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ORIGINAL ARTICLE

Betahistine Prevents Memory Loss in Pentylenetetrazole-Kindled Mice: The Role of Acetylcholinesterase and Glycogen Synthase Kinase-3β Inhibition

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ABSTRACT

Background: Recurrent seizures are usually associated with memory deficits. **Objectives**: This study assessed the effect of betahistine, histamine (H3) receptor antagonist, on memory deficits in pentylenetetrazole (PTZ)-kindled mice. **Methods**: 24 mice were allocated into saline, PTZ, and betahistine 5 mg-PTZ and betahistine 10 mg-PTZ equal groups. PTZ, 40mg/kg, was injected i.p. on alternate days while betahistine, 5 and 10 mg/kg doses, were i.p. injected once daily till 19th day. Seizure score was recorded after each PTZ injection. Finally, Y-maze and elevated plus maze tests were done then mice were sacrificed followed by brain excision for assessment of histamine, acetylcholine (Ach), acetylcholinesterase (AchE), phosphorylated glycogen synthase kinase-3βS9 (pGSK-3βS9) and phosphorylated Akt (p-Akt) levels. **Results:** In PTZ group, maximum seizure score was approached with decreased time spent and number of entries in the novel arm of Y-maze as well as increased transfer latency in the elevated plus maze. Histamine, Ach, pGSK-3βS9 and p-Akt were decreased with increased AchE level. Betahistine treatments decreased seizure score with increments in time spent and number of e ntries in the novel arm of Y-maze and decreased transfer latency in elevated plus maze in dose dependant-manner. The drug increased histamine, Ach, pGSK-3βS9 and p-Akt levels while decreased AchE activity. **Conclusion:** Betahistine prevented PTZ-induced memory loss; an effect could be in part attributed to the enhanced cholinergic activity and GSK3-β inhibition.

Key words: Betahistine, Acetylcholinesterase, Spatial, Memory, Hippocampus, phosphorylated glycogen synthase kinase-3βS9.

INTRODUCTION

Epilepsy, a chronic neurological disorder, requires antiepileptic drugs (AEDs) therapy for long time or even for life [1]. The process of epileptogenesis includes series of events that lead to neuronal dysfunction and even neuronal damage [2]. The disturbed neuronal functions and neuronal loss result in learning and memory deficits [3]. The hippocampus and entorhinal cortex are the primary regions in the brain which mediate spatial navigation. Consequently, lesions in the hippocampus result in impairment of spatial memory [4]. Additionally, cognitive impairment is one of the adverse drug reactions to the prolonged use of AEDs [5].

Histamine, as a central neurotransmitter, modulates arousal, attention, and memory via four types of histamine (H1, 2, 3& 4) receptors. Presynaptic H3 receptors regulate the release of histamine, acetylcholine (Ach), norepinephrine, dopamine, and serotonin [6]. Blockade of H3-receptor promotes the release of these neurotransmitters, consequently improving cognitive functions such as attention, working memory, and memory consolidation [7]. Preclinical studies reported that H3-receptor antagonists improved social recognition and spatial learning [8& 9]. The clinical trials have demonstrated the ability of H3-receptor antagonists to enhance cognition [10& 11].

Betahistine, strong H3 receptor antagonist and weak H1 receptor agonist, is approved for treatment of vertigo of vestibular origin and vertigo-associated symptoms [12]. The drug has the ability to pass blood brain barrier and enter central nervous system with subsequent enhancement of histaminergic and cholinergic pathways [13]. Pentylenetetrazole (PTZ) kindling model is a preclinical paradigm utilized to screen the antiepileptic drugs as well as to investigate post repetitive seizures consequences on memory, cognition and behavioral disturbances associated with epilepsy [14].

The present study aimed to assess the effect of betahistine administration on memory in PTZ-kindling mice and the possible mechanistics involved.

MATERIALS AND METHODS

Drugs: Betahistine powder (Sigma-Aldrich, USA); Pentylenetetrazole: powder (Sigma-Aldrich, USA). Both betahistine and PTZ were dissolved in normal saline before each injection.

Animals

Male albino mice, weighing 25–30 g, purchased from the faculty of pharmacy, Zagazig University, Egypt and kept in colony cages with free access to food and tap water, under standardized housing conditions. All animal experiments comply with the ARRIVE guidelines and carried out in accordance with the U.K. animals. The experimental protocol has been reviewed and approved by Zagazig University - Institutional Animal Care and Use Committee (ZU-IACUC); Approval number: ZU-IACUC/3/F/ 392 /2022.

Kindling Model

To induce kindling, PTZ was injected in a dose of 40 mg/kg, intraperitoneal (i.p.) on alternate days. Mice were observed for 30 minutes after each PTZ injection. Seizures were evaluated using the scoring scale: 0, no response; 1, jerks; 2, Straub's tail; 3, clonus [15]. The maximum kindling score of 6 (sum of 1 + 2 + 3) is approached if the animal showed all the phases of convulsions (i.e. jerks, Straub's tail, and full-blown clonus). PTZ injections were given by an investigator while scoring was done by an investigator blind to the experimental grouping.

Experimental Design

Animals were randomly allocated into four groups of six animals per each. *Saline control group* (received i.p. injections of normal saline 1mL/kg/day), *PTZ group*, *Betahistine 5mg-PTZ group* and *Betahistine 10mg-PTZ group*. Except for saline control group, every animal was administered ten PTZ (40 mg/kg) i.p. injections on alternate days. Betahistine doses, were selected according to Yazdi et al. [3], were i.p. injected once daily and 30 minutes before PTZ doses. In the 20th day Y-maze test and the learning session of elevated plus maze tests were done. By the 21st day the memory test session of elevated plus maze was done then the animals were sacrificed. The whole brain was dissected and homogenized for estimation of histamine, acetylcholine (Ach), acetylcholinesterase (AchE), phosphorylated glycogen synthase kinase-3β on serine 9 (pGSK-3βS9) and p-Akt levels.

Y-maze test

The principle of assessment of memory by Y-maze is based on the innate curiosity of mice to explore the previously unvisited places. A mouse with intact working spatial memory will remember the arms previously visited and enter a less recently visited arm of the Y-maze. In the training session, one arm of the maze is closed off by a divider and the mouse is placed into distal end of one of the open arms facing the center of the maze. The mouse is left for 15 minutes to explore the maze then returned to the cage and the divider is removed. After 1 hour, the testing session starts by placing the same mouse into the distal end of the same arm used in the training session and left for 5 minutes. The number of entries and the time spent in the novel arm, from which the divider is removed, are recorded using canon 600D camera and stop watch by an investigator who is blind to the experimental groups. A mouse that shows no preference for any of the arms during the testing session has impaired spatial memory [16].

Elevated plus maze (EPM) test:

The EPM has two closed arms (with 12 cm height walls) perpendicular to another two arms without side walls (open arms). In learning session, the mouse was placed on the distal part of an open arm, and the time (in seconds) taken by the animal to transfer to any of the closed arms by his four limps, transfer latency (TL), was recorded. The cutting point is 90 sec, if the mouse still in the open arm, it was manually directed to one closed arm and left for two min to explore the maze. In the memory session, on the next day, the TL was recorded for each animal [17].

Tissue Preparation

The brain was homogenized in KCl buffer (Tris–HCl, 10 mM NaCl, 140 mM KCl, 300 mM EDTA, 1 mM Triton-X-100 0.5 %) at pH 8.0 supplemented with protease and phosphatase inhibitor. The homogenate was centrifuged at 10,000Xg for 30 minutes. The supernatant was used for estimation of histamine, Ach, AchE, pGSK-3βS9 and p-Akt levels [18].

Estimation histamine level

A colorimetric assay kit (Abcam, Cambridge, UK) was used to assess the histamine concentration in the brain. Samples and standards were added to the wells in 96-well plate (10 µL). The working reagent (histamine assay buffer, histamine probe, and histamine enzyme mix) were added (50 µL) and incubated for 30 min. The absorbance was recorded at 450 nm. Histamine concentration was expressed as nmol/mg.

Estimation of Ach level

A colorimetric assay kit (Abcam, Cambridge, UK) was utilized for assessment of ACh level according to manufacturer instructions. Samples and

standards were added to the wells in 96-well plate (10 µL). 50 µL of the working reagent (choline assay buffer, choline probe, and choline enzyme mix) was to start reaction and plate was incubated for 30 min. The absorbance was recorded at 570 nm. ACh concentration was expressed as nmol/mg.

Estimation of AchE level

Acetylcholinesterase ELISA Kit (RTEB0741) is utilized to estimate AchE activity according to manufacturer (ELISA Genie, Dublin, Ireland) instructions. The assay kits use a quantitative sandwich ELISA technique and each kit comes with highly specific antibodies pre-coated on a 96-well microtiter plate. The micro ELISA plate has been pre-coated with an antibody specific to AchE. Standards or samples are added to the appropriate micro ELISA plate wells and combined with the specific antibody. Then a biotinylated detection antibody specific for AchE and Avidin-Horseradish Peroxidase (HRP) conjugate is added to each micro plate well successively and incubated. The plate is washed to remove all non-specific binding. The substrate solution is added to each well. Only those wells that contain AchE, biotinylated detection antibody and Avidin-HRP conjugate will exhibit a change in color. The reaction is terminated by the addition of a sulphuric acid solution and the change in the optical density is measured at 450 nm. AchE level was expressed as ng/mg protein

Estimation of pGSK3β (S9) level

Glycogen Synthase Kinase-3 beta (GSK3B) (S9) Phosphorylation ELISA (EGSK3B-100, BioAssay Systems, USA)Kit was utilized for assessment of pGSK3β (S9) according to Vlastaridis [19]. The absorbance was read at 450 nm. The pGSK3β (S9) level was expressed as pg/mg protein**.**

Determination of phosphorylated Akt (p-Akt) level

The sandwich enzyme-linked immunosorbent assay p-Akt [pS473, DRG International Inc., USA] kit was utilized for determination p-Akt [20]. The absorbance was read at 450 nm. The p-Akt activity level is expressed as U/mg protein.

STATISTICAL ANALYSIS

The results were statistically analyzed using the SPSS 23.0 software package for Windows (SPSS Inc., Chicago, IL). Statistical analysis of the obtained data was performed with one-way analysis of variance (ANOVA) followed by the post hoc Tukey test for multiple comparisons. Dunnett T3 test was utilized for analysis of non-parametric data. Differences were considered statistically significant if $p < 0.05$.

RESULTS

Effects of betahistine on seizure score

In PTZ group, full kindling was approached and seizure score was significantly ($p<0.001$) increased compared to saline group which showed no seizure at all. In betahistine (5 and 10 mg)-PTZ interaction groups, seizure scores were significantly ($p<0.001$) decreased compared to PTZ group but significantly ($p<0.001$) increased compared to saline group. There was insignificant ($p=0.998$) decrease in seizure score in betahistine 10 mg-PTZ group compared to betahistine 5 mg-PTZ group (Fig. 1). Case fatalities were not recorded in all groups. .

Effects of betahistine on number of entries and time spent in the novel arm of Y-maze

PTZ significantly ($p<0.001$) decreased number of entries into the novel arm compared to saline group. In betahistine (5 and 10 mg)-PTZ interaction groups, the numbers of entries were significantly ($p<0.001$) increased compared to PTZ group and insignificantly different from saline ($p=0.957$) group as well as to each other ($p=0.744$) **(Fig. 2A)**. As regarding time spent in the novel arm of Y-maze, it was significantly ($p<0.001$) decreased in PTZ group compared to saline group. In betahistine (5 and 10 mg)-PTZ groups, this time was significantly ($p<0.001$) increased compared to PTZ group and insignificantly ($p=0.999$ and 0.272 respectively) changed compared to saline group and to each other ($p=0.216$) **(Fig. 2B)**.

Effects of betahistine on TL in elevated plus maze

In the learning session, TL was significantly ($p<0.001$) increased in PTZ group compared to saline group. In betahistine (5 and 10 mg)-PTZ interaction groups, TL was significantly ($p<0.001$) decreased compared to PTZ group but insignificantly ($P= 0.938$ and 0.907 respectively) increased compared to saline group and insignificantly ($p=1.000$) changed compared to each other **(Fig. 3A)**. In memory test session, PTZ significantly ($p<0.001$) increased TL compared to saline group. In betahistine 5 mg-PTZ interaction group, TL was significantly ($p<0.001$) decreased compared to PTZ group and significantly ($p<0.001$) increased compared to saline group. In betahistine 10 mg-PTZ group, TL was significantly ($p<0.05$) decreased compared to PTZ and betahistine 5 mg-PTZ groups and insignificantly ($P= 0.086$) increased compared to saline group **(Fig. 3B)**.

Effect of betahistine on Ach and histamine levels in the brain

Acetylcholine, histamine, and p-Akt levels in the brain were significantly ($p<0.001$) decreased in PTZ group compared to saline group. In betahistine

5 mg-PTZ interaction group, Ach, histamine, and p-Akt concentrations were significantly ($p<0.001$) increased in relation to PTZ group while compared to saline group, Ach and histamine level were insignificantly ($p=0.938$ and 0.896 respectively**)** decreased but p-Akt was insignificantly ($p=0.170$) increased. In betahistine 10 mg-PTZ interaction group, Ach, histamine, and p-Akt levels were significantly ($p<0.001$) increased in relation to saline, PTZ, and betahistine 5 mg-PTZ groups **(Fig. 4A, B and C)**.

Effect of betahistine on AchE and pGSK3β (S9) levels in the brain

In PTZ group, AchE level was significantly ($p<0.001$) increased compared to saline group. In betahistine (5 and 10 mg)-PTZ interaction groups, AchE level was significantly ($p<0.001$) decreased compared to saline and PTZ groups but was insignificantly ($p=0.490$) changed compared to each other **(Fig. 5A)**. Regarding to pGSK3β (S9), PTZ significantly ($p<0.001$) decreased its level compared to saline group. In betahistine (5 and 10 mg)-PTZ interaction groups, pGSK3β (S9), levels were significantly ($p<0.001$) increased compared to saline and PTZ groups but insignificantly ($p=0.843$) changed compared to each other **(Fig. 5B)**.

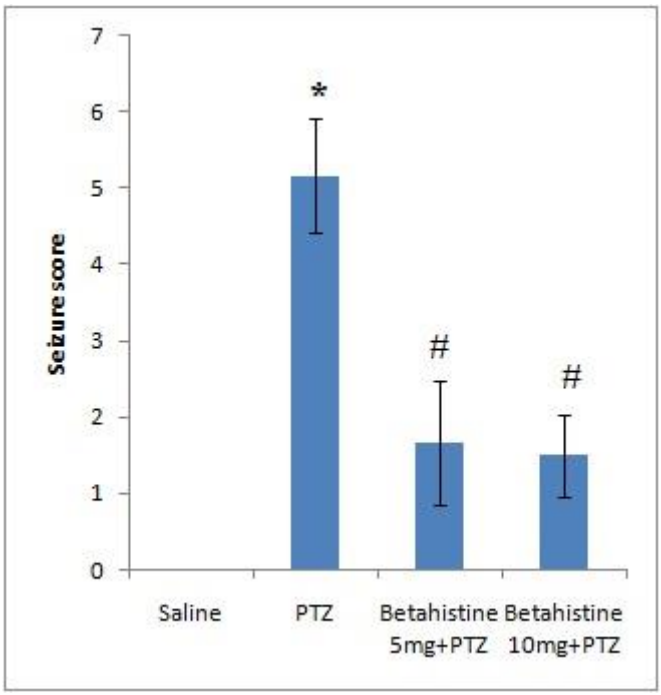

Fig. 1: Effects of betahistine on seizure score. The represented results are mean ± standard deviation (n=six mice per each group). One way ANOVA (F=74.928), * significant from saline group, # significant from saline and PTZ group.

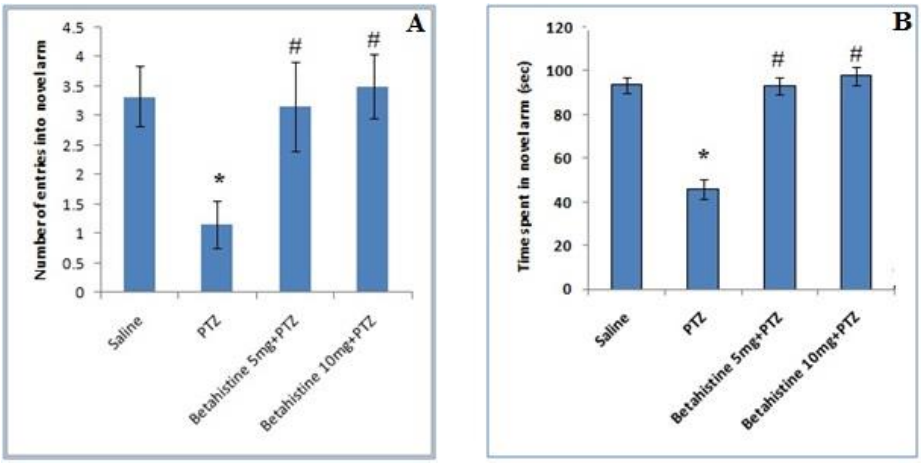

Fig. 2 (A& B): Effects of betahistine on number of entries (A) and time spent in the novel arm (B) of Y-maze. The represented results are mean ± standard deviation (n=six mice per each group). One way ANOVA (F= 22.009 and 225.380 respectively), * significant from saline control group, # significant from PTZ group. PTZ: Pentylenetetrazole.

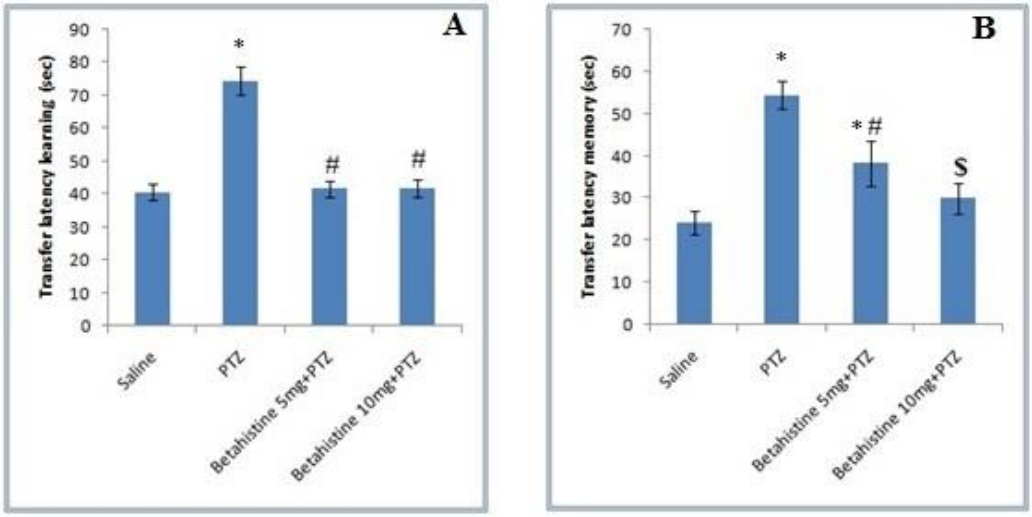

Fig. 3 (A& B): Effects of betahistine on transfer latency in learning (A) and memory (B) test sessions of elevated plus maze**.** The represented results are mean ± standard deviation (n=six mice per each group). One way ANOVA (F= 181.891 and 68.479 respectively), * significant from saline control group, # significant from PTZ group, $ significant from PTZ and betahistine 5mg-PTZ groups. PTZ: Pentylenetetrazole.

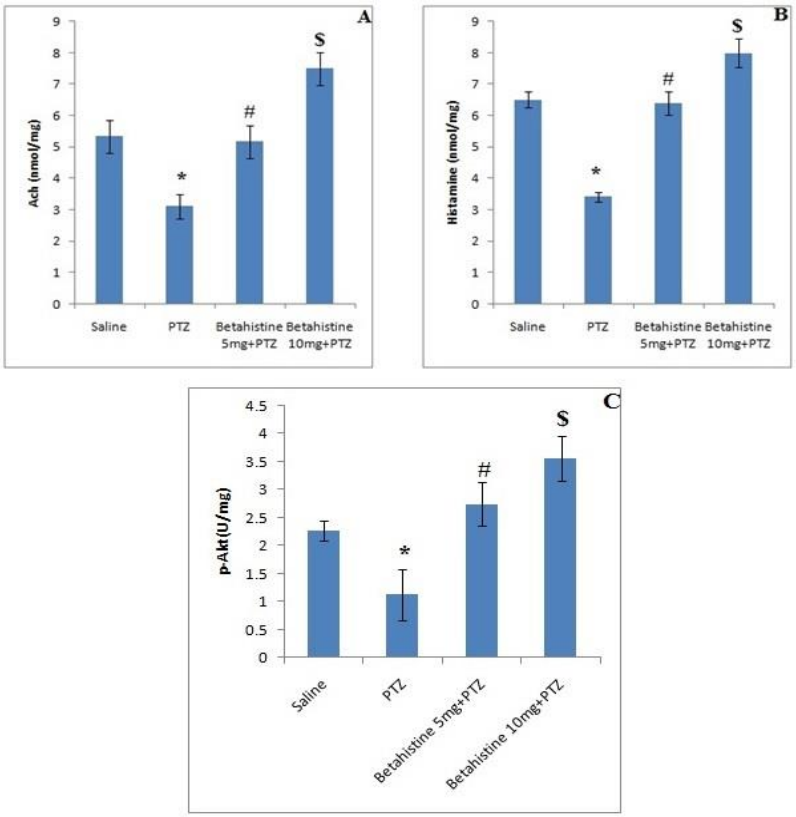

Fig. 4 (A, B and C): Effects of betahistine acetylcholine (A), histamine (B), and Akt (C) levels in the brain**.** The represented results are mean ± standard deviation (n=six mice per each group). One way ANOVA (F= 76.762, 203.871, and 43.061 respectively), * significant from saline control group, # significant from PTZ group, $ significant from saline, PTZ and betahistine 5mg-PTZ groups. PTZ: Pentylenetetrazole, Ach: Acetylcholine.

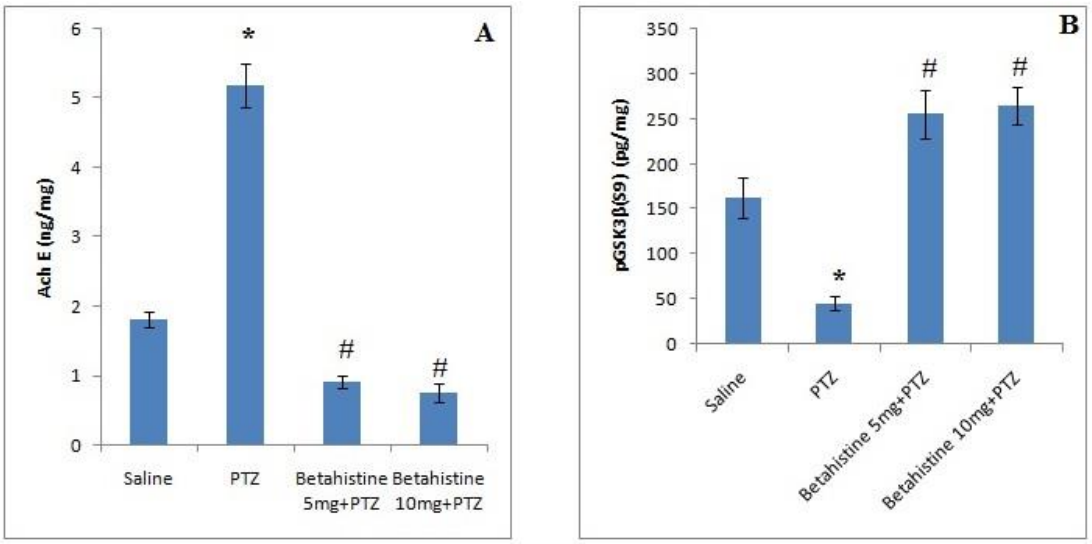

Figure 5 (A& B): Effects of betahistine AchE (A) and pGSK3β (S9) (B levels in the brain**.** The represented results are mean ± standard deviation (n=six mice per each group). One way ANOVA (F= 740.396 and 145.996 respectively), * significant from saline control group, # significant from saline and PTZ groups. PTZ: Pentylenetetrazole, AchE: Acetylcholinesterase. pGSK3β (S9): phosphorylated glycogen synthase kinase 3β at serine 9.

DISCUSSION

Kindled mice are considered a model of chronic epilepsy with permanent structural and functional changes in the forebrain structures [21]. Memory loss is attributed to synaptic dysfunction and neuronal degeneration [22]. Spatial information acquisition impairment is correlated with the degree of synaptic loss in cornu ammonis (CA1) of the hippocampus [23]. In the present results, betahistine administration conferred protection from clonic seizures and kindling induction after repeated PTZ administration. Similar results were obtained by Yazdi et al. [3] who found that betahistine administration prevented tonic-clonic seizures in PTZ-kindled mice.

In the present finding, PTZ kindled mice had memory impairment. In betahistine-PTZ interaction groups, betahistine prevented memory impairment. These findings were consistent with Jia et al. [24] who reported that thioperamide, the oldest H3 receptor antagonist, displayed memory enhancing effects in PTZ-kindled mice. Moreover, in a randomized double blind placebo controlled trial, Wang et al. [25] reported that betahistine effectively improved cognitive function in patients with schizophrenia.

In the present study, the mechanistics of the procognitive effect of betahistine were in part clarified after assessment of some neurotransmitters and enzymes related to memory and cognition. In this context, the present findings showed a decrement of the histamine level in the brain by PTZ administration while betahistine treatment increased its level. Similar results were obtained with Zhang et al. [26] who reported that brain histamine level was decreased in fully PTZ-kindled rats. In addition, Alachkar et al. [27] found that H3 antagonism by E177 increased histamine level in PTZ kindled rats. Increment in histamine level after betahistine administration could be explained by blockade of presynaptic H3 receptors. These receptors are autoreceptors which inhibit histamine release and their block by betahistine enhances histamine release [28].

The present results showed that PTZ increased AchE activity and decreased Ach level. However, betahistine treatment inhibited AchE and increased Ach level. AchE is the enzyme responsible for acetylcholine catabolism. In parallel with these findings, Mani [29] found that betahistine protected from doxorubicin-induced spatial impairment through increasing acetylcholine level in mice brain. Indeed, AchE inhibitor drugs improve memory in mild to moderate for Alzheimer Dementia (AD) [30]. Ach has an important role in cognitive functions such as learning and memory [31]. In addition, cholinergic receptor types including α7- and β2-containing subtypes are involved neuronal survival [32].

The present finding showed that PTZ decreased phosphorylation of glycogen synthase kinase GSK3β at its inhibitory serine 9 (S9) while betahistine treatment enhanced S9 phosphorylation resulting in GSK3β inhibition in the brain. In addition, p-Akt activity level was decreased by PTZ administration while increased by betahistine treatment. Indeed, GSK3-β is activated by

phosphorylation on tyrosine-216, while inhibited by phosphorylation on serine 9 residues. Protein kinase A and p-Akt phosphorylate GSK3β on serine 9 [33]. Based on histamine receptors signaling, PKA is increased by betahistine administration as the increased histamine level on the present results will activate H2 receptors which are linked to G-stimulatory (Gs) protein that activate adenylyl cyclase–cyclic AMP–PKA pathway [34].

Hyperactive GSK3-β causes neurodegeneration resulting in memory impairment as GSK3-β promotes tau hyperphosphorylation which in turn dissociates from the microtubules to impair axonal transport with induction of synaptic dysfunction [35]. Spatial information acquisition impairment is correlated with synaptic loss in the hippocampus [23]. In parallel with the present results, Hooper et al. [36] concluded that induction of long term potentiation (LTP) in the dentate gyrus and CA1 of the hippocampus is associated with phosphorylation of GSK3-β at the inhibitory Ser 9 site. In addition, transgenic mice with over-expressing GSK-3β develop impairment in LTP and deficits in hippocampus-dependent spatial memory formation [37]. Moreover, GSK3β inhibition is associated with preservation of β-catenin which influence the synaptic size and strength. Consequently, the increased GSK3-β activity in brain leads to inhibition of β-catenin signaling pathway with development of learning and memory deficits [38].

CONCLUSION

Betahistine treatment prevented PTZ-induced memory loss. These effects could be attributed in part to enhanced cholinergic activity through AchE inhibition and blockade of presynaptic H3 heteroreceptors as well as its neuroprotective effects mediated by GSK3-β inhibition. Thus betahistine could be an effective adjunct to prevent epilepsy-associated dementia.

Conflict of interest: no

Financial disclosure: no

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