

Ameliorating effects of proglumide on neurobehavioral and biochemical deficits in animal model of status epilepticus

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Abstract: Status epilepticus (SE) is a recurrent generalized convulsion condition and is regarded as a medical emergency with around 50% of the cases occurring in children. Besides neurobehavioral and motor deficits, SE is reportedly associated with imbalance in a number of neurochemicals in several areas of the brain. Furthermore, neuronal hyperactivity and/or excitotoxicity in such brain areas have been associated with excessive generation of free radicals. Proglumide (Pgm) is a known cholecystokinin (CCK) antagonist and any changes in the level of CCK and in the number of CCK receptors has been linked with SE. The present study was designed to investigate the possible neuroprotective effects of Pgm (0, 250, 500 and 750mg/ml/kg i.p.) on epileptic seizure activities, some neurobehavioral tests, and on some oxidative stress related parameters like lipid peroxides measured as thiobarbituric acid-reactive substance (TBARS) and total glutathione (GSH) in brain (hippocampus and striatum) of young rats that were experimentally induced with SE by lithium (Li) in 3mEq/ml/kg dose, i.p. followed 20h later by pilocarpine (Pc) in 20mg/ml/kg dose, s.c.). Besides significant anti-epileptic effect, Pgm significantly ameliorated SE-induced deterioration in cognitive behavior (in water-maze), motor performance (on rotarod), and biochemical changes in brain. It is concluded from the present study that Pgm has significant neuroprotective effects against SE and this effect may probably be due to its antioxidant activity. Pgm may prove to be a potentially effective antiepileptic drug, however, further studies are needed to ascertain this possibility.

Keywords: Proglumide; pilocarpine; status epilepticus; neurobehavior; oxidative stress.

INTRODUCTION

Status epilepticus (SE) is an emergency condition where recurrent generalized convulsions when lasting for more than 30 minutes causes neuronal injury if not controlled timely (Chen *et al.*, 2010, Tariq *et al.*, 2008). Children are more susceptible to SE than adults and 50% of such cases occur in age group of 2 years and less (Shinnar *et al.*, 1997). Learning, memory and motor deficits are the main problems in children suffering from SE (Hernandez *et al.*, 2002). A wide range of neurochemical imbalance has also been associated with neuronal hyperactivity in various brain regions during SE (Freitas *et al.*, 2004, Tariq *et al.*, 2008), and such hyperactivity of neurons has been reported to result into excessive production of free radicals (Freitas *et al.*, 2005). Furthermore, the brain which on one hand contains large quantities of oxidizable lipids and metals, on the other, has fewer antioxidation mechanisms as compared to the other tissues (Naffah-Mazzacoratti *et al.*, 2001), thus, making it highly vulnerable to oxidative stress (Freitas, 2009).

Furthermore, SE is reported to result into significant brain damages in human (Duncan 2002) as well as in animal models (Haut *et al.*, 2004) where subsequent seizures do cause neuronal cell loss in the hippocampus area. Also, importantly, Pc-induced epilepsy in rodent models can

provide useful information regarding epileptic activity related oxidative stress (Freitas *et al.*, 2003; Smith and Shibley, 2002). Most of the clinical and temporal features of SE can easily be reproduced by the Li-Pc model of SE (Tariq *et al.*, 2008). Depletion in neurochemicals that are involved in neuronal excitability and behavioral alterations may provide beneficial clues for searching new therapeutic compounds in treating SE. In many experimental studies, it has been experienced that antioxidants are helpful in diminishing the excitotoxicity induced by agents like kainic acids, glutamate and Pc (Silva *et al.*, 2008, Tariq *et al.*, 2008, Wu *et al.*, 2009, Dong *et al.*, 2009). Thus, antioxidants appear to have a preventing potential against the excitotoxicity induced seizures. The anticonvulsant effect of several agents having antioxidant property such as curcumin, transresveratrol, melatonin, adenosine, alpha lipoic acid, pentoxifylline and buspiron, has been demonstrated in various studies (Tariq *et al.*, 2008, Gupta *et al.*, 2009; de Freitas *et al.*, 2010).

The cholecystokinin (CCK) receptors have been evidently proven for having multiple role in various physiological pathways including the opiodergic pathways (Rastegar *et al.*, 2002; Homayoun and Dehpour, 2004), anticonvulsant effects (Legido *et al.*, 1995), stress-induced increase in the expression of CCK mRNA in hippocampus (Giardino *et al.*, 1999) and stress-induced amnesia also (Dauge *et al.*, 2003). CCK plays an important role in

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communication among neurons as a neuromodulator or neurotransmitter (Crawley, 1994). Proglumide (Pgm) is known CCK antagonist (Bunney *et al.*, 1985) and changes in the receptor population of CCK and/or in CCK level has been associated with SE (Schwarzer *et al.*, 1995). These findings do suggest that in the pathogenesis of drug-induced neurobehavioral toxicity, CCK is most likely involved in the process. However, the role of CCK in LiPc-induced SE in young rats has not been elucidated. The present study was designed to explore the possible neuroprotective effects of Pgm in epileptic seizures, motor performance, cognitive behavior, and to assess the role of brain TBARS and GSH in identifying the antioxidant property of this drug in Li-Pc induced SE in young rats.

MATERIALS AND METHODS

Experimental Animals

The animals used in the present study were young male Sprague Dawley rats (20 days old), and were housed under controlled conditions with 12 hours light-dark diurnal cycle at $22\pm 1^\circ\text{C}$, humidity at 50-60% and free access to food and water except during experimental handlings. All study protocols and experimental animal handling procedures were approved by the Review Committee and were in accordance with the Research and Ethics Committee of King Saud University, Riyadh, Saudi Arabia.

SE induction

Animals were divided into seven groups at random. Groups 1, 2 and 3 served as controls and received saline, Li (3 mEq/ml/kg, i.p.) and Pc alone (20mg/ml/kg, s.c.) respectively. Groups 4 to 7 were administered an aqueous (saline) solution of Li (BDH Laboratory Supplies, Poole, England in a dose as in control), followed by (20 h later) Pc (Sigma Chemical Co., St. Louis, MO, USA, in the dose as used for control) for inducing SE. Group 4 served as the experimental control of SE group and groups 5 to 7 served as the drug test groups. Pgm (Sigma, USA), was dissolved in saline, and was administered at doses of 0, 250, 500 and 750mg/ml/kg i.p. (one hour before Pc injection) to groups 4 to 7 respectively. These doses of Pgm were selected on the basis of our pilot studies and also on the basis of earlier studies where similar doses were used in rats i.p. (Tariq *et al.*, 1998) as well as orally (Al Moutaery, 2005). After Pc injections, the animals ($n=20$ per group) were observed for stereotyped movements (STM) and peripheral cholinergic signs (PCS), clonic movements of forelimbs, tremors, head bobbing, and seizures, which developed into SE progressively within 1–2 h (Persinger *et al.*, 1993). All seizure activities were presented as latencies to develop seizure and SE and any mortality within 24h after SE was also recorded.

Behavioral observations

After induction of SE, the animals ($n=10$ to 12 per group) were subjected to behavioral studies. All animals were allowed to acclimatize to the observation room for 2h before testing. The tests were performed between 10:00 and 15:00 hours of the lighted phase. The rotarod test was followed by Morris water-maze test for each animal.

Rotarod test

The rotarod test is a standard procedure to test the motor performance and limbic motor coordination aspects for balancing the body (Hamm *et al.*, 1994). Rats were placed on the rotating rod or drum (Ugo, Basile, Italy), with a diameter of 6 cm (speed, 6rpm). The time spent on the rod maintaining the equilibrium was recorded for a maximum of 180 seconds without falling down. Average of three consecutive measurements was recorded as a single score for each animal.

Morris water-maze test: The test has been extensively used for assessing cognitive functions in epilepsy models (Faverjon *et al.*, 2002). The rats were tested for visual-spatial memory using a water-maze (Morris, 1984). To mention the method in brief, a galvanized white circular water tank (117 cm diameter, 55 cm height) was filled with clear tap water ($26\pm 1^\circ\text{C}$) to a depth of 30 cm. A stainless steel escape platform measuring 10 cm diameter, was placed 1 cm below the water level and the water was made opaque by addition of milk (one litre), which prevented visualization of the platform. North (N), south (S), east (E) and west (W), were designated on the rim of the tank, thus dividing the pool into four quadrants (NW, NE, SE and SW). Each rat was allowed to swim freely on the first day in the pool for 60 sec without the platform present in the pool. This enabled the rat to habituate to the training environment of the swimming. On days 2-5, rats were trained for 24 trials (six trials a day, with an inter-trial interval of 30 sec) to locate and escape onto the submerged (hidden) platform. Each day, at the start of each trial, the rat was held facing the perimeter of the water tank and dropped into the pool. The time (latency) from immersion into the pool to climb onto the hidden platform (maximum trial duration 120 sec) was recorded. Each rat was given a 30 sec inter-trial interval for rest on mounting the platform and for learning and memorizing the spatial cues (prominent objects on the walls of the test room) to reach the platform for escape.

On 6th day, the rats were subjected to a 120 sec probe trial in which the platform was removed from the pool. The time spent in each quadrant (within 120 sec probe test) was recorded. In such probe trials, normal animals typically spend more time in the quadrant where the platform had been primarily located (days 2-5) than in other quadrants. Such probe trial is a measure of the strength of spatial learning or memory recall, the closest parallel to episodic memory in humans (Jeltsch *et al.*, 2001).

Biochemical studies

On the basis of our pilot studies and from the literature survey (Nascimento *et al.*, 2005, Frietas *et al.*, 2006), the biochemical investigations were carried out 1h after Pc injection. The animals (n=8 to 10 from each group) were sacrificed by decapitation and the striatum and hippocampus from the cerebral area were removed on iced surface and frozen immediately in liquid nitrogen and stored at -70°C for the determination of the oxidative stress indices like lipid peroxides (TBARS) and total glutathione (GSH).

Determination of lipid peroxides

Lipid peroxides (LP) were estimated in striatum and hippocampus tissues spectrophotometrically in the form of thiobarbituric acid-reactive substances (TBARS) following the method of Ohkawa *et al.*(1979). All tissues were homogenized in 1.15% cold KCl, centrifuged at 3000xg for 5 min and the aliquot from the clear supernatant was mixed with 2ml of reaction mixture (containing 15% trichloroacetic acid and 0.375% thiobarbituric acid solution in 0.25 N HCl) and heated in a boiling water bath for 5 min. Thereafter the samples were cooled at room temperature and centrifuged at 1000xg for 10 min. The absorbance of supernatant was read at 535 nm for absorbance against a blank that contained all reagents except the tissue samples. Tissue lipid peroxide levels were quantified and expressed as nanomoles of TBARS formed per g tissue weight in for 5 min.

Determination of Glutathione

Total glutathione (GSH) level in striatum and hippocampus was estimated by a modified method of Mangino *et al.* 1991). Briefly, the isolated brain tissues (about 50 mg) were homogenized in 1ml 0.1 M perchloric acid containing 0.005% EDTA. After centrifugation of the homogenates, at 4000 rpm for 10 min the GSH was assayed in the supernatants in a dual beam Shimadzu UV160 UV-VIS spectrophotometer at 30°C. The absorbance was monitored for 3 min at 412 nm and the change in absorbance (slope of change) was used to quantitate total GSH by comparing the slope with a standard curve prepared with pure glutathione (Sigma) on the same spectrophotometer.

STATISTICAL ANALYSIS

All data were statistically analyzed by Gaussian-shaped distribution for normality using the Kolmogorov – Smirnov goodness-of-fit test and Bartlett’s test for equal variance. The data passing the normality test (p>0.01) were compared with the ANOVA with post-hoc testing using Tukey-Kramer Multiple Comparisons Test or Student-Newman-Keuls Multiple Comparisons Tests. The results were expressed as the mean value ± standard error of the mean (SEM) and the significance were defined as p<0.05 for all tests.

RESULTS

Characteristics of SE induced by Li-Pc

A significant and gradual change in behavior started appearing within 5 min after Pc injection in all the animals. Changes were observed in PCS (piloerection, miosis, mild tremors, diarrhoea, salivation, scratching, and STM (paw licking, sniffing, and rearing) followed by seizures in 100% of the animals with a mean latency of 9.62 ± 1.2 min to develop seizure (table 1). The signs of convulsions consisted of head bobbing with intermittent forelimb and hindlimb clonus, loss of posture, hyperextension of tails, falling back and myoclonic jerks building up to a status epilepticus (SE) in 100% of pups. The mean latency to onset of SE was 23.86 ± 1.54 min (table 1), and on the average, the SE lasted for more than one hour in all the animals. In all a mortality of 10% was observed over a period of 24h following Pc injections (table 1).

Effect of Pgm pre-treatment on Li-Pc induced SE

A dose-dependent and significant increase in the latencies to seizure and SE and decrease in the percentages of seizures and SE was observed in the SE animals pretreated with Pgm (table 1). Furthermore, a reduction in the intensity and frequency of seizure, PCS and STM were also observed in these animals (data not shown in table 1). The young rats pretreated with Pgm, showed no mortality as compared to 10% mortality in the Li-Pc treated group (table 1). All control groups that received only Li or the drugs alone did not show any signs of seizure or SE.

Table 1: Dose dependent antiepileptic activity of proglumide (Pgm) against Li - Pc induced Status Epilepticus (SE).

Behavioral Parameters Observed	Control	Pgm (mg/kg)		
	0	250	500	750
Latency to seizures (min)	9.62±1.20	12.46 ^{ns} ±1.17	22.47 [*] ±1.59	35.33 ^{**+} ±1.64
Seizures (%)	100	85.2	61.3 [*]	21.3 ^{**+}
Latency to SE (min)	23.86±1.54	32.52 [*] ±1.07	44.61 ^{**+} ±1.22	56.11 ^{***+} ±2.63
SE (%)	100	61.4 [*]	36.8 ^{**+}	23.5 ^{****}
Mortality (%) Within 24 hours	10	0	0	0

Animals were observed for more than 1h after Li-Pc (Lithium-Pilocarpine) injections for SE in all groups, and for more than 24 hours for mortality. ^{ns}statistically non-significant. * and + significant with respect to control and within treated groups respectively.

Behavioral consequences

Rotarod test

The control rats walked normally on the rod and maintained equilibrium, whereas the Li-Pc (SE) treated rats were unable to maintain a significant equilibrium (fig. 1). The animals attempted to escape in right or left directions resulting in early fall from the rod. Overall, Li-Pc (SE) animals could not stay longer on the rotating drum and spent lesser time on the rotarod as compared to the rats in control group. However, pretreatment with Pgm significantly and dose-dependently improved the equilibrium performance of animals on rotarod (fig. 1).

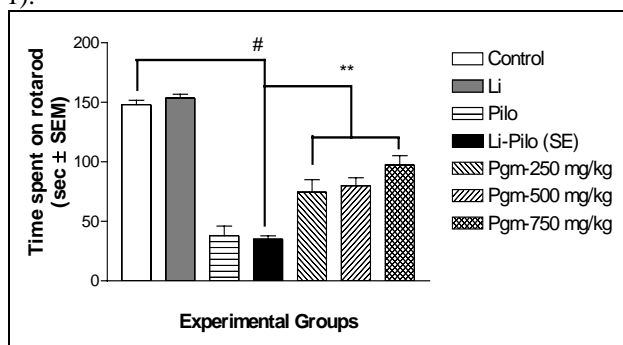


Fig. 1: Effect of proglumide (Pgm) in varying doses on the motor performance in the rotarod test expressed as the time spent on the rod maintaining equilibrium without falling down.

Morris water-maze test

Rats treated with Li-Pc (SE), took a longer time to reach the platform as compared with control group ($p < 0.01$; fig. 2), however, a gradual improvement in performance over the 4 days of testing (training) period (day 2 to day 5) was noticed in all groups of animals. Overall, the number of successful trials to reach the platform was significantly higher in the Pgm pre-treated groups as compared to Li-Pc (SE) group on all the four testing days ($p < 0.001$; fig. 2). Observations on the sixth day of training (probe trial) showed that Pgm pretreatment caused the animals to spent more time in the target (platform) quadrant as compared to the Li-Pc only (SE) group ($p < 0.001$; fig. 3).

Biochemical Studies

Lipid peroxidation levels (TBARS) in the striatum and hippocampus. The lipid peroxidation level (TBARS) in the striatum and hippocampus were significantly ($p < 0.001$) increased after 1h of Li-PC (SE) treatment (fig. 4). Pretreatment with Pgm significantly ($p < 0.001$) and dose-dependently attenuated Li-Pc induced effects and an increase in the levels of TBARS in the striatum and hippocampus was observed as compared to Li-Pc (SE) group (fig. 4).

Glutathione (GSH) in striatum and hippocampus

The Li-Pc (SE) group showed a highly significant ($p < 0.001$) depletion of striatal and hippocampal GSH (fig. 5). However, pretreatment with Pgm attenuated Li-Pc

induced depletion in GSH significantly and dose dependently as compared to Li-Pc (SE) group (fig. 5).

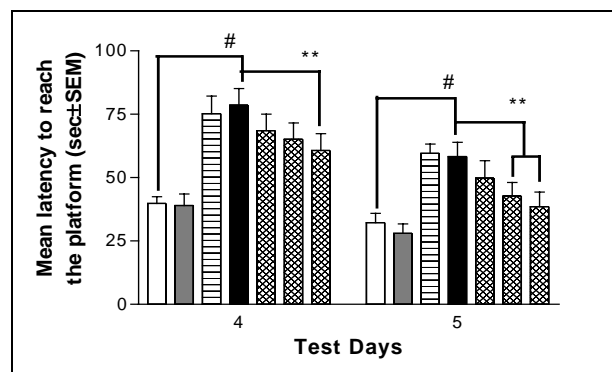
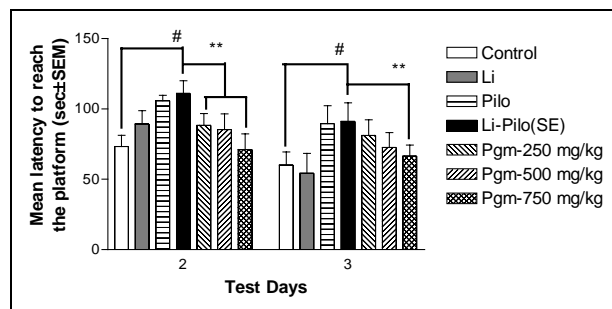


Fig. 2: Performance in water-maze of animals that experienced SE along with their controls as well as the SE groups that were pretreated with proglumide (Pgm). Mean latency to reach the hidden platform (y-axis) on each testing day (x-axis) shows that animals subjected to SE were slower in finding the platform (cognitive effect) than the controls on all four testing days. Treatment with Pgm in varying doses was effective in improving the cognitive function.

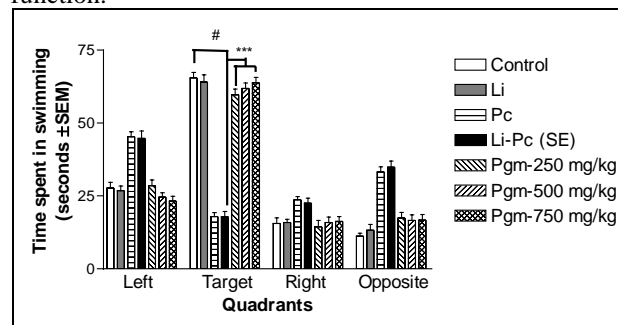


Fig. 3: Performance in water-maze of animals that experienced SE along with their controls as well as the SE groups that were pretreated with proglumide (Pgm). The probe test shows that the SE-induced animals spent less time in the target quadrant than the control group. Pretreatment of SE-induced animals with Pgm had an attenuating effect and the animals spent more time in the target quadrant.

Abbreviations: Li=lithium chloride; Pilo=pilocarpine; SE=status epilepticus; # represents significance as compared to control ($p < 0.001$), whereas *, ** and *** show $p < 0.01$, $p < 0.05$ and $p < 0.001$ respectively, as compared to SE group by ANOVA.

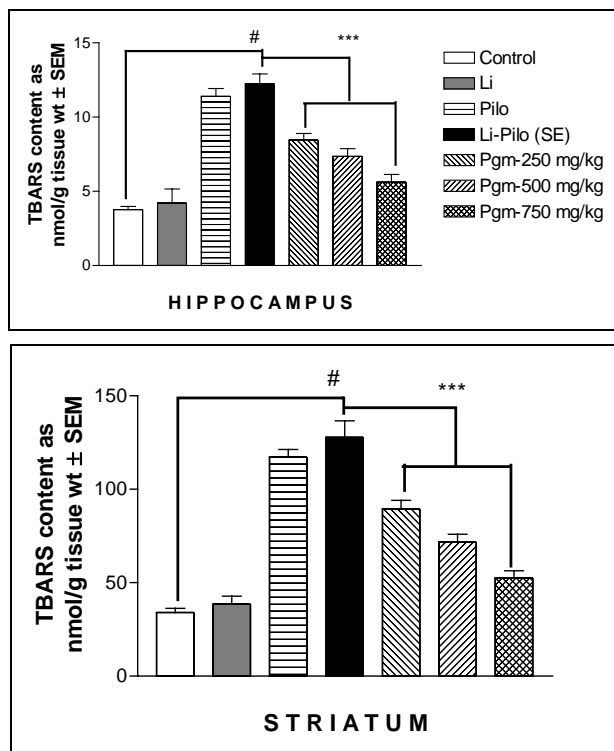


Fig. 4: Effect of proglumide (Pgm) on lipid peroxidation content (TBARS) in hippocampus and striatum of young rats after Li – Pc induced SE.

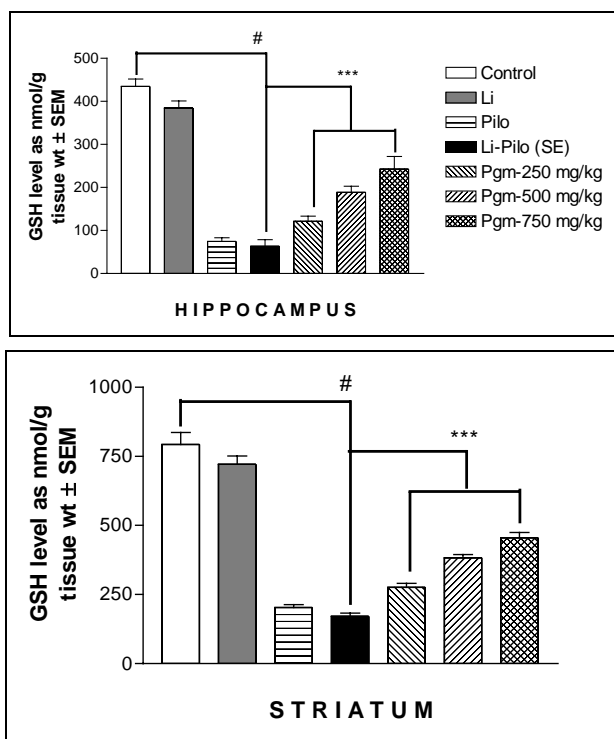


Fig. 5: Effect of proglumide (Pgm) on total glutathione level (GSH) in hippocampus and striatum of young rats after Li – Pc induced SE.

DISCUSSION

The present findings indicate that Pc administration to rats pretreated with Li, showed all symptoms of PCS including piloerection, miosis, mild tremors and diarrhea followed by seizures. Later on, SE was developed in the animals between 20 to 30 minutes after Pc administration showing almost all signs of STM like head bobbing, intermittent hind limb and forelimb clonus, hyperextension of hind limbs and tail along with loss of body posture. The result of present neurobehavioral studies using rotarod indicated impaired motor coordination and the results are in agreement with earlier reports in rats with SE (Treit *et al.*, 1993). Results of Morris water-maze test are also in agreement with earlier study (Wu *et al.*, 2001) which suggested that animals with SE either took a longer time to reach the escape platform or completely failed to reach the platform suggesting for an impaired visual-spatial memory and cognitive deficit. The present deficits in neurobehavioral findings of young rats are further supported by clinical reports also where 20 % motor deficit and 33 % intellectual impairment have been reported in children with SE (Aicardi and Chevrie, 1983). The specific cause of motor and cognitive deterioration following SE is far from clear. However, according to recent reports, neurochemical imbalance following SE might be responsible for neurobehavioral changes (Nascimento *et al.*, 2005; Morris *et al.*, 2003). Furthermore, Pc has recently been shown to cause a selective reduction in the CCK positive basket cell innervations resulting into inhibition of pyramidal cells which ultimately decreases seizures in a mouse model of epilepsy (Wyeth *et al.*, 2010).

The present results showed evidently the antiepileptic activity of Pgm against Li-Pc induced seizure, as revealed by highly significant decrease in frequency of epileptic activities, increase in the latency to SE and causing no mortality (table 1). Furthermore, our study consistently demonstrated that pharmacological intervention with Pgm, significantly and dose-dependently attenuated SE induced deficits in motor performance and impaired memory. Pgm has also been reported to reduce the stress-induced increase of pentylenetetrazole (PTZ) induced seizure threshold (Homayoun and Dehpour, 2004).

The present biochemical studies indicated a significant and dose-dependent increase in TBARS and decrease in GSH levels in the striatum and hippocampus of the rats treated with Li-Pc (figs. 3 and 4) suggesting for a preliminary and significant level of oxidative stress in these brain areas. Pretreatment of SE animals with Pgm attenuated significantly and dose dependently the Li-Pc induced oxidative stress in the striatum and hippocampus (figs. 3 and 4) and this finding is supported by the earlier studies also (Xavier *et al.*, 2007; Tariq *et al.*, 2008). However, further studies involving measurement of GSH/

GSSG (oxidized glutathione) ratio might be interesting since glutathione is a putative neurotransmitter (Abe *et al.*, 1999). Studies involving more antioxidant parameters like superoxide dismutase, catalase and glutathione peroxidase estimation may further support for the antioxidant capability of Pgm.

The present findings clearly indicate that lipid peroxidation levels (TBARS) in the striatum and hippocampus of young rats increase whereas the reduced glutathione (GSH) concentration decrease after seizure activities of SE induced by Li-Pc. It is likely that this pathomechanism of oxidative stress in brain may be the possible reason at least in part to the pathophysiology of the seizure activity. The present study suggests for the antioxidant activity of Pgm at a preliminary level and emphasize that further studies are needed to establish this possibility.

In conclusion, the neurobehavioral deficits and the imbalance in oxidative stress activities (TBARS and GSH) in the present study may probably be involved in the induction of SE through propagation of seizures. Pgm shows a promising anticonvulsant and antioxidant activity against SE and draws our attention for further detailed studies on these lines.

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