Effect of lithium on the cerebellum of adult male albino rat and the possible protective role of selenium (Histological, Histochemical and immunohistochemical study)

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Abstract: Introduction: Lithium is a widespread therapeutic agent used for the treatment of manic depressive and bipolar mood disorders. Clinical and experimental studies have widely demonstrated the cerebellum as well as other organ toxicity caused by lithium. Aim of the work: This work aimed to clarify the toxic effect of lithium on rat cerebellum and the possible protective role of selenium. Material and Methods: Forty adult male albino rats were subjected to experiment for twenty-one days. The animals were divided randomly into four equal groups: control; selenium treated; lithium treated and protective (lithium and selenium treated). Control group, rats in this group were given orally the same amount of vehicle (distilled water and normal saline) and Selenium group, received selenium at a dose of (0.5 mg/kg b.wt/day) orally daily. Lithium treated group, was administered lithium with daily dose of (150 mg/kg b.w/day). Protective group, received selenium at a dose of (0.5 mg/kg b.wt/day) orally daily 1 hour before oral administration of lithium. All the animals were sacrificed at day twenty-two. Cerebellum of each animal was processed for histological, histo-chemical and immuno-histochemical studies. Results: In lithium treated group, histologically and histochemically, rat cerebellar cortex showed pathological changes in the form of distorted, various shaped Purkinje cells, which was degenerated in some areas and appearance of vacuoles replacing these cells. Cerebellar medulla showed some degenerated neurons and dilated congested capillaries. There was mild PAS reaction in the degenerated neurons in the cerebellar medulla. On the other hand, weak reaction for Nissel granules in the perikarya of degenerated and some Purkinje cells were seen. Immunohistochemically, in cerebellar medulla of lithium treated group, neuroglial cells and their processes showed strong positive immunoreactivity for GFAP (Glial Fibrillar Acidic Protein). On the other hand, immuno-histochemical study on the cerebellar cortex of lithium treated group, Purkinje cells cytoplasm showed strong positive immunoreactivity for iNos (isoform Nitros Oxide). Additionally, histological, histochemical and immunohistochemical examination of the protective group displayed normal appearance of most of Purkinje cells in cerebellar cortex and neurons in cerebellar medulla, but still some neurons appeared degenerated. Conclusion: toxic effect of lithium should be kept in mind during chronic usage. Selenium advised to be administered in concomitant with lithium treatment as it could ameliorate lithium toxicity on cerebellum.

Key Words: Lithium – Selenium – rat cerebellum.

1. Introduction:

Lithium is a monovalent cation. It is a widespread therapeutic agent which is currently used for the treatment of manic depressive and bipolar mood disorders (Markowitz et al., 2000; Niethammer and Ford, 2007). Additionally, it produced an anti-suicide effect (Cerqueira et al., 2008). It is a double edged sword; it is a unique drug with an invaluable psychoactive potential on one hand and a drug which can cause multisystem toxicity and even death on the other hand (Kumarguru et al., 2013).

Previous studies documented that, long term use of therapeutic dose of lithium could cause irreversibly damage to the brain, heart & kidneys and in some cases, be fatal (Delva and Hawken, 2001). Cerebellar toxicity was demonstrated as uncommon but potentially irreversible side effect on sequence of lithium therapy (Cerqueira et al., 2008). Lithium is present in most of body tissues; kidney, thyroid and brain. It’s uptake by central nervous system is not uniform. This may lead to high lithium concentration in the brain while it is in therapeutic level in plasma. This fact elucidated that, high serum concentration of lithium is not mandatory for it’s neurotoxicity. Lithium neurotoxicity mechanism seems to be multifactorial (Heidari and Sagheb, 2012).

Persistent neurological deficits have been reported in some cases of lithium carbonate intoxication. In these cases, cerebellar signs such as ataxia, dysarthria, dysmetria and tremor of the extremities are predominant and in the more severe cases encephalopathy and coma. Neuromuscular manifestations include proximal muscle weakness,
rhabdo-myolysis, a myasthenia gravis like syndrome and axonal neuropathy (Niethammer and Ford, 2007).

Some researcher reported, psychiatric patients with cerebellar degeneration secondary to lithium intoxication (Cerqueira et al., 2008). Others, provided evidence that, chronic lithium treatment of Wistar rats for 6 weeks inhibits total activity of phosphoglucomutase (PGM) from rat cortex, hippocampus, striatum, brainstem and cerebellum and subsequent inhibit glycogen synthesis in brain (Souza et al., 2010). More-over, Heidari and Sagheb, (2012) observed reduction of cerebellar white matter volume as a complication of long period of lithium therapy. On the other hand, Sassi et al., (2002) reported increase in brain grey matter contents and N-acetyl-aspartate levels in lithium-treated patients when compared to healthy individuals and untreated patient. This may represent neurotropic effect of lithium.

The mechanism of action of lithium is not completely understood. It exerts multiple actions on brain cells by affecting several cellular signaling pathways (Lenox and Hahn, 2000). It inhibits glycogen synthase kinase-3, which is involved in a wide range of signal transduction pathways. However, this lithium effect occurs at high concentrations and may be more relevant for its toxic effect (Shaldubina et al., 2001).

Selenium is an "essential" trace element which has a variety of functions. It is an essential micronutrient with a brain specific physiology. It is a key component of several functional selenoproteins which play important roles in brain development and metabolism as recently reviewed (Brauer & Savaskan, 2004).

Selenium also presents a potent antioxidant which acts through its various selenoenzymes to control the level of cellular hydro-peroxides and redox tone of the cell that can damage protein, cell organelle and DNA (Beck et al., 2004). It is involved in the regulation and control of the body’s antioxidant glutathione as well as peroxidase system, which plays a major role in the control of ROS (Chinoy and Patel, 2000).

Many previous studies have demonstrated that, selenium had protective effects in lead toxicity in brain regions (Moshtagie et al., 2007) and had ability to protect brain against fluoride induced oxidative stress (Reddy et al., 2009).

The aim of the present study was to assess the possible protective effect of selenium with lithium induced cerebellum toxicity in adult rat model using simple histological, histochemical and immunohistochemical methods.

2. Material and methods:

Material:

Animals:

Forty adult male Albino rats of an average weight (170-200 grams) were selected for this study. The animals obtained from breeding animal house, Faculty of Medicine, Menofiya University (Menofiya, Egypt). The animals were acclimated to room temperature (22-25°C) for one week before start of experiment and kept under good hygienic condition. During the study, they were fed standard animal food and tap water ad libitum.

Drugs and chemicals:

Lithium carbonate (Prianil CR): The drug used in the form of tablets. Each tablet contains 400 mg of lithium carbonate. It was obtained from the Nile Company for Pharmaceuticals and Chemical industries (Cairo, Egypt). The calculated dose was dissolved in 20 ml distilled water.

Selenium:

0.1 mg Selenium-ACE tablets were obtained from Sigma for interpharma UK under license of Wassen international LTD. The calculated dose was dissolved in 2 ml 0.9% normal saline.

Experimental protocol:

Animal experimentations were carried out in an ethical manner following guidelines set by Ethical Committee of Menofiya University. The animals were divided into 4 groups, each of 10 rats:

Group I: Rats in this control group were given the same amount of vehicle (distilled water and normal saline) orally by intragastric gavage along the time of the experiment (twenty-one days).

Group II: Rats in this group received Selenium (0.5 mg/kg b.wt./day) orally for twenty-one days (Amara et al., 2010)

Group III: Rats in this group were administered (150 mg/kg b.wt./day) of lithium carbonate orally (Vijaimohan et al., 2010) dissolved in distilled water for twenty-one days and were sacrificed after 24 hrs of last dose. This dose was reported to be toxic for rats.

Group IV: Rats in this group were received Selenium (0.5 mg/kg b.wt./day) orally 1 hr before the administration of same dose of lithium carbonate as group III daily for 21 days. Lithium carbonate and Selenium as antioxidant were administered orally to each animal using curved needle-like tube to be introduced directly into the stomach (a gavage process). On day twenty-two, 24 hrs after last dose of drug administration, the rats were anaesthetized with ether inhalation, their hearts were exposed and then intracardiac perfusion was done.

For light microscope study, each animal perfused intracranially with 10% formaline. After
perfusion, skull was opened and the cerebellum was removed carefully. Mid sagittal sections of cerebrum were obtained then immersed in normal saline. The tissues were divided and subjected to the following studies:

I- Histopathological & histochemical study:

The cerebellum of each animal was dissected out then fixed in 10% formal saline. The specimens were processed to obtain paraffin blocks from which 5 μm thick sections were cut and stained with: haematoxylin & eosin (H&E) for routine histological stain (Stevens and Wilson, 1996). Periodic acid Schiff’s reaction (PAS) for detection of mucopolysaccharides (Clayden, 1971) and toluidine blue stain for detection of Nissel granules (Kiernan, 2008).

Immunohistochemical study:

Immunostaining was performed using the avidin-biotin peroxidase technique for localization of Glial Fibrillary acidic Protein (GFAP) and same technique for the demonstration of the Inducible Nitric Oxide synthetase enzyme (iNos). Five μm paraffin sections mounted on coated slides were deparaffinized and treated with 0.1 M citrate buffer for minutes to unmask antigens (Cattoretti et al., 1993). Sections were incubated in H2o2 for 10 minutes to abolish endogenous peroxidase activity. Then, sections hydrated, washed, heated in buffered citrate & incubated for 30 minutes with horse serum for 2 hours at room temperature to inhibit non specific immunoreactions. Primary monoclonal anti-GFAP serum (AM020-5M Bio-genex) was applied at 1:5000 dilutions or iNOS (M-19): Rabbit polyclonal antibody raised against rat iNOS (Santa Cruz Biotechonology). Sections were incubated with 1ry monoclonal antisemur for 36 hours at 4°C. After washing, they were incubated with biotinylated secondary antibodies (REF85-9043-Zymed 1:200) for 5 hours, then followed by avidin-biotin peroxidase complex. Finally, the immune reaction was visualized with 0.05% diaminobenzidine. Then slides were counter stained with Mayer’s hematoxyline before mounting (Kiernan, 1999).

3. Results:

Group I (control group):

Light microscopic examination of H&E stained sections of cerebellum of the control group revealed that, cerebellar cortex was made up of three layers: molecular layer, Purkinje cell layer and granular layer (Fig. 1). The molecular layer formed of nerve fibers and scattered cells. The Purkinje cell layer was the middle layer and consisted of large pyriform cells with clear vesicular nuclei arranged in one row along the upper margin of the granular layer. The granular layer was the inner most layer of the cerebellar cortex, immediately adjacent to the cerebellar medulla (white matter). This layer composed of tightly packed small rounded cells with deeply stained nuclei (Fig. 1). Scattered neurons with their nerve fibers and capillary lined with endothelium were found in the cerebellar medulla (white matter) (Fig. 2).

Cerebellar medulla stained with Periodic Acid Chief (PAS) revealed strong PAS reaction on normal neurons (Fig. 3). Toluidine blue stain revealed, strong blue staining of Nissel granules in the perikarya of normal Purkinje cells in the cerebellar cortex (Fig. 4).

Immunohistochemical staining of Cerebellar medulla (white matter) showed positive immunoreactivity for Glial Fibrillary Acidic Protein (GFAP) in star shaped glial cells and their processes (Fig. 5).

For iNOS Immunostaining, cerebellar cortex sections revealed mild positive reaction in the molecular layer, moderate immunoreactivity in some areas of granular layer while Purkinje cells cytoplasm were non immunoreactive (Fig. 6).

Group II (Selenium group):

Showed the same light microscopic appearance like control group I.

GroupIII (Lithium treated)

Examination of H&E stained sections of the cerebellar cortex of lithium treated rats showed distorted Purkinje cells with various shapes. Some Purkinje cells arranged in two layers and others appeared either degenerated or with karyolytic nuclei (Figs. 7&8). Degenerated Purkinje cells appeared with shrinkage of their cytoplasm and pyknosis of nuclei. Some areas of vacuolation were present indicating cell loss (Fig. 9). In the region of cerebellar medulla some neurons appeared degenerated with dilated congested capillary (Fig. 10).

PAS reaction of section of cerebellar medulla of this group showed mild PAS reaction in the degenerated neurons and moderate reactions in some normal neurons (Fig. 11). Toluidine blue stained cerebellar cortex sections showed weak reaction for Nissel granules in the perikarya of some Purkinje cells and in degenerated cells (Fig. 12).

Immunohistochemical stain for GFAP in cerebellar medulla sections revealed strong positive immunoreaction in star shaped neuroglial cells and their processes with relatively increased branching of neuroglial cell processes and apparent increase in neuroglial cells number especially besides the degenerated neurons (Fig. 13).

For iNOS Immunostaining, cerebellar cortex sections revealed strong immunoreaction in most of Purkinje cells cytoplasm while minimal cells were non immunoreactive. Mild immunoreactivity was
detected in the molecular layer while granular cell layer exhibits moderate reaction in some areas (Fig. 14).

**Group IV protective group (Lithium and selenium treated):**

Light microscopic examination of H&E stained section of cerebellum of this group revealed, the Purkinje cell layer with almost near normal cells (Fig. 15). Most of neurons in cerebellar medulla retained their normal appearance but still some neurons appear degenerated with slightly congested capillary (Fig. 16).

Cerebellar medulla stained with (PAS) revealed strong PAS reaction on almost normal neurons (Fig. 17). Toluidine blue stain revealed, strong blue staining of Nissel granules in the perikarya of almost normal Purkinje cells in the cerebellar cortex, while weak reaction was detected in one degenerated Purkinje cell (Fig. 18).

Immunohistochemical staining of Cerebellar medulla (white matter) showed positive immunoreaction for GFAP in glial cells and their processes nearly similar to control group with some neuroglial cell processes still retained more branching (Fig. 19).

In cerebellar cortex sections stained with iNOS Immunostaining, mild positive immunoreaction was detected in the molecular layer, moderate immunoreactivity in some areas of granular layer while Purkinje cells cytoplasm were non immunoreactive. Almost immunostain reaction was similar to control group (Fig. 20).

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**Fig. 1:** A photomicrograph of a control adult rat cerebellar cortex showing molecular layer (M), Purkinje cell layer (P) and the granular layer (G). The Purkinje cell layer contains large pyriform cells with vesicular nuclei (p) and the granular layer contains crowded small cells with deeply stained nuclei (g) (H&E, X400)

**Fig. 2:** A photomicrograph of a control adult rat cerebellar medulla showing scattered neurons (N), nerve fiber (F) and capillary (C) lined with endothelium. (H&E, X400)

**Fig. 3:** A photomicrograph of a control adult rat cerebellar medulla showing strong PAS reaction of normal neurons (→). (PAS, X1000)

**Fig. 4:** A photomicrograph of a control adult rat cerebellar cortex showing strong reaction for Nissel granules in the perikarya of Purkinje cells (→). (Toluidine Blue, X1000)
Fig. 5: A photomicrograph of a control adult rat cerebellar medulla (white matter) showing positive immunoreactivity for GFAP in star shaped glial cells and their processes (A). (GFAP, X1000).

Fig. 6: A photomicrograph of a control adult rat cerebellar cortex showing mild iNOS immunoreactivity in the molecular layer (M). Purkinje cells cytoplasm (P) are non immunoreactive. Granular layer exhibits moderate immunoreactivity in some areas (→). (iNOS immunostain, X1000)

Fig. 7: A photomicrograph of lithium treated adult rat cerebellar cortex showing molecular layer (M), Purkinje cell layer (P) and the granular layer (G). Most of Purkinje cells appear distorted with various shapes. Some Purkinje cells arranged in two layers (→) and others with karyolytic nuclei (K). (H&E, X400)

Fig. 8: A photomicrograph of lithium treated adult rat cerebellar cortex showing the three cortical layers. Most of Purkinje cells appear degenerated (D) while some appeared within normal (P). (H&E, X400)

Fig. 9: A photomicrograph of lithium treated adult rat cerebellar cortex showing degenerated Purkinje cells (D). They exhibit shrinkage of cytoplasm and pyknosis of nuclei. Some areas of vaculations (V) denoting cell loss. (H&E, X400)

Fig. 10: A photomicrograph of lithium treated adult rat cerebellar medulla showing degenerated neurons (D) and dilated congested capillary (C). (H&E, X1000)
Fig. 11: A photomicrograph of lithium treated adult rat cerebellar medulla showing moderate PAS reaction of some normal neurons (→) and mild PAS reaction in the degenerated neurons (D). (PAS, X1000)

Fig. 12: A photomicrograph of lithium treated adult rat cerebellar cortex showing weak reaction for Nissel granules in the perikarya of some Purkinje cells (→) and also in degenerated cells (D). (Toluidine Blue, X1000)

Fig. 13: A photomicrograph of lithium treated adult rat cerebellar white matter showing strong positive immunoreactivity for GFAP in star shaped neuroglial cells and their processes (A). Note relatively increased branching of neuroglial cell processes and apparent increase in neuroglial cells number especially besides the degenerated neurons (→). (GFAP, X1000)

Fig. 14: A photomicrograph of lithium treated adult rat cerebellar cortex showing mild iNOS immunoreactivity in the molecular layer (M). Most of Purkinje cells cytoplasm (→) are strong immunoreactive while minimal cells non immunoreactive (arrow head). Granular layer (G) exhibits moderate immunoreactivity in some areas. (iNOS immunostain, X1000)

Fig. 15: A photomicrograph of lithium and selenium treated adult rat cerebellar cortex showing molecular layer (M), Purkinje cell layer (P) and the granular layer (G). The Purkinje cell layer contains almost near normal cells. (H&E, X400)

Fig. 16: A photomicrograph of lithium and selenium treated adult rat cerebellar medulla showing normal neurons (N) and slightly congested capillary (C). Note: other neurons appear degenerated (→). (H&E, X 400)
Fig. 17: A photomicrograph of lithium and selenium treated adult rat cerebellar medulla showing strong PAS reaction of almost normal neurons (→). (PAS, X1000)

Fig. 18: A photomicrograph of lithium and selenium treated adult rat cerebellar cortex showing strong reaction for Nissel granules in the perikarya of almost normal Purkinje cells (→) nearly similar to control group. Note: weak reaction in one degenerated Purkinje cell (arrowhead). (Toluidine Blue, X1000)

Fig. 19: A photomicrograph of lithium and selenium treated adult rat cerebellar white matter showing strong positive immunoreactivity for GFAP in neuroglial cells and their processes (A) nearly similar to control group. Note: Some neuroglial cell processes still retained more branching. (GFAP, X1000)

Fig. 20: A photomicrograph of lithium and selenium treated adult rat cerebellar cortex treated showing mild iNOS immunoreactivity in the molecular layer (M). Purkinje cells cytoplasm (→) are non immunoreactive. Granular layer exhibits moderate immunoreactivity in some areas. (iNOS immunostain, X1000)

4. Discussion

Lithium is one of the first-line drugs for acute and maintenance treatment of bipolar disorder (Lyoo et al., 2010). The organ toxicity associated with lithium therapy may limits it use as therapeutic agent. Cerebellar toxicity was demonstrated as uncommon but potentially irreversible side effect on sequence of lithium therapy (Cerqueira et al., 2008). Lithium exerts it’s multiple actions on brain cells by affecting several signal pathways (Lenox and Hahn, 2000).

The present study aimed to assess the possible protective effect of selenium on lithium induced cerebellar toxic model using simple histological, histochemical and immuno-histochemical methods.

The present study revealed the normal general structure of cerebellar cortex and medulla of the control rats. These observations correlated with the histological organization of the normal cerebellum structure as reported by (Eltony et al., 2010). This denoted that, the investigated rats were healthy.

In the current study, histopathologic examination of the cerebellar cortex of lithium treated rats revealed different shaped, distorted Purkinje cells which sometimes arranged in two layers. Some Purkinje cells were degenerated with shrinkage of their cytoplasm, pyknosis of their nuclei and with areas of vacuolation indicating cell loss. Purkinje cell degeneration could be referred to cerebellar dysfunction. These findings were in agreement with Cerqueira et al., (2008) who proved in their neuropathologic study association between lithium toxicity and cerebellar degeneration. They reported reduction in the number of Purkinje cells with preservation of the basket cells of the cerebellar cortex, spongiform changes in white matter and changes in the dentate nucleus. Several experimental studies attributed this effect to the direct and indirect
of glial cells can be explained as a compensatory response to neuronal damage by lithium. This increase in astrocytes agreed with Keshavarz et al., (2013) who reported that, glial cells are markedly affected by stress.

The apparent increased number of neuroglial cells in lithium treated rats could be attributed to the role of these cells in the degenerative diseases of CNS as has been reported by Reali et al., (2005) who stated that, central nervous system degenerative diseases are often characterized by an early and strong reaction of astrocytes. Song et al., (2013) explained that, lithium may act indirectly to improve the function of neurons by protecting astrocytes from apoptosis by inhibiting glycogen synthetase kinase-3β which elevate Bcl-2 level.

In addition, recent research has demonstrated that, astrocytes release several molecules that fulfill many of criteria for a transmitter including (glutamate, ATP, D-serine, adenosine, eicosanoids and cytokines) and when activated may be responsible for the drug induced neurotoxicity (Volterra and Meldolesi, 2005).

Nitric oxide is a toxic free radical gas which produced in biological tissues by nitric oxide synthetase enzyme (NOS) (Voet and Voet, 1994). There are three distinct isoforms of NOS: nNOS, iNOS and eNOS. Neuronal (nNOS) produced within the cerebellum mediates normal synaptic transmission (Yu et al., 2000). Inducible (iNOS) which produces high amount of nitric oxide that has been hypothesized as a major contributor in the disease pathway, stress and toxicity. It is involved in immune response, bind with calmodulin and produces NO as an immune defense mechanism (Gusarov and Nudler, 2005). Endothelial (eNOS) which is expressed in blood vessels (Drew and Leeuwenburgh, 2002; Gouda et al., 2010). NO is a messenger molecule with different functions in the body including the vascular integrity, haemostasis, synaptic plasticity, long term potentiation, learning and memory (Floyd and Hensley, 2002).

Examination of the cerebellar cortex of lithium treated rats that were stained immunohistochemically for iNos immunostain, revealed strong positive immunoreactions in most of Purkinje cells cytoplasm with mild immunoreactivity in molecular layer and moderate immunoreaction in granular layer. This is consistent with the findings of Siles et al., 2002; Gouda et al., 2010) who reported significant increased expression in rat cerebellum under stress and age. They mentioned that, under basal conditions, iNOS is expressed at low levels, but when induced by stimuli such as pro-inflammatory cytokines and endotoxins high level of iNOS expression was detected.
The distribution pattern of iNOS expression in lithium treated rat cerebellar cortex, detected in the present work was similar to the pattern of distribution of nitrotyrosine reported by Chung et al., (2002) who concluded that, nitrotyrosin is a marker of nitrosative damage; it indicates the presence of high concentration of NO.

In the current study, examination of the cerebellar cortex of the rats of the protected group which received both lithium and selenium, showed that, most of Purkinje cell layer looks near normal, granular and molecular cell layers were almost normal. Additionally, most of neurons in the cerebellar medulla retained their normal appearance but still some degenerated neurons and slightly congested capillary was reported. This could be attributed to the selenium protective effect as its supplementation inhibited dopaminergic toxicity and protect neurons from oxidative stress (Chen and Berry, 2003). Selenium is known to provide protection from reactive oxygen species (ROS) induce cell damage (Smith and Cass, 2007) and so attenuate the toxic effect on rat cerebellum. This is in line with Moshtaghe et al., (2007) who stated that, selenium administration could ameliorate cerebellum toxicity caused by lead.

The antioxidant protective effect of selenium on brain cells was due to its role in elevation of glutathione, reduction of lipid peroxidation and protection from cell death (Savaskan et al., 2003). Thus selenium plays a critical role in the maintenance of proper functioning of the nervous system through expression of selenio-proteins, which are mostly involved in regulation of redox status under physiological conditions and in antioxidant defense (Reddy et al., 2009).

Thus, in this work selenium may effectively prevented tissue damage by decreasing oxidative stress and restoring antioxidant status.

Conclusion:

Toxic effect of lithium should be kept in mind during chronic usage. Selenium advised to be administered in concomitant with lithium treatment as it could ameliorate lithium toxicity on cerebellum.

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