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Vitamin C as an antioxidant stabilizes the depressive brain

ABSTRACT:

Lithium chloride treatment of neural diseases, such as manic-depressive disorder, causes impairment of anti-oxidative defence. It induces opposite effects on nitric oxide-related systems. Antioxidant supplementation (Ascorbic acid) with Li Cl could have a therapeutic effect in unstable patients. The present study investigates the modulation of NO-producing cells in the rat brain, due to Li Cl administration to experimental animals. We report here the potential that Vitamin C administration along with Li Cl has in counteracting anti-oxidative stress. In control animals and in animals injected with low dose Li Cl together with Vitamin C, there were large numbers of NO-producing neurons. This number significantly decreased in animals treated only with the low dose of Li Cl and those injected with the high dose of Li Cl together with vitamin C. Moreover, the smallest number of NO-producing cells appeared in animals treated with high dose Li Cl only. Therefore, we suggest that Vitamin C reduces Li Cl noxious effects (reduction of NO levels) and recommend administering it together with mood stabilizing drugs.

KEY WORDS:

Psychiatric disorders, Lithium Chloride, Vitamin C, Antioxidants, Nitric Oxide.

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INTRODUCTION:

Lithium has been successfully used as a treatment for psychiatric disorders, such as bipolar disorder (Belmaker, 2004), schizophrenia, Alzheimer's (Nunes *et al.*, 2013) and depression for about 50 years with controversial mechanisms of action (Barchas *et al*., 1994; Cade, 1999). It possesses potential neuroprotective action by releasing neurotrophic factors, reducing pro-inflammatory status, and decreasing oxidative stress (Diniz *et al.*, 2013), in various experimental models (Nonaka *et al.*, 1998; Xu *et al.*, 2003) and in clinical observations (Machado-Vieira *et al.*, 2009; Forlenza *et al.*, 2012 & 2014; Diniz *et al.*, 2013) and animal models (Nonaka *et al.*, 1998; Senatorov *et al.*, 2004). It also exerts some potent anti-inflammatory properties on the brain (Nassar and Azab, 2014). However, it inhibits the key enzyme in the metabolism of amyloid precursor protein and tau protein phosphorylation (Klein and Melton, 1996; Lovestone *et al*., 1999), which are critical steps in the formation of pathological hallmarks of Alzheimer's disease.

Nitric oxide (NO) is an atypical neurotransmitter in its chemical nature, biosynthesis, mechanism of action, and cellular localization. It diffuses into adjacent neurons (Barañano *et al*., 2001), not only it blocks cellular enzymes required in metabolism, but it also activates the soluble enzymes present in the cytoplasm. This activation mediates many of the physiological actions of NO in mammalian cells (McCann *et al*., 2005). In fixed tissues, reactive neurons have NADPH-diaphorase activity (González-Soriano *et al*., 2002) used as a histochemical detection method for neuronal NO-producing structures (Bredt *et al*., 1991). This is evident in cells positive to NADPH-d histochemistry and/or to the neuronal nitric oxide synthase (nNOS) immunohistochemistry in different species of vertebrates (Arévalo *et al.*, 1995; Münoz *et al*., 1996; Smeets *et al*., 1997; Alonso *et al*., 2000; Giraldez-Perez *et al*., 2008). The interest in detecting NADPH-d by histochemistry arose when researchers identified NADPH as a marker for nNOS (Hope *et al.*, 1991) and a colocalization of NADPH activity and NOS immunoreactivity was relatively easy to document, repeatedly,

in distinct sets of neurons (Briñon *et al*., 1998; Giraldez-Perez *et al*., 2008).

Ascorbic acid is an important antioxidant playing a role as an enzymatic cofactor (Park and Levine, 1996; Himmelreich *et al.*, 1998; Ishikawa *et al.*, 1998) in detoxification processes (Frei *et al.*, 1989; Harrison and May, 2009; May, 2012). Among other functions, Vitamin C also modulates synaptic activity and neuronal metabolism (Castro *et al.*, 2007, 2008, & 2009; Harrison and May, 2009). It is concentrated in the brain (Spector and Lorenzo, 1973), the organ responsible for 25% of glucose use of the whole body (Attwell and Laughlin, 2001; Alle *et al.*, 2009). The brain, therefore, is dependent on antioxidants for its own protection against pathological conditions (Wilson, 1997; Harrison and May, 2009). Consequently, finding a way to prevent or even fight oxidative stress would ensure the correct supply of antioxidants, such as ascorbic acid, and the development of new therapeutic strategies. This study aims at examining if Vitamin C has a favourable effect on NO-producing cells when administered with Li Cl.

MATERIAL AND METHODS:

Experimental Animals:

Animals (forty adult male Wistar rats, 150 – 200 g), obtained from the animal house facility, Faculty of Science, Alexandria University were under standard conditions of light and temperature, and allowed *ad libitum* access to food and water. The local ethics committee approved the present study's experimental procedures. Animal acclimatization for one week before experimentation minimized their stress. The author performed drugs administration and animal perfusion of all experimental groups between 9: 00 a.m. and 5: 00 p.m.

Experiments consisted of five groups of rats (8 animals each). Group I received 1 ml of physiological saline by intraperitoneal (*ip*) injection to represent controls. Groups II and III received two different Li Cl doses, namely low (50 mg / kg Body Weight "BW") and high (100 mg/kg BW), respectively. Groups IV and V received two different Li Cl doses, namely low (50 mg/kg BW) and high (100 mg/kg BW), respectively together with 250 mg/kg BW vitamin C.

Histological preparation for NADPH-d histochemistry:

Animals were anesthetized using sodium pentobarbital and perfused with 4% paraformaldehyde in Phosphate Buffer Saline (PBS). Diencephalon specimens were post-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) (pH 7.4) for 2 h at room temperature then immersed overnight in a solution of 12% sucrose in PB at 4°C. Specimens were transferred into tissue Tek (Sakura Finetek U.S.A. Inc) and kept in − 80°C until processed. Blocks were then transferred to −20°C for 2 h before being cut on a cryostat at 14 µm thickness.

Collected free-floating sections rinsed in a fresh PB solution were treated according to previously published methods (Hope *et al.*, 1991; Moreno *et al.*, 2002). In brief, free-floating sections were incubated in a medium containing 1 mM β-NADPH (Chematics, U.S.A), 0.8 mM nitroblue tetrazolium (sigma, Germany) and 0.06% triton X-100 (Sigma-Aldrich) in PB; at 37°C for 1 to 2 h. Successive rinses in cold PB stopped the reaction. All sections were finally mounted using 0.25% gelatine in 0.1 M Tris buffer, pH 7.6 then dried overnight and cover slipped.

Image Acquisition:

The author used a series of serial transverse sections to illustrate the distribution of nitric oxide positive cells in the rat diencephalon. The image of each section was recorded with a digital camera wide zoom, operating on a microscope and reversed on a computer by the Analysis Life Science Series software (Soft Imaging System, Japan) and Life View software (Animation Technologies Inc., Taiwan).

Quantification and statistical analysis:

For data quantification procedures, the author drew all sections using camera Lucida, and then manually counted all stained cells. All data were represented as means ± SEM and were statistically analysed with One-Way ANOVA using "Statistical Package for the Social Sciences" SPSS software (SPSS Inc., U.S.A.) and student's two-tailed t-test to compare every two groups together. Differences were considered to be significant when $p \leq 0.05$.

RESULTS:

The effects of Lithium Chloride on forty Wistar rats' brains were investigated. The study was further extended to explore the potential therapeutic effects of Vitamin C when co-administered with the drug intended to cure psychiatric diseases. In each experimental group, GP (I) rats received physiological saline by intraperitoneal (*ip*) injection. These rats were the control ones. GP (II) rats got the low dose of Li Cl (50 mg / kg BW). Rats from GP (III) had high Li Cl dose (100 mg / kg BW). GP (IV) and (V) received the same Li Cl doses as GP (II) and (III) respectively in addition to 250 mg / kg BW vitamin C.

The present results show that GP (I), that is the control, has the highest number of cells labelled with NADPH-d (Fig. 1, left panel). The cell number reactive to NADPH-d immunohistochemistry in GP (II) significantly decreased ($p \leq 0.03$) (Fig. 1, right panel) compared to controls.

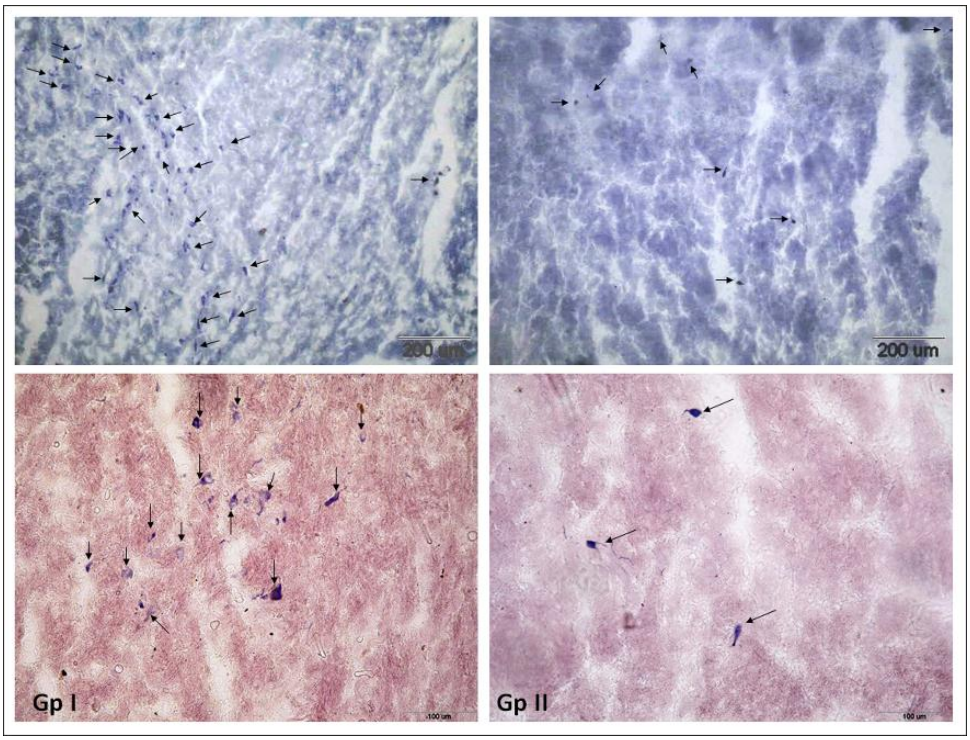

Fig. 1. Treatment with Li Cl at 50 mg / kg BW (Gp II animals) caused a highly significant decrease (right panel arrows) in NO – producing cells ($p \leq 0.03$) compared to Gp I control animals (left panel arrows). Scale bars: 200 µm (upper panels), 100 µm (lower panels).

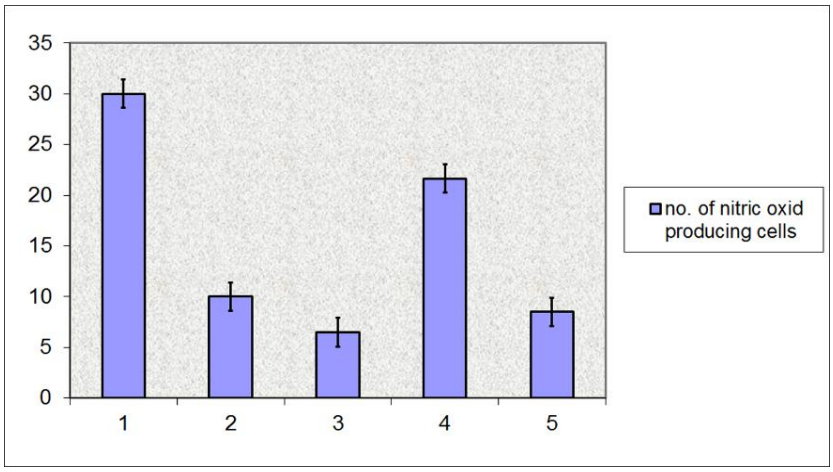

Fig. 2. Mean number of NO-producing cells in the different experimental groups. Control animals (column 1) have the highest number of NO-producing cells. The low dose of Li Cl (column 2) significantly decreased this number ($p \leq 0.03$) and the high dose (column 3) went on decreasing NO cells ($p \leq 0.02$). This effect is reversed with Vitamin C treatment more with the low dose of Li Cl (column 4: $p \leq 0.02$) than with the high dose (column 5: $p = 0.07$).

The mean number of labelled cells in the different groups is in figure 2 while table 1 represents SEM significance.

GP (III) animals, treated with the high Li Cl dose of 100 mg / kg BW, shows the smallest number of NO-producing cells (Fig. 3, right panel), compared to both controls ($p \leq 0.02$) (Fig. 3, left panel) and low dose-treated rats of group (II) ($p \leq 0.05$) (Fig. 1, right panel). This decrease in NO-producing cells is statistically significant, as seen in table 1 and figure 2.

Table 1. Mean number of NO-producing cells in all groups, also represented in the Graph. Note the significant decrease in these cells in GP (II) compared to GP (I) ($p \leq 0.03$) and the lowest number of cells in GP (III) ($p \leq 0.02$). The addition of Vitamin C to the low dose of Li Cl in GP (IV) rebounded the NO-producing cells number to almost the control level ($p \leq 0.02$). GP (V) shows the therapeutic effect of Vitamin C that caused the number of cells to surpass those in GP (III) ($p = 0.07$) and almost reach those of GP (II) ($p = 0.08$).

	GPI	GPII	GPIII	GPIV	GPV
Mean No of NO-producing cells	30 ± 2	10 ± 1.4	6.5 ± 0.5	21.66 ± 2.867	8.5 ± 0.408

GP (IV), in which animals were intraperitoneally-injected with the low dose of Li Cl together with Vitamin C, shows a significant increase in NO-producing cells (Fig. 4, right panel) compared to those receiving the same low concentration of Li Cl

but without Vitamin C supplementation ($p \leq 0.04$) (Fig. 1, right panel & Fig. 2, column 2). These animals also showed a slight decrease compared to the control group ($p = 0.07$) (Fig. 4, left panel & Fig. 2, column 1).

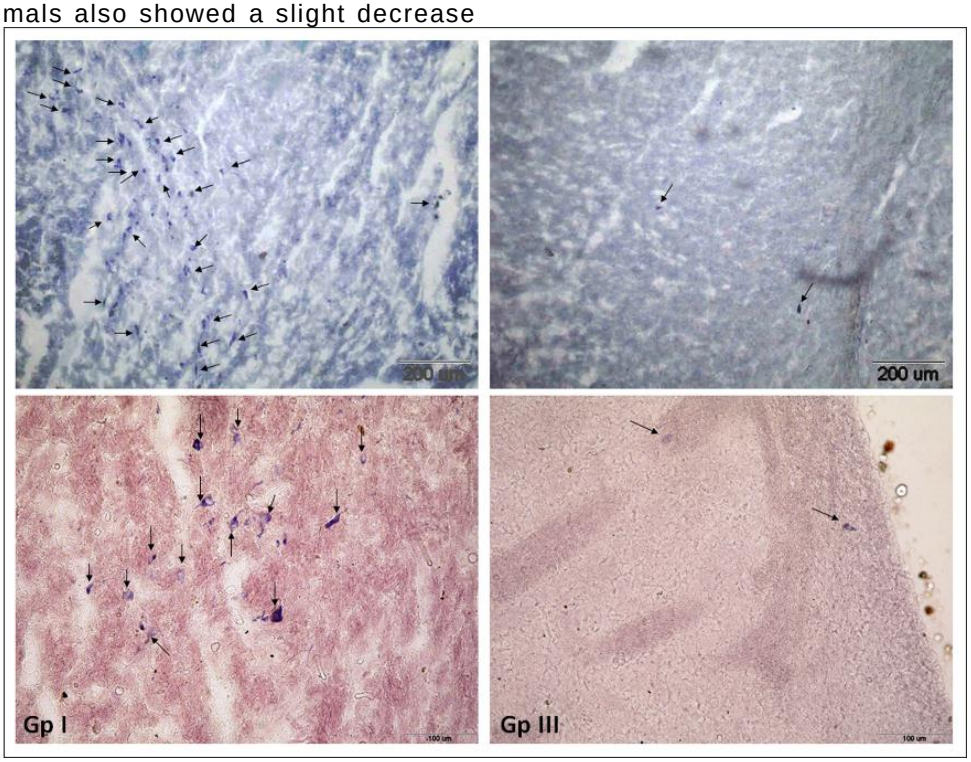

Fig. 3. Li Cl at 100 mg / kg BW (group III animals) caused a highly significant decrease ($p \leq 0.02$) in NO – producing cells (arrows in right panel) compared to controls (arrows in left panel). Note the obvious reduction ($p \leq 0.05$) caused by the high dose Li Cl (group III animals) compared to the low dose injection in animals of group II (right panel in figure 1). This group shows the smallest number of NO – producing cells. Scale bars: 200 µm (upper panels), 100 µm (lower panels).

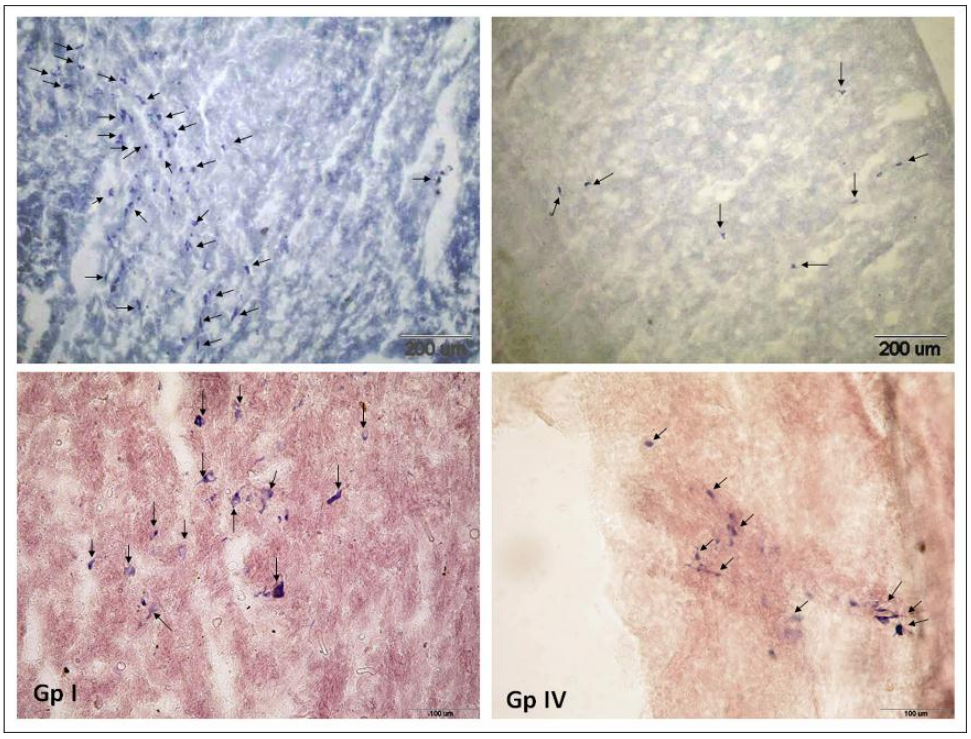

Fig. 4. Li Cl at 50 mg / kg BW supplemented with 250 mg / kg BW Vitamin C (group IV animals) caused a small not dramatic decrease in NO – producing cells ($p = 0.07$) (arrows in right panel), compared to group I control animals (arrows in left panel). The presence of Vitamin C doubled the number seen in group II ($p \leq 0.02$) (right panel of figure 1) showing that it brought back the number of cells almost to the control level ($p \leq 0.04$). Scale bars: 200 µm (upper panels), 100 µm (lower panels).

Animals from GP (V), treated with Li Cl high dose together with Vitamin C, showed a significant decrease in cells producing NO, compared to GP (IV), those treated with Li Cl low dose together with Vitamin C ($p \leq 0.04$) (Fig. 4, right panel & Fig. 2, column 4) and

controls (p ≤0.03) (Fig. 5, left panel & Fig. 2, column 1). This effect is midway between group (II), animals treated with the low dose Li Cl and (III), those treated with the high dose, both without Vitamin C (Figs 1 & 3, right panels). It is slightly less than the former (p = 0.08) and more than the latter (p = 0.07). These results show the therapeutic effect of Vitamin C as it caused the increase in NO production in animals that was supposed not to produce this neurotransmitter under the effect of the high dose of Li Cl.

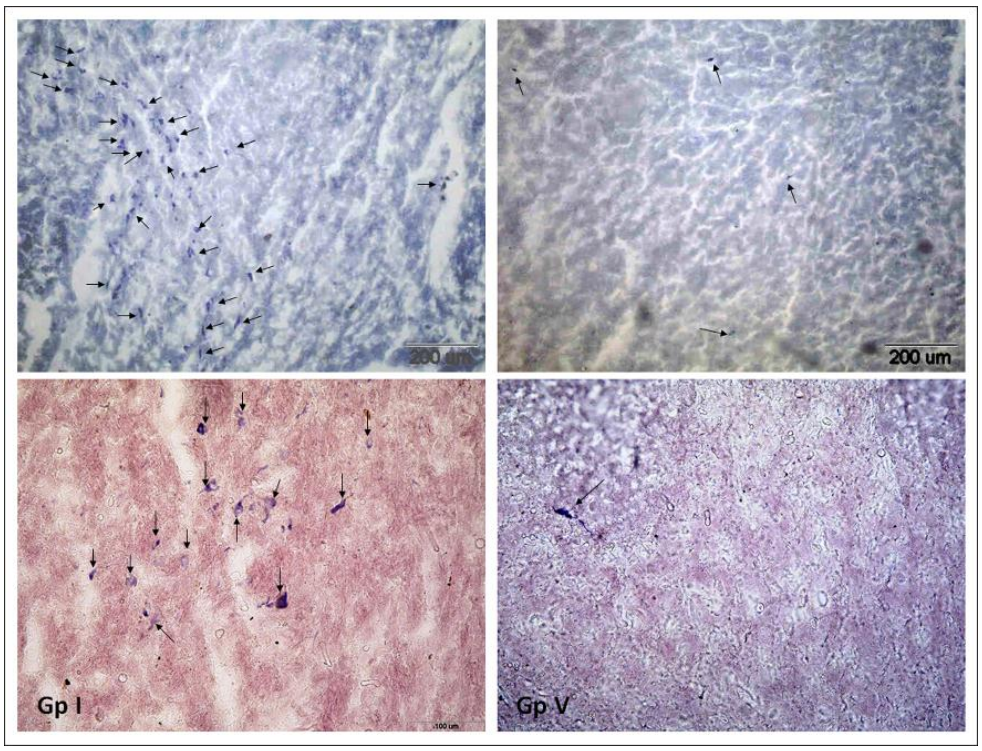

Fig. 5. Li Cl at 100 mg / kg BW supplemented with 250 mg / kg BW Vitamin C caused a highly significant decrease in NO – producing cells (p ≤0.03) (arrows in right panel), compared to controls (arrows in left panel). Note that this is almost the same number of cells as in group II (p = 0.08) (right panel of figure 1). The presence of Vitamin C recovered the decrease seen in group III (p = 0.07) (right panel of figure 3). Scale bars: 200 µm (upper panels), 100 µm (lower panels).

DISCUSSION:

Lithium is a mood-stabilizing agent used for the treatment of manic disorder and depression. In the present study, the author shows for the first time that *in vivo* intraperitoneal injection with low and high doses of Li Cl, namely 50 and 100 mg/kg BW, respectively, caused a significant decrease in the number of NO-producing cells (Figs 1 & 3, right panels & Fig. 2).

Previous studies demonstrated that long-term lithium treatment modifies brain gene expression in astrocytes, having effects on members of the G protein family (Li *et al.*, 1991), adenylate cyclase (Colin *et al.*, 1991), and glucocorticoid receptor mRNAs (Peiffer *et al.*, 1991). These studies also suggested that in the major cell type, in mammalian CNS, Lithium's beneficial effects require chronic administration. Even after withdrawal, one can still see these effects. It is thus possible that chronic treatment with lithium can affect astroglial gene expression *in vivo* (Feinstein, 1998) through increases in the expression or activation of several transcription factors or cyclic AMP response element-binding proteins (Williams and Jope, 1995; Miller and Mathe, 1997; Ozaki and Chuang, 1997).

Moreover, in the mammalian brain, NO is synthesized by the enzyme NOS (Stuehr and Griffith, 1992), in neurons (type 1 NOS) and by an inducible, calcium-independent isoform, type 2 NOS (NOS-2) in glial cells; astrocytes and microglia (Galea *et al.*, 1992; Simmons and Murphy, 1993). Disruption of NO pathways triggers many of the mechanisms behind brain injury and influences the evolution of brain damage in different ways, dependent on the site, mechanism and timing of NO synthesis and concentration (Toda *et al.*, 2009).

Relative NO reduction appears to occur in the early stages of cerebral injury, e.g. after traumatic brain injury (Cherian *et al.*, 2000; Tuzgen *et al.*, 2003; Ahn *et al.*, 2004).

The present results support these findings as treatment with Lithium depletes the number of NO-producing cells especially in high doses. We suggest that high dose Lithium treatment, although beneficial for mood stabilization, can cause brain injuries. However, this aspect remains to be more deeply investigated in future studies.

Despite the controversies surrounding NO dysfunction after brain injury, there is

strong evidence from animal experimentations that by increasing cerebral NO levels directly or indirectly, using respectively, inhaled NO or NO donors, one can see neuroprotective effects of the neurotransmitter (Garry *et al.*, 2015). In the present study, we show that by adding Vitamin C to Lithium treatment, we decrease its noxious effect seen in the increase of the number of NO-producing cells (Fig. 4, right panel & Fig. 2).

Nevertheless, NO interacts with many signalling pathways. This makes it difficult to translate results from animal models where the control of conditions is very tight, to the patient population where the evolution of injury is unpredictable (Garry *et al.*, 2015). This is the reason why, in the present study, the author used intact rats so as she can measure the effect of Li Cl on NO-producing cells, with no interference with any other stimulus to the gaseous neurotransmitter. Although Li Cl causes a decrease in the number of cells producing NO, Vitamin C has the power to revert the effect of the drug and almost recover the number of NO-producing cells. Nonetheless, in order to apply these findings on manic-depressive disorder patients, we need to undergo more experiments, in conditions similar to the human microenvironment. Another option would be to create a mathematical model for these results using modelling equations and software in order to predict the recovering effect of Vitamin C on the decrease of NO-producing cells after Lithium treatment in humans (in preparation).

In conclusion, lithium induces opposite effects on NO – related systems in the brain. The present study shows that it significantly reduces NO levels as compared with controls. However, Vitamin C succeeds to decrease its noxious effects on experimental animals, except in the case of high dose treatment probably through lipid peroxidation. We suggest that Vitamin C and lithium are good candidates for concurrent administration during the treatment of depression and other neural diseases.

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دور فيتامين سي كمضاد للأكسدة في استقرار المخ القابل للاكتئاب

ماري زكريا مفتاح

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يتسبب علاج كلوريد الليثيوم للأمراض العصبية ولا سيما اضطراب الهوس الاكتئابي في تلف العمليات الحيوية الدفاعية المضادة للأكسدة. فلقد وجد أن له تأثيرات متضاربة على الأجهزة التي لها صلة بأكسيد النيتريك. ولذلك فالعلاج بحمض الأسكوربك المضاد للأكسدة مع كلوريد الليثيوم قد يعطي نتائج علاجية عند المرضي الغير مستقرين. تهدف هذه الدراسة إلى بحث التغيرات المحتملة في الخلايا المنتجة لأكسيد النيتريك في مخ الجرذان بعد حقن حيوانات التجارب بكلوريد الليثيوم. أظهرت نتائج هذه الدراسة أن لفيتامين سي طاقة كامنة في مواجهة التوتر الناتج عن مضادات الأكسدة عند استخدامه مع كلوريد الليثيوم. ولقد اتضح ذلك في العدد الكبير للخلايا العصبية المنتجة لأكسيد النيتريك عند الحيوانات الطبيعية وتلك التي عولجت بجرعة كلوريد الليثيوم المنخفضة بالإضافة إلى فيتامين سي. انخفض عدد هذه الخلايا بشدة عند الحيوانات المعالجة بجرعة كلوريد الليثيوم المنخفضة فقط وتلك التي عولجت بجرعة كلوريد الليثيوم المرتفعة بالإضافة إلى فيتامين سي. بالإضافة إلى ذلك، فقد وجد أصغر عدد من الخلايا المنتجة لأكسيد النيتريك عند الحيوانات التي عولجت بجرعة كلوريد الليثيوم المرتفعة فقط. ولذلك فاقتراحنا هو أن فيتامين سي يقلل الآثار المضرة لكلوريد الليثيوم وهي تخفيض مستوي أكسيد النيتريك في المخ. ونوصي بدمج فيتامين سي مع كلوريد الليثيوم عند علاج بعض الأمراض النفسية.