depletion (as GSSG efflux) as another marker of cellular oxidative stress.

We concluded that Li causes neurotoxicity in a dose-dependent manner. Besides, Li-induced neurotoxicity is a result of the generation of ROS and LP, which leads to mitochondrial/lysosomal toxic cross-talk.

Keywords: lithium; neurotoxicity; neuronal cortical culture; mitochondria; lysosome; oxidative stress

1 Introduction

Biologicals, chemicals, metals as well as medications could be considered as the neurotoxic agents (Clausen et al., 2019; Garza-Lombo et al., 2019; Horta et al., 2019; Richardson et al., 2019; Staff et al., 2019). These factors, depending on whether they affect the central or peripheral part of the brain, cause complications on the structure and function. This health issue can lead to serious problems such as memory impairment, learning difficulties, and visual and hearing impairment (Costa et al., 2020). Many efforts were conducted to find ways for preventing many diseases such as neurotoxicity; for example, the use of different types of antioxidants (Baradaran Rahimi et al., 2019; Huang et al., 2019; Ji et al., 2019; Mansoori et al., 2011; Sohrabvand et al., 2017; Yousefsani et al., 2019). Neurotoxicity is usually irreversible; therefore, it is very important to identify the causes of neurotoxicity and their mechanisms for finding ways for prevention (Ferraro et al., 2019; Kordafshari et al., 2017; Zhang et al., 2020). Lithium (Li) is a well-known medication for the treatment of patients with bipolar mood disorder. Recent researches demonstrated that Li could be helpful for the treatment of Alzheimer's disease (Hampel et al., 2019). It also could be effective for the treatment of Parkinson's disease (Arraf et al., 2012). This widely used medication has a narrow therapeutic index. This means that this medication is

* **Corresponding author: Jalal Pourahmad**, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran, P. O. Box: 141556153 tel: +98-2122558786, fax +98-2188209620, e-mail: j.pourahmadjaktaji@utoronto.ca

Bahareh Sadat Yousefsani, Research Institute for Islamic and Complementary Medicine, Iran University of Medical Sciences, Tehran, Iran, School of Persian Medicine, Iran University of Medical Sciences, Tehran, Iran

Romina Askian, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran

potentially toxic (Salimi et al., 2017a). Therefore, the small errors in prescription, drug interactions or patient mistakes in taking medications can lead to poisoning. Furthermore, some diseases can also increase Li plasma concentrations and cause toxicity, like impaired renal function, chronic causes of volume depletion, and infections (de Cates et al., 2017; Vodovar and Megarbane, 2017). Li is easily absorbed through the intestines, distributed in the body, and then almost completely excreted through the kidneys. Although Li neurotoxicity mainly occurs due to Li plasma levels above 1.0 mmol/L, in some cases, neurotoxicity could happen in the normal plasma concentration (Megarbane et al., 2014; Mesquita et al., 2010; Soriano-Barcelo et al., 2015). Therefore, it is important to note that plasma concentrations of Li should not only be considered but also, it is necessary to consider any clinical signs as a warning factor for the onset of toxicity. Most of the Li-induced toxicity occurs at therapeutic doses. Lethal concentrations of Li is above 3.5 mmol/L, suggesting that hemodialysis is the only treatment (Decker et al., 2015).

The symptoms of Li neurotoxicity include tremor, delirium, drowsiness, ataxia, muscle weakness and twitching, slurred speech, pseudodementia, psychomotor slowing, disorientation, seizures, coma, and death (Soni, 2019). There are important factors considered as risk factors for Li-induced neurotoxicity. These include: hypertension, heart failure, rapid correction of hyponatremia and hyperlipidemia, neurologic illness, enteric fever, acute gastroenteritis, and epilepsy (Lang and Davis, 2002; Malhotra et al., 1985; Mani et al., 1996; Swartz and Jones, 1994).

Fever is usually one of the common signs of Li intoxication (Donaldson and Cuninghame, 1983; Hansen and Amdisen, 1978). High body temperature induces protein degradation, therefore, pathologic changes would be observable in the nerve and glial cells (Hancock and Vasmatzidis, 2003). Usually, in the early stages of lithium intoxication, body temperature is normal, but as the toxicity progresses, body temperature increases. Increasing body temperature can also cause dehydration and further poisoning (Schou et al., 1968).

Researches revealed that oxidative stress production is the most important reason for Li toxicity (Salimi et al., 2017a). Li can increase the Ca^{2+} level in the cultured cerebellar granule neurons and causes neurotoxicity (Yao et al., 1999). Besides, Li induced lipid peroxidation (LP) in synaptosomes, which leads to the modification of synaptic endings and the lipid content of synaptoplasmatic membranes that consequently leads to severe disturbances in the function of neurotransmitter uptake systems and depolarization-dependent calcium channels (Efendiev

and Kerimov, 1994; Sawas and Gilbert, 1985; Sawas et al., 1986). This can lead to depression, sleep disorders, and other important neurotoxic signs (Taranova and Nilova, 1986).

Although neurotoxicity signs of Li have been recognized recently, the exact sub-cellular mechanisms are still unclear. For this purpose, the rat primary neuronal cortical culture was used for evaluation of mechanisms behind the Li-induced neurotoxicity.

2 Materials and methods

2.1 Chemicals

10×Hank's balanced salt solution (HBSS), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) buffer, fetal bovine serum (FBS), neurobasal medium, minimal essential medium (MEM) with Earle's salts and L-glutamine, penicillin-streptomycin, gluta MAX-I supplement, B27 serum-free supplement, trypsin, cell-freezing medium from was purchased Invitrogen (Burlington, ON). Sodium pyruvate, Li^+ carbonate, D-glucose, selenium dioxide, putrescine, progesterone, bovine transferrin, insulin from Sigma (Oakville, ON, Canada).

2.2 Animals

All experiments and procedures in this study were performed in full compliance with the NIH Guide for the Care and Use of Laboratory Animals guidelines for animal research and Shahid Beheshti University of Medical Sciences Animal Ethics Committee. Experiments were held on young (1-day-old pups) male Wistar rats.

2.3 Isolating the neurons

Isolating the neurons were done based on Meberg and Miller method (Meberg and Miller, 2003). Under a laminar flow hood, the brains of postnatal rat pups were removed and placed in a dish containing CMF-HBSS (calcium-, magnesium-, and bicarbonate-free Hank's balanced salt solution). Under a microscope, the cerebral hemispheres and hippocampus were removed carefully and transferred to a drop of CMF-HBSS and chopped the tissues as finely as possible and then transferred to a 50 mL conical centrifuge tube in a final volume of 12 mL CMF-HBSS containing 1.5 mL each of 2.5% trypsin and 1% (wt/vol) DNase and

incubated in a 37°C water bath for 5 min. After triturating the cell suspension, the chunks of undissociated tissue were removed by passing through a cell strainer. The centrifugation was done (10 min, 1000 rpm) to remove the enzymes and lysed the cells and the supernatant was discarded. Hemocytometer was used to calculate cell density. Cells were suspended with the Glial Medium (430 mL DMEM, 50 mL FBS, 5 mL penicillin, 5 mL sodium pyruvate) and the cell density was adjusted to 10^6 cells/mL in the flasks. Each flask contained 10 mL of isolated neurons suspension. The neurons were used for toxicological tests after one day of incubation (Yousefsani et al., 2020).

Before performing each test, we centrifuge the cell culture (1 min, 1000 rpm) to separate cells from the culture medium and other substances in it. Isolated neurons were separated in the same volume of incubation buffer (Krebs 750 mL and HEPES 2.25 g, pH 7.4). This was done to reduce interference (Pourahmad et al., 2011).

2.4 The viability of neurons

The trypan blue (0.2% w/v) exclusion test was performed for determination of the survival of isolated neurons with the intactness of the plasma membrane. Taking the samples of the incubated neurons was done at different time points during 24 h (Pourahmad and O'Brien, 2000).

2.5 Determination of reactive oxygen species

Dichlorofluoresceindiacetate (DCFH-DA, 1.6 μ M) was used for determination of Li-induced ROS formation. 3 mL of isolated neurons suspension (3×10^6 cells/mL) were centrifuged (1 min, 1000 rpm) then, the supernatant was discarded. The isolated neurons were incubated in the test tube containing dye (10 min, 37°C). DCFH-DA penetrated isolated neurons and hydrolyzed to non-fluorescent dichlorofluorescein (DCFH). The fluorescence intensity of DCF was measured using a Shimadzu RF5000U fluorescence spectrophotometer. Excitation and emission wavelengths were 500 nm and 520 nm, respectively. The results were expressed as fluorescent intensity per 10^6 cells (Shen et al., 1996).

2.6 Lipid peroxidation assay

The amount of TBARS (thiobarbituric acid-reactive substances) was measured during the decomposition

of lipid hydroperoxides. 3 mL phosphoric acid (1%) and 1 mL thiobarbituric acid (0.6%) was added to 0.5 mL of centrifuged neurons (1 min, 1000 rpm). After that the mixture was heated in a boiling water bath (45 min). 4 mL of n-butanol was added to the mixture and centrifuged (10 min, 5000 rpm) after cooling. The organic layer was transferred to a quartz cuvette. The PG Instruments T60V spectrophotometer was used for measuring the absorbance (Khansari et al., 2017).

2.7 Intracellular GSH and extra cellular GSSG assessment

The spectrofluorometric method was used for evaluation of GSH and GSSG (Hissin and Hilf, 1976). In this method a reaction between orthophthalaldehyde (OPT) (1 mg/mL) and GSH (in pH = 8) and GSSG (in pH = 12) in incubation buffer (Krebs 750 mL, HEPES 2.25 g, pH 7.4) formed a fluorescent substance. Therefore, for evaluating GSH, after centrifugation (1 min, 1000 rpm) of 1 mL of neuron suspension, the culture medium was excluded. Then, 1 mL trichloroacetic acid (10% w/v) was added to the neurons and centrifuged (10 min, 5000 rpm) again. 4.4 mL of incubation buffer (pH = 8) was added to 0.5 mL of supernatant. After that, 100 μ L OPT (1 mg/mL) was added to the above-mentioned solution and left for 15 min (at room temperature) and then transferred to a quartz cuvette. For evaluating GSSG, 200 μ L of N-ethylmaleimide (0.04 N) was added to 0.5 mL of supernatant to prevent oxidation of GSH to GSSG. 4.2 mL of incubation buffer (pH = 12) was added to this mixture, after that, 100 μ L OPT (1 mg/mL) was added to the above-mentioned solution and left for 15 min (at room temperature). Then, it was transferred to a quartz cuvette. Each sample was measured using a fluorometer (350 nm excitation and 420 nm emission wavelengths) (Salimi et al., 2017b).

2.8 MMP assay

Mitochondrial uptake of the cationic fluorescent dye, rhodamine123, has been used for estimation of MMP (Kiani et al., 2017). For exclusion the culture medium, 0.5 mL of neuron suspension was centrifuged (1 min, 1000 rpm) and then 2 mL of rhodamine123 1.5 μ M was added to the cells. After 10 min of incubation (37°C), the suspension was centrifuged (1 min, 1000 rpm) again. Then, the supernatant was transferred to a quartz cuvette. Each sample was measured using a fluorimeter (490 nm

excitation and 520 nm emission wavelengths). Our data were shown as the percentage of MMP collapse ($\%\Delta\Psi_m$) in all groups.

2.9 LMI assay

Isolated neuron lysosomal membrane stability was evaluated by redistribution of the fluorescent dye, acridine orange (AO) (Pourahmad et al., 2005). For exclusion the culture medium, 0.5 mL of neuron suspension was centrifuged (1 min, 1000 rpm) and then, 2 mL AO (5 μ M) was added to the cells. After 10 min of incubation (37°C), the suspension was centrifuged (1 min, 1000 rpm) again. Then, the supernatant was transferred to a quartz cuvette. The absorbance was measured using a fluorimeter (495 nm excitation and 530 nm emission wavelengths). Our data were shown as the percentage of lysosomal membrane leakiness in all groups.

In all tests, α -Tocopherol succinate as lipid antioxidant, mannitol as hydroxyl radical scavenger, carnitine as MPT pore sealing agents and chloroquine as lysosomotropic agent was added to the isolated neuron culture 1 h before Li administration. These concentrations were chosen based on previous researches (Pourahmad et al., 2011; Yousefsani et al., 2018). None of these protective agents showed any toxic effects or interaction on isolated neurons or experiments at concentrations used (data not shown).

2.10 Evaluation of apoptosis

For quantification the percentage apoptosis versus necrosis by flowcytometric analysis (Cyflow Space-Partec), Annexin V fluorescein isothiocyanate (FITC)/propidium iodide (PI) stained cells was applied. Annexin V/PI assay was done using a commercial kit (Immunotech; Beckman Coulter, Dubai, United Arab Emirates) according to the manufacturer's instructions (Salimi et al., 2015).

2.11 Statistical analysis

The statistical analysis was done using the Graph Pad Prism software, version 8.0.2 (Graph Pad Software, San Diego, CA, USA). Data are reported as mean $\pm$ standard deviation of three separated tests. Data were analyzed using one-way analysis of variance followed by Tukey-Kramer test as the post hoc test. The minimal level of significance chosen was $p < 0.05$.

3 Results

3.1 Effect of Li on cell viability in isolated neural culture

As shown in Figure 1, Li-treated neurons after 2 h showed a significant decrease in cell viability in a dose-dependent manner ($p < 0.001$). IC₅₀ for Li after 2 h exposure was 1.98 ± 0.87 mM.

3.2 Effect of Li on ROS formation in isolated neural culture

As shown in Figure 2, Li produced a marked increase in ROS generation ($p < 0.001$) in a dose-dependent manner.

3.3 Effect of Li on lipid peroxidation in isolated neural culture

As demonstrated in Figure 3, Li significantly increased the TBARS ($p < 0.001$) in a dose-dependent manner.

3.4 Effect of Li on MMP in isolated neural culture

Because of neuronal ROS formation, Li induced a rapid decline of MMP, an apparent marker of mitochondrial dysfunction ($p < 0.001$) (Figure 4).

3.5 Effect of Li on LMI in isolated neural culture

When the isolated neurons were loaded with a lysosomotropic agent (acridine orange), severe oxidative damage to lysosomal membrane demonstrated a significant ($p < 0.001$) release of acridine orange into the cytosolic fraction ensued within 2 h of incubation with Li (Figure 5).

3.6 Effect of Li on intracellular GSH and efflux GSSG

As demonstrated in Figure 6, incubation of neurons with Li caused another marker of cellular oxidative stress, rapid neural glutathione (GSH) depletion. Most of the Li-induced

Figure 1: Effect of different concentrations of Li on neuronal death using primary neuronal cortical culture. Determination of cytotoxicity was done as the percentage of cells that absorb trypan blue. Values are shown as mean $\pm$ SD of three separate experiments ($n = 3$). ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). $\phi\phi\phi$ $p < 0.001$, significant difference in comparison with Li (1 mM)-treated neurons.

Figure 2: Effect of different concentrations of Li on ROS formation using primary neuronal cortical culture. Reactive oxygen species were determined spectrofluorometrically by the measurement of highly fluorescent DCF. Values are shown as mean $\pm$ SD of three separate experiments ($n = 3$). ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). $\phi\phi\phi$ $p < 0.001$, significant difference in comparison with Li (1 mM)-treated neurons.

GSH depletion could be ascribed to the dismissal of GSSG (Pourahmad et al., 2011).

4 Discussion

The results of this study revealed that 50% of the neural cells were lysed at a concentration of 1 mmol/L

(IC₅₀). In addition, Li significantly increased the ROS formation at EC₅₀ concentration in neuronal cortical culture. Furthermore, a significant amount ($p < 0.05$) of TBARS formed in the third hour of incubation because of Li-induced lipid peroxidation. Li-induced cytotoxicity, ROS and TBARS generation were prevented by α -Tocopherol succinate as lipid antioxidant, mannitol as hydroxyl radical scavenger, carnitine as MPT pore sealing

Figure 3: Effect of different concentrations of Li on lipid peroxidation using primary neuronal cortical culture. TBARS formation was measured spectrophotometrically and expressed as μM concentrations. Values are shown as mean $\pm$ SD of three separate experiments ($n = 3$). *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). $\phi\phi\phi$ $p < 0.001$, significant difference in comparison with Li (1 mM)-treated neurons.

Figure 4: Effect of different concentrations of Li on MMP collapse using primary neuronal cortical culture. The difference in mitochondrial uptake of the rhodamine 123 between the untreated control and Li treated cells is the biochemical basis for the measurement of the percentage of MMP decline. Values are shown as mean $\pm$ SD of three separate experiments ($n = 3$). ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). $\phi\phi\phi$ $p < 0.001$, significant difference in comparison with Li (1 mM)-treated neurons.

agent and chloroquine as lysosomotropic agent. Previous studies demonstrated that chemicals and metals, as well as some medications, which stimulate ATPases, could inhibit lipid peroxidation. It seems that there are close relationships between the stimulation of ATPase activities and inhibition of lipid peroxidation in cerebral cortex synaptosomes (Gilbert and Sawas, 1983; Sawas and Gilbert, 1984).

Li causes mitochondrial membrane potential collapse that was prevented by radical scavengers (mannitol) and lipid antioxidants (α -Tocopherol succinate). This indicates

that the decline of mitochondrial membrane potential is a consequence of ROS formation and lipid peroxidation. One of the most important organelles, which play an exclusive role in neuronal cell survival or death, is mitochondria. Mitochondria supply energy for metabolism in the neurons (Clark and Nicklas, 1970). They are responsible for more than 90% of the cellular ATP generation. Mitochondria also regulate cellular death pathways (Santos et al., 2010). It is thought that mitochondrial dysfunction causes increased production of ROS through damaged respiratory chain, increased lipid peroxidation and impairment of beta-

Figure 5: Effect of different concentrations of Li on lysosomal membrane degradation using primary neuronal cortical culture. The redistribution of acridine orange from lysosomes into cytosol in acridine orange loaded neurons was assigned as a biochemical basis for the measurement of lysosomal membrane injury. Highly fluorescent acridine orange redistribution was determined spectrofluorometrically in treated neurons and shown as the percentage of neurons lysosomal membrane leakage in all groups in three different time intervals. Values are shown as mean $\pm$ SD of three separate experiments ($n = 3$). ** $p < 0.01$, *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). $\phi\phi\phi$ $p < 0.001$, significant difference in comparison with Li (1 mM)-treated neurons.

oxidation. This can trigger the release of pro-apoptotic (TNF- α) and pro-fibrotic (TGF- β) cytokines by Kupffer cells leading to cell death, inflammation and fibrosis (Sepahi et al., 2016). In the previous studies, the effect of Li on isolated heart mitochondria was evaluated. Li increased mitochondrial dysfunction by increasing ROS formation and lipid membrane peroxidation. Li also inhibited the activity of succinate dehydrogenase and reduced ATP production in mitochondria (Salimi et al., 2017a). There are medications that induce ROS formation by increasing Fe^{2+} accumulation or redox cycling (Castro et al., 2011). This may cause mitochondrial nitrative and/or oxidative changes as a triggering factor for harmful alteration, such as unfolding, aggregation, and fragmentation of proteins or catalytic enzymes. All promoting degradations of modified proteins lead to autophagy and/or mitophagy (Bayeva et al., 2013). Damages to mitochondria can lead to necrotic/apoptotic cellular and neuronal damages; therefore, the MPT pore sealing agents such as carnitine potentially reduces ROS formation and neural deaths.

Li-induced AO release was also prevented by hydroxyl radical scavengers (mannitol), lipid antioxidant (α -Tocopherol succinate), and MPT pore sealing agent (carnitine). This means that any damage to the lysosomal membrane of nerve cells occurs due to increased ROS and TBARS formation. The results of this study revealed that Li induced lysosomal membrane damage leading to protein degradation, lipid peroxidation, and mitochondrial dysfunction. This is similar to the damages caused by the formation of intracellular ROS such as H_2O_2 , as a result of CYP450 mediated metabolic activation (Razavi-

Azarkhiavi et al., 2014; Seglen and Gordon, 1982). H_2O_2 is one of the most abundant and stable ROS molecules in organisms. H_2O_2 has an intermediate oxidation number (-1) so it can possess reducing and oxidizing properties that are important for its cellular multi-functionality. H_2O_2 can directly or indirectly (via the formation of other ROS) contribute to lipid peroxidation, protein carbonylation, DNA and the oxidation of methionine residues and thiol groups. H_2O_2 acts as a signaling molecule in various cellular processes (Imlay, 2003). Specific aquaporins isoforms facilitate the diffusion of H_2O_2 across biological membranes and, therefore, crucially impact on the membrane permeability of H_2O_2 (Bienert and Chaumont, 2014). Hydroxyl radical damages the lysosomal membrane and consequently digestive enzymes (cathepsins) enter the plasma. Cathepsins could target the mitochondrial outer membranes or activate Bid, Bax, and other lytic enzymes including phospholipase A_2 to open the MPT pores. This leads to the release of cytochrome c and the collapse of mitochondrial inner membrane potential. Cytochrome c release cause further oxidative stress induction through increasing mitochondrial H_2O_2 diffusion into the lysosomes and generating more lysosomal hydroxyl radicals (Pourahmad et al., 2012; Salimi et al., 2019). Therefore, we conclude that mitochondrial and lysosomal damages are interdependent and any damage to mitochondria ultimately causes damage to lysosome and vice versa. Therefore, this is the reason why lysosomotropic agents (chloroquine) prevents mitochondrial damage. On the other hand, carnitine as MPT pore sealing agents prevented damage to the lysosomes

Figure 6: Effect of different concentrations of Li on GSH depletion and the net values of GSH (a) and GSSG (b) using primary neuronal cortical culture. Intracellular GSH and extra cellular GSSG were measured spectrofluorometrically. Values are shown as mean $\pm$ SD of three separate experiments ($n = 3$). *** $p < 0.001$, significant difference in comparison with non-treated neurons (control). φφφ $p < 0.001$, significant difference in comparison with Li (1 mM)-treated neurons.

The incubation of isolated neurons with Li induced intracellular GSH depletion. Most of the GSH depletion could be justified to the efflux of GSSG. In this test as in previous experiments intracellular GSH depletion and increased extracellular GSSG prevented by lipid antioxidant (α -Tocopherol), hydroxyl radical scavenger (mannitol), MPT pore sealing agent (carnitine), and lysosomotropic agent (chloroquine). These results suggested that neural cortical cells would be survived by reducing ROS and TBARS production or protecting

neural lysosomes and mitochondria. GSH is an important intracellular antioxidant, which plays a critical role in maintaining cellular redox homeostasis (Lash, 2006). It is a non-enzymatic thiol antioxidant capable of protecting cells against oxidative stress (Razavi-Azarkhiavi et al., 2016). When isolated neurons were incubated with Li, glutathione depletion occurred due to ROS generation. Results of this study demonstrated that depleting of isolated neural GSH, potentiated all Li induced ROS generation, mitochondrial membrane potential collapse,

lysosomal membrane leakiness, and neural cytotoxicity. Therefore, it is confirmed that the cytotoxic mechanism of Li is mediated through oxidative stress.

5 Conclusion

Our results propose that add-on therapy or nutritional consumption of antioxidant agents (especially antioxidants that protects lysosomes and mitochondria) could reduce the chance of Li-induced neurotoxicity.

Acknowledgements: The data provided in this article was extracted from the Pharm. D. thesis of Dr. Romina Askian. The thesis was conducted under supervision of Prof. Jalal Pourahmad at Department of Toxicology and Pharmacology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Disclosure statement: The authors declare that they have no conflicts of interest.

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