Neurobehavioral and biochemical effects of magnesium chloride ($MgCl_2$), magnesium sulphate ($MgSO_4$) and magnesium-L-threonate (MgT) supplementation in rats: A dose dependent comparative study

Sadia Sadir[1], Saiqa Tabassum[1,2], Shaista Emad[1,3], Laraib Liaquat[1], Zehra Batool[1,4], Syeda Madiha[1], Sidrah Shehzad[1], Irfan Sajid[5] and Saida Haider[1]*

[1]Neurochemistry and Biochemical Neuropharmacology Research Unit, Department of Biochemistry, University of Karachi, Karachi, Pakistan
[2]Department of Biosciences, Shaheed Zulfikar Ali Bhutto Institute of Science and Technology (SZABIST), Karachi, Pakistan
[3]Department of Biochemistry, Jinnah Medical & Dental College, Karachi, Pakistan
[4]Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi, Pakistan
[5]Department of Biochemistry, Federal Urdu University, Karachi, Pakistan

Abstract: Magnesium (Mg) is an essential biomineral that acts as an intracellular cofactor for more than 300 enzymes. It is an important modulator of the N-methyl-D-aspartate (NMDA) receptor which is involved in memory function and depression. The purpose of this study was to compare the dose dependent effect of oral supplementation of Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4$) and Magnesium-L-threonate (MgT) on memory and depression-related behaviors in rats. Rats were orally administered with different doses (50 mg/kg, 100 mg/kg and 150 mg/kg) of each Mg salt. Following 28 days of oral supplementation, animals were subjected to behavioral tests. After completion of behavioral test, rats were decapitated. Brain and plasma samples were used for neurochemical and biochemical analysis. Assessment of behaviors in elevated plus maze (EPM) test and forced swim test (FST) showed that MgT more significantly improved memory of rats and decreased depression-like symptoms in healthy rats as compared to controls. Biochemical analysis indicated significant increase in plasma Mg levels dose dependently following MgT administration. This increase might be related to observe enhanced cholinergic functions and decline in oxidative stress in rats in the present study. This comparative study highlights that MgT (100mg/kg) is the most appropriate Mg salt and dose for oral treatment that strengthens cholinergic system and improves brain related functions through attenuation of oxidative burden in adult healthy rats.

Keywords: Magnesium, memory, acetylcholine, acetylcholinestrase, oxidative stress, depression.

INTRODUCTION

Nutrients are the vital factors that are required by the body for the survival of living organisms. The importance of minerals in nutrition of humans is well known. Minerals are inorganic nutrients, distributed in all tissues and fluids and their existence is essential for the body to perform specific cellular functions (Bourre, 2006). Magnesium (Mg) is the fourth most abundant mineral in the living body. It is a crucial intracellular cation involved in a broad variety of biochemical functions. Mg is a blocker of the N-methyl-D-aspartate (NMDA) receptor (the ionotropic glutamate receptor) (Slutsky *et al.,* 2010; Eby and Eby, 2010). Mg deficiency has been related to various biochemical, neurochemical and molecular alterations that are associated with variety of neurological and neuromuscular dysfunctions including behavior disturbances, hyperexcitability, depression, tetany, cardiac arrhythmias, muscular weakness, tremors, increased stress susceptibility, ataxia, anxiety, irritability, memory disturbances and psychotic behaviors while each of these symptoms were reported to be attenuated by Mg administration (Sartori *et al.,* 2012; Serefko *et al.,* 2013; Mickley *et al.,* 2013). Recently, a highly bioavailable Mg composition, Magnesium-L-threonate (MgT) was developed that was shown to elevate brain Mg via chronic oral supplementation (Slutsky *et al.,* 2010). Researches on MgT revealed that increased levels of Mg in the brain increased NMDA receptors signaling and the expression of brain-derived neurotrophic factor (Lou *et al.,* 2017; Abumaria *et al.,* 2011). These structural alterations in the rat brain enhance synaptic plasticity, which is responsible for improvement in learning and memory in young as well as aged rats (Slutsky *et al.,* 2010). Age-associated memory disorders are also associated with the cholinergic dysfunctions (Haider *et al.,* 2015). Acetylcholine (ACh), a key neurotransmitter stimulates cholinergic neurons and has a major role in memory process (Deiana *et al.,* 2011, Puri *et al.,* 2014). Acetyl cholinesterase (AChE) is the crucial enzyme mainly found at cholinergic neuronal synapses in the central nervous system, catalyzes the hydrolytic breakdown of ACh to acetate and choline. AChE is reported to be a specific biomarker for the assessment of cholinergic functions (Zhong *et al.,* 2009).

**Corresponding author:* e-mail: saida-h1@hotmail.com

Memory disturbances are also associated with depression (Puri *et al.,* 2014). Numerous studies have demonstrated the antidepressant effect of Mg in various models of depression in mice and rats (Poleszak *et al.,* 2004, 2005a, 2005b, 2006). Many authors have reported that Mg also enhances the efficacy of classic antidepressants (Cardoso *et al.,* 2009; Poleszak *et al.,* 2005b) and NMDA antagonists (Poleszak *et al.,* 2007) in behavioral investigations. However, literature studies showed that Mg in the form of MgT has not been evaluated for antidepressant profile except for a single study (Abumaria *et al.,* 2009). Oxidative stress has been postulated to have an important role in the pathogenesis of depression and memory disturbances (Ng *et al.,* 2008; Parletta *et al.,* 2013). Oxidative stress is the disturbance of oxidants-antioxidants balance and its equilibrium shifts toward the oxidants, impairs redox signaling and responsible for cellular and molecular damages (Chung *et al.,* 2013). Previous studies have linked elevated oxidative stress markers with brain-associated disorders (Chung *et al.,* 2013, Haider *et al.,* 2015; Tonnies and Trushina, 2017). Free radicals are produced as a result of oxidative imbalance and leads to lipid peroxidation (LPO) and consequently causes tissue damage. The brain is more compromised in oxidative damage because of its high rate of oxygen consumption, its modest antioxidant defenses and its lipids-rich composition that provides greater substrate for lipid oxidation (Ng *et al.,* 2008; Parletta *et al.,* 2013). Oxidative damage in tissues is mainly monitored by the estimation of malondialdehyde (MDA) levels which is a well-known secondary product of LPO under the conditions of oxidative stress (Zhong *et al.,* 2009). Mg has been reported to have antioxidant potential. Researchers demonstrated that Mg treatment decreases LPO and increases antioxidant enzymes in rat plasma, liver and brain (Hans *et al.,* 2003; Safar *et al.,* 2010) that could be linked to its antagonistic activity towards NMDA receptors (Safar *et al.,* 2010). Moreover, evidence suggest that pretreatment with Mg was protective against cadmium induced oxidative stress in rat plasma (Buha *et al.,* 2012).

Extensive work has been done on the effects of $MgCl_2$ and $MgSO_4$ on memory and depression related behavior but research studies using MgT is scarce. Based on the above discussed findings, the present work is designed to compare the dose-related behavioral effects of commonly used chloride and sulfate salts of Mg to commercially available new organic form of Mg, available as Mg-L-Threonate and its possible involvement in cholinergic functions and oxidative stress.

MATERIALS AND METHODS

Animals

Sixty male Albino Wistar rats weighing 180±50g purchased from HEJ Institute of Chemistry, University of Karachi, Karachi, Pakistan were used in the study. All the experimental animals were housed in separate cages at constant temperature (22±2°C) and humidity (60±10%) under a 12h light-dark cycle (light on at 06:00 h). Animals were supplied with standard rodent diet and tap water freely. All experimental procedures were performed in a balanced manner to nullify the effect of order and time. All experiments were approved by institutional Board of Advance Studies and Research (BASR no: 01768/Sc), University of Karachi and performed in strict accordance with National Institute of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). Before the start of experimental work, all animals were given 1 week of acclimation period and subjected to various handling methods in order to avoid the stress of novelty and handling.

Drug preparations

Magnesium chloride ($MgCl_2$, Merck, Darmstadt, Germany), Magnesium sulphate ($MgSO_4$, Merck, Darmstadt, Germany) and Magnesium-L-threonate (MgT, Magceutics Inc. USA) solutions were prepared in distilled water and administered orally to experimental animals. Doses of elemental Mg were chosen on the basis of our previous experiments and literature (Slutsky *et al.,* 2010; Abumaria *et al.,* 2011; Haider *et al.,* 2016).

Experimental protocol

Experimental animals were randomly assigned into ten groups of six rats and treated for 28 days respectively: (1) Control (Tap water), (2) $MgCl_2$ (50mg/kg), (3) $MgCl_2$ (100 mg/kg), (4) $MgCl_2$ (150mg/kg) (5) $MgSO_4$ (50 mg/kg) (6) $MgSO_4$ (100mg/kg) (7) $MgSO_4$ (150mg/kg) (8) MgT (50mg/kg) (9) MgT (100mg/kg) (10) MgT (150mg/kg). Following 28 days of oral supplementation animals were subjected to Elevated Plus Maze (EPM) test and Forced Swim Test (FST) for assessment of memory and depression. At the end of behavioral assessments, rats were decapitated to collect their plasma and brains. Plasma and brains were immediately preserved for further biochemical and neurochemical assays.

Behavioral methods

Elevated plus maze (EPM) test

EPM is usually conducted to assess acquisition and retention process of memory (Batool *et al.,* 2016). In this study the test was used to assess memory of rats in terms of % memory retention. The test comprised of two trials (1st day: Learning and 2nd day: Retention of memory). On the 1st day rats were individually placed at one end of open arm, facing away from the central platform and the transfer latency (time taken in seconds by the rat to move into one of the closed arm with all its four paws) was recorded. The cutoff time was 2 min for the rat to explore the maze. On the 2nd day test session was performed to evaluate the retention of memory and same procedure was repeated. The % memory retention was calculated by

using the formula (TL_1- TL_2)/TL_1 × 100 [TL_1= transfer latency on the 1st day; TL_2=transfer latency on the 2nd day]. Significant increase in % memory retention was a sign of memory improvement in rats. The method for EPM was same as described previously (Haider *et al.*, 2016).

Forced swim test (FST)

This behavioral test is widely used to assess the depression like symptoms in rats. In this test the depression like behavior in the rat was judged by the immobile posture i.e., the rat remained floating in water without any struggling or efforts for escape and making only those movements needed to keep its head above the surface of water. Test session was conducted for 5 min of each rat. The time of immobility (sec) during 5 min of test period was recorded and the procedure for behavioral assessment was same as described earlier (Haider *et al.*, 2015).

Neurochemical methods

Neurochemical analysis was done to monitor the changes in cholinergic functions. Brain homogenates were used to determine concentration of whole brain content of ACh and presented as nmol/g of brain tissue (Batool *et al.*, 2016). Activity of AChE in rat brain was also determined and expressed as µmol/min/g of brain tissue (Haider *et al.*, 2015).

Biochemical methods

Estimation of plasma Mg

Analysis of plasma Mg levels was done by using commercially available kit (abcam, ab102506).

Estimation of oxidative status

Oxidative status of brain was monitored in terms of LPO by measuring MDA levels, the ultimate end product of LPO. Results of LPO were expressed in terms of µmoles of MDA/g of brain (Haider *et al.*, 2015).

STATISTICAL ANALYSIS

The statistical analysis was performed by using one-way analysis of variance (ANOVA) via SPSS version 20 software. Post-hoc analysis was performed using Bonferroni test. A probability (p) less than 0.05 was considered significant. Values are expressed as mean ± SEM.

RESULTS

Dose related effects of Mg salts on Memory function

Dose response effects of three different salts of Mg on memory function were evaluated by EPM (fig. 1) following 28 days of oral administration. Results for EPM analyzed by one-way ANOVA displayed significant effect of Mg treatment on % memory retention [$F(9,50) = 4.237$, $p<0.01$]. Post hoc test showed that administration of MgT at the doses of 100 mg/kg and 150 mg/kg for 28 days resulted in a significant increase in % memory retention ($p<0.01$) as compared to control rats. Data of % memory retention revealed no significant change in rest of the Mg groups as compared to that of controls. However, there was a tendency of improvement in % memory retention in all Mg treated groups as compared to control rats.

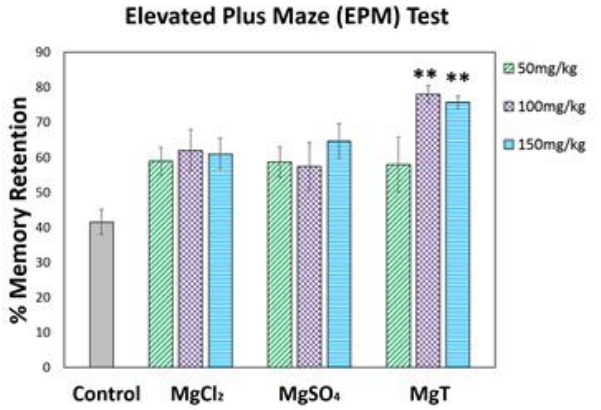

Fig. 1: Effect of oral supplementation of Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4$) and Magnesium-L-threonate (MgT) on % memory retention assessed by elevated plus maze (EPM) test. Data is presented as mean ± SEM (n = 6). Analysis was done by one-way ANOVA. Significant differences were obtained by Bonferroni test: **p < 0.01 with respect to controls.

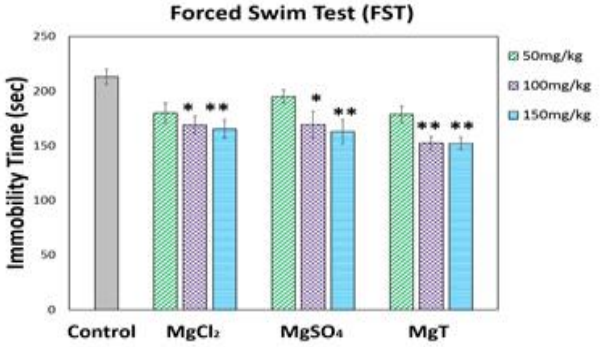

Fig. 2: Effect of oral supplementation of Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4$) and Magnesium-L-threonate (MgT) on immobility time in forced swim test (FST). Data is presented as mean ± SEM (n = 6). Post-Analysis was done by Bonferroni test following one-way ANOVA. Significant differences were obtained *p < 0.05, **p < 0.01 with respect to controls.

Dose related effects of Mg salts on depression-like symptoms

The dose related effects of Mg administration on depression-like symptoms in rats was evaluated by FST. Data for FST (fig. 2), which was analyzed by one-way ANOVA revealed significant effect of Mg administration [$F(9,50) = 5.022$, $p<0.01$]. Post hoc analysis indicated that

administration of $MgCl_2$ and $MgSO_4$ at the dose of 100 mg/kg ($p<0.05$) and 150mg/kg ($p<0.01$), significantly decreased immobility time of rats in FST as compared to controls whereas MgT supplementation at both the doses of 100 mg/kg and 150 mg/kg ($p<0.01$) for 28 days resulted in a more significant decrease in immobility time as compared to control rats.

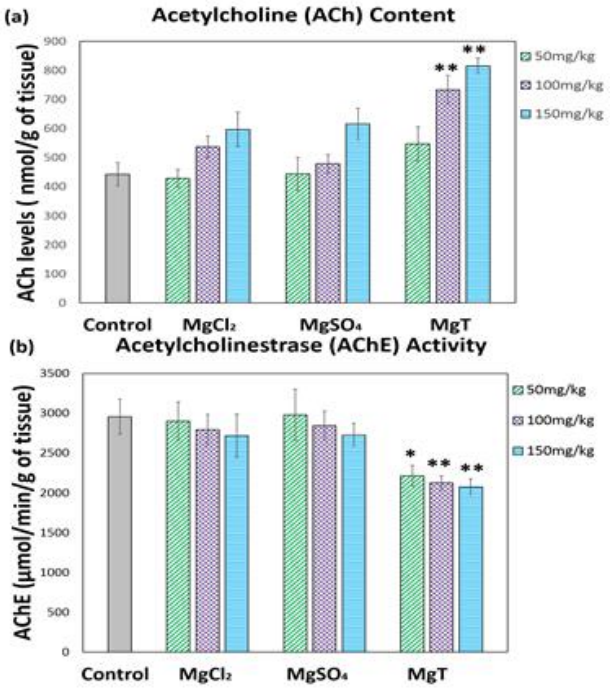

Fig. 3: Effect of oral supplementation of Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4$) and Magnesium-L-threonate (MgT) on (a) brain acetylcholine (ACh) levels and (b) acetylcholinesterase (AChE) activity in whole brain. Data is presented as mean ± SEM (n = 6). Post hoc analysis following one-way ANOVA was done. Significant differences were obtained *p<0.05, **p<0.01 from control rats.

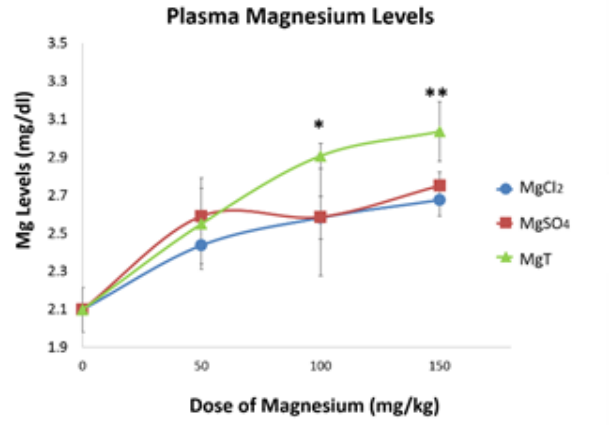

Fig. 4: Dose response curve of oral supplementation of Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4$) and Magnesium-L-threonate (MgT) for plasma magnesium (Mg) levels Data is presented as mean ± SEM (n=6). Post-Analysis was done by Bonferroni test following one-way ANOVA. Significant differences were obtained *p<0.05, **p<0.01 with respect to controls.

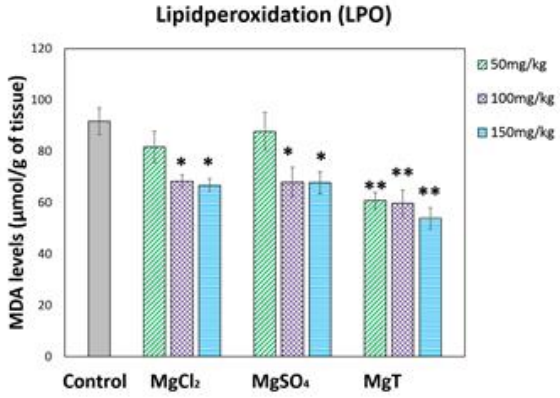

Fig. 5: Effect of oral supplementation of Magnesium chloride ($MgCl_2$), Magnesium sulphate ($MgSO_4$) and Magnesium-L-threonate (MgT) on brain lipid peroxidation (LPO) represented as Malondialdehyde (MDA) levels in brain tissue. Data is presented as mean ± SEM (n = 6). Analysis was done by one-way ANOVA. Significant differences were obtained by Bonferroni test: *p<0.05, **p<0.01 with respect to controls.

Dose related effects of Mg salts on brain cholinergic function

Brain cholinergic functions were assessed by measuring ACh content and AChE activity. Data analysis by one way ANOVA showed significant effect of Mg supplementation on ACh content [$F(9,50) = 7.953$, $p<0.01$] and AChE activity [$F(9,50) = 6.858$, $p<0.01$] in rat brain. Regarding brain ACh content (fig. 3a), data analyzed by Bonferroni test displayed that only supplementation of MgT at the doses of 100 and 150 mg/kg raised the brain ACh content significantly as compared to control rats. However, brain ACh content in other Mg treated groups was found comparable to controls. Post hoc analysis of results for AChE activity (fig. 3b) showed that MgT following 28 days of administration at the doses of 50 ($p<0.05$), 100 and 150 mg/kg ($p<0.01$) significantly decreased the brain AChE activity as compared to that of controls. However, effect of $MgCl_2$ and $MgSO_4$ treatment was not found significant in post-hoc comparison.

Dose related effects of Mg salts on plasma Mg levels

Analysis of plasma Mg levels by one way ANOVA showed significant difference between groups [$F(9,50) = 2.610$, $p<0.05$]. Fig. 4 shows dose dependent curves for each Mg salt. However, post-hoc comparison showed that only MgT at a dose of 100 mg/kg ($p<0.05$) and 150 mg/kg ($p<0.01$) significantly increased plasma Mg levels dose dependently as compared to controls. Whereas dose dependent increase in plasma Mg levels by other Mg formulae were found non-significant as compared to that of controls.

Dose related effects of Mg Salts on brain oxidative status
Effect of Mg on oxidative status of brain was determined by MDA levels indicating LPO. Results of LPO (fig. 5), showed significant effect of Mg treatment following 28 days [$F(9,50) = 6.772$, $p<0.01$] analyzed by one-way ANOVA. Post analysis by Bonferroni test demonstrated that $MgCl_2$ and $MgSO_4$ at the dose of 100 mg/kg and 150 mg/kg ($p<0.05$) significantly decreased LPO as compared to controls, as indicated by a decrease in MDA levels. Moreover, post-hoc analysis demonstrated that all three doses of MgT ($p<0.01$) significantly decreased MDA levels in brain as compared to controls. This decline in brain LPO was more pronounced following MgT treatment as compared to other salts. Thus, MgT is more effective in reducing oxidative stress in healthy rats since all three doses of MgT produced significant effect on LPO as evident by a decrease in MDA levels.

DISCUSSION

The present study shows the dose related memory improving and antidepressant effects of Mg salts. Mg, an important biomineral has been largely removed from daily diet of individuals due to extensive use of processed foods, which was considered as potentially harmful for human physiology (Eby and Eby, 2010). Novel synthetic form of Mg, MgT was recently developed and has gained much attention due to its maximum bioavailability in brain. In previous studies, MgT was typically administered to experimental animals via drinking water (Slutsky *et al.,* 2010; Abumaria *et al.,* 2011; Mickley *et al.,* 2013), however in this current study MgT and other Mg salts were administered orally by feeding tube. The aim of this study was to compare the behavioral effects of oral supplementation of this highly bioavailable Mg compound with other Mg salts focusing on its role in cholinergic system and effects on oxidative stress. Findings of this study revealed that MgT among all salts produced significant beneficial effects in behavioral investigation of both memory and depression related to significant enhancement in cholinergic neurotransmission and antioxidant status.

Dietary pattern and lifestyle can affect the human brain health and has a significant role in the formation of brain cognitive and defensive capacity. Age associated brain illnesses can also be due to inability of brain protective mechanism because of dietary deficiencies (Parletta *et al.,* 2013). So, it is highly important to determine the dietary elements that are capable of maintaining the neuronal health and help in proper brain functioning. Extensive literature emphasizes on the protective role of Mg in various disease conditions particularly with brain associated disturbances (Safar *et al.,* 2010; Buha *et al.,* 2012; Serefko *et al.,* 2013; Haider *et al.,* 2016). The role of Mg in memory and depression is well reported earlier (Hoane, 2007; Serefko *et al.,* 2013) but it is still emerging due to its involvement in variety of physiological and biochemical functions in the body.

In this study memory was assessed in EPM by calculating % memory retention. Findings showed that oral supplementation of MgT at a dose of 100 mg/kg and 150 mg/kg significantly improved memory of healthy rats while $MgCl_2$ and $MgSO_4$ failed to do so. These results are in accordance with previously reported data (Slutsky *et al.,* 2010), since oral treatment of MgT was previously shown to increase 7-15% Mg in rat CSF while other Mg compounds did not raise Mg in CSF as compared to controls (Slutsky *et al.,* 2010). We monitored plasma Mg concentration by chronic oral administration of Mg salts to rats and similar results obtained that only MgT supplementation significantly increased plasma Mg levels in a dose dependent manner. Though serum Mg level was found to be significantly increased following administration of $MgCl_2$ in previous literature but route of administration used was different in the study (Poleszak *et al.,* 2005a). $MgCl_2$ and $MgSO_4$ have been previously reported to attenuate memory loss induced by traumatic brain injuries. But, however the route of administration of Mg treatment was not oral in these studies (Hoane, 2007). Improved memory function in the present study following chronic supplementation of MgT may be attributed to the enhancement of cholinergic function. Administration of MgT decreased AChE activity and increased ACh levels in the brain tissue. To our knowledge this is the first study to show the dose-related effect of MgT on cholinergic function of brain. Brain cholinergic system is highly implicated in the process of learning and memory (Puri *et al.,* 2014). Mg is an inorganic NMDA receptor antagonist, and its nootropic effect is attributed to this antagonistic property previously (Slutsky *et al.,* 2010; Abumaria *et al.,* 2011). Our study clearly demonstrated the involvement of the cholinergic function in the memory improving effects of MgT. The enhancement in brain ACh levels by inhibition of AChE may strengthen the process of learning and memory and its positive implication in neurodegenerative disorder has been demonstrated earlier (Puri *et al.,* 2014). It may be suggested from our study that MgT could play a significant role in the maintenance of synaptic levels of ACh by inhibiting its catalytic enzyme (AChE) activity, thus it can be used as better preventive therapy for age-linked cognitive and memory dysfunctions.

In current investigation, dose dependent effect of different salts of Mg in depression like symptoms was evaluated by FST. Administration of $MgCl_2$, $MgSO_4$, and MgT significantly decreased immobility time in FST. Findings of FST in this study are in line with previously reported data that administration of Mg salts produce an antidepressant-like effect in the FST, a widely-accepted behavioral model (Poleszak *et al.,* 2004, 2005a, b, 2007). The above findings also highlight the coping ability of

MgT in healthy rats. Various neurochemical and biochemical alterations in the brain are associated with behavioral and cognitive deficits (Ng *et al.*, 2008; Stasiak *et al.*, 2014). Oxidative stress has been involved in the pathogenesis of various disease states and may be a common underlying mechanism in various brain associated disorders (Chung *et al.*, 2013; Liaquat *et al.*, 2018). Under normal physiological conditions, cellular organelles including mitochondria, peroxisomes, endoplasmic reticulum, are the main source of free radicals' production (Tonnies and Trushina, 2017). Free radicals act as oxidizing agent that cause the stimulation and destruction of sensitive macromolecules responsible for cellular death (Dong *et al.*, 2009). To evaluate the effect of MgT on oxidative stress by chronic MgT supplementation we monitored the MDA levels in brain. It has been reported that Mg modulates the oxidants-antioxidants equilibrium and elevates antioxidant compounds to decrease LPO in plasma and brain in various disease and toxicity models (Hans *et al.*, 2003; Buha *et al.*, 2012). Similar findings were observed in this current study that Mg in all the forms; $MgCl_2$, $MgSO_4$ and MgT decreased brain LPO in rats. Eby and Eby (2010) previously reported that Mg deficiency with excessive Ca and glutamate levels increases oxidative burden responsible for various brain disabilities including depression and memory loss in human. In this study, improvement in brain oxidative profile could be due to increased plasma Mg levels observed following MgT supplementation and may be attributed to its antidepressant-like effects in healthy rats.

CONCLUSION

This study highlights that administration of Mg significantly increased the memory function and enhanced the coping ability against depression like symptoms in rats possibly by improving cholinergic function and antioxidant mechanism of brain. However, this improvement was more pronounced following oral supplementation of MgT which among all three different salts of Mg showed the maximal beneficial effects in brain associated functions in healthy rats. Therefore, this route and dosage regimen of MgT (100 mg/kg) can be suggested as a moderate dose for its protective effects in brain functions.

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