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Neuroprotective effects of Huperzine-A in aluminum induced neurotoxicity in hippocampus of mice

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ABSTRACT

Exposure to aluminum has been correlated with the epidemiology of Alzheimer's disease. Huperzine A (HupA), derived from the Chinese herb Huperzia serrata, is an inhibitor of acetylcholinesterase (AChE). However, its use in aluminum induced neurotoxicity has not been reported. The objective of the study was to examine the effect of HupA on aluminum-induced changes in AChE activity and hippocampal neurotoxicity. Aged female swiss albino mice were divided into five groups (n=10). Aluminum treated group (Al-S) received 30 mg/Kg of aluminum chloride ($AlCl_3$) intraperitoneally (IP) for two weeks followed by IP normal saline. Three HupA treated groups received similar IP dosage of $AlCl_3$ for two weeks followed by HupA 0.1 mg/Kg, HupA 0.2 mg/Kg and HupA 0.5 mg/kg. Control group received IP normal saline. At the end of week 4, AChE was estimated, and pyramidal neurons of hippocampus were counted. Al-S group showed an increase in mean brain AChE and Al-HupA 0.1 reduced it significantly ($p<0.05$). Reduction in mean neuronal density in Al-S group, was comparatively more in CA3 hippocampal area ($p<0.001$). Quantitative study found that 0.2mg/Kg of synthetic Huperzine A given intraperitoneally, was able to increase neuronal count significantly compared to 0.1mg/Kg and 0.5mg/Kg of Huperzine A.

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INTRODUCTION

Aluminum is a potential neurotoxic metal. The link between oral and environmental exposure to aluminum and Alzheimer's disease (AD) was found using epidemiological studies (Kobayashi *et al.*, 1987; Flaten, 1990). A study suggested that a concentration of aluminum in drinking water above 0.1 mg/L might be a risk factor for dementia (Rondeau *et al.*, 2000; McLachlan *et al.*, 1996). Soluble mineral forms of aluminum can appear in drinking water. Aluminum can also appear in the drinking water following water-purification, as a by-product of water purification procedure. Aluminum content in the food can be traced to compounds added to the food as preservatives and coloring agents. Food additives such as cheese, baked goods and grain products can also contain aluminum (Soni *et al.*, 2001). A study comparing intraperitoneal administration of the aluminum with oral administration, found greater concentration of aluminum in the hippocampus, cerebral cortex, striatum, midbrain and cerebellum of rats receiving intraperitoneal administration (Sánchez-Iglesias *et al.*, 2007). Linkage of aluminum to AD was highlighted in the

study which found an elevated level of aluminum in necropsy samples of patients with histopathologic confirmation of AD as compared to samples of normal brains (Crapper *et al.*, 1973). A period of deficit in the connectivity of the hippocampal formation is usually reported to be followed by loss of episodic memory in AD (Lazarov and Hollands, 2016). Acetylcholine causes neuronal synapses to adapt and change in their communications in the hippocampus and cerebral cortex, thereby playing an important role in the learning process. Aluminum has been proven to produce cholinotoxic effects on the brain (Gulya *et al.*, 1990).

Huperzine A (HupA) is a lycopodium alkaloid found in Chinese clubmoss, Huperzia Serrata. In traditional Chinese medicine, this herb has been used for the treatment of swellings, contusions and schizophrenia (Zangara, 2003). HupA selectively inhibits acetylcholinesterase (AChE) and can cross the blood-brain-barrier (Wang and Tang, 1998). Study has shown that HupA can cause improvement in memory deficit in elderly people (Xu *et al.*, 1999). However, HupA has not been used as a neuroprotective agent in aluminum induced neurotoxicity. The objective of the study is to evaluate the effect of HupA on the AChE activity and histomorphometry of hippocampal neurons in aged albino mice pretreated with aluminum chloride.

MATERIALS AND METHODS

Animal Care and Ethics

The experimental part related to the animal use was carried out following the guidebook of Laboratory Animal Care Committee of the International Medical University. Ethical approval was taken from the University Joint Committee on Research and Ethics. Any potential suffering was minimized using precautionary measures. Female swiss albino mice, purchased from the Institute of Medical Research, Malaysia, were used in this study. The mice were provided with standard pellet food and water ad libitum. The mice were housed in two mice per cage with automatic temperature control and maintenance of 12 hour light and 12-hour dark cycle. All mice were observed for their diet and water intake and any significant change in weight daily. Well being of the mice under experiment, was continuously monitored by measuring daily body weight, food and water intake.

Aluminum Chloride

Aluminum chloride (Merck, Darmstadt, Germany) solution was prepared (w/v) in normal saline. Body weight of the mice was weighed daily, and concentration of the salt was adjusted from a stock solution to prepare 0.3 mL of the solution containing the dose of aluminum chloride (30 mg/kg bodyweight). Intraperitoneal (IP) injection of 0.3 mL of aluminum chloride was administered for 14 days using 0.5 mL insulin Terumo® needle and precautions were taken to prevent damage to the abdominal organs.

Huperzine A

Huperzine A (Sigma-Aldrich) solution was prepared (w/v) in normal saline. Body weight of the mice was weighed daily, and fresh HupA solutions were prepared with desired concentrations per kg of body weight (0.5mg/kg, 0.2mg/kg and 0.1mg/kg in 0.3mL of solutions). IP injections of 0.3 mL of HupA were administered to the different groups of mice from 15^{th} day to 28^{th} day of the experiment.

Animal Experiment

Female swiss albino mice, 25 weeks old (30–35 g), were divided into five groups (n = 10). Control (group C) group of mice was treated with IP injections of 0.3 mL saline for 28 days. Sham treated (group Al-S) group of mice was treated with IP injections of 0.3 mL of aluminum chloride (30mg/kg) for 14 days followed by 0.3 mL of normal saline for 14 days. Experimental groups (Al-HupA 0.5, Al-HupA 0.2, Al-HupA 0.1) were treated with IP injections of 0.3 mL of aluminum chloride (30mg/kg) for 14 days followed by 0.3 mL of Hup A, 0.5 mg/kg bodyweight, 0.2 mg/kg bodyweight and 0.1 mg/kg bodyweight respectively.

Estimation of AChE in Serum

At the end of 28 days, the mice were anesthetized with intraperitoneal administration of ketamine hydrochloride (80 mg/kg) and xylazine hydrochloride (10 mg/kg). Blood samples were collected by cardiac puncture. Using amplex red AChE assay kit (Invitrogen), AChE concentration of the serum was estimated (Krishnan *et al.*, 2012) with the help of fluorescence microplate reader. Amplex red reagent is a selective probe for hydrogen peroxide and fluorogenic substrate for peroxidase. The serum samples were treated with amplex red reagent. The stock solutions were prepared following the guidebook of the kit. Serum sample of the mice in different dilutions were prepared in triplicate. Wells of the Nunc plate were filled up with 100 μL of the diluted samples and controls. Using 100 μM acetylcholine (ACh), 0.2 U/ml choline oxidase and, 2 U/ml horse radish peroxidase (HRP), a working stock solution of amplex red reagent was prepared. The reaction was initiated by adding 100 μL of the working solution to each well containing the samples and controls. Incubation was done in dark for

30 minutes. Fluorescence emitted by the individual samples was measured with the microplate reader. The excitation was measured at 560 nm and emission was measured at 590 nm. Subtracting the values derived from the negative control, background fluorescence was corrected. Readings of mean fluorescence (log10) was plotted against AChE concentration of the positive controls (log10) and linear regression was done in Microsoft excel. With ascending concentration of the sample, AChE activity in a sample increased exponentially. Equation of linear regression line (positive control) was substituted with log10 of the mean value of fluorescence readings. Then log10 of AChE concentration of the experimental samples was calculated. Serum AChE concentration of individual animals of group C, group Al-S, group Al-HupA 0.5, group Al-HupA 0.2 and group Al-HupA 0.1 was calculated and compared groupwise.

Estimation of AChE in Brain Homogenate

The part of the brain between optic chiasma in front and infundibulum behind, containing the hippocampus was removed from five animals in each group. Using PBS solution at -80°C temperature, brain samples were stored. Homogenized brain samples in PBS solution were used for AChE estimation. Estimate of AChE concentration in the brain homogenate samples (U/mL), was performed using the same reagent, equipment and guideline used in the kit used to estimate serum AChE. However instead of reaction buffer, PBS solution was used for dilutions of positive controls and brain homogenate samples. PBS solution was also used as negative control.

Qualitative and Quantitative Histological Studies

Buffered formal saline (10%) was used for fixation of the brain samples in five animals from each group. Hippocampal area was identified and carefully dissected in the mouse brain between optic chiasma and infundibulum. Histological staining of the serial coronal sections (8 micron) of the hippocampal area, processed with paraffin, were done using Nissl stain (0.2% thionin). Qualitative observations of pyramidal layer of CA1 and CA3 areas of hippocampus were done. Every 10th section (5 sections in each animal) showing the hippocampus was selected from each animal. The sections were photographed under 400X objective using Nikon compound microscope (attached with a camera). Two areas of CA1, and two areas of CA3 were randomly selected in each section. A measured rectangular area was selected over the photomicrograph and with the help of a software (Image-pro express), normal neurons were counted. Mean diameter of the neuronal nucleus was measured for each region. The neuronal density (P) per unit area of section was measured using Abercrombie formula P = A. M / L+M; A= Neuronal count, L = Mean nuclear diameter of the area, M= Section thickness in micron (8 microns) (Krishnan *et al.*, 2012). The neuronal density per unit area (cm2) was then calculated.

Statistical Analysis

Estimation of serum and brain AChE were conducted in triplicates and were repeated at least three times with similar results. The mean serum and brain AChE levels of the control, Al-S, Al-HupA0.1, Al-HupA 0.2 and Al-HupA 0.5 groups of mice were subjected to Mann–Whitney U test followed by Kruskal-Wallis test to estimate inter-group variation. Using SPSS 20, mean neuronal count per square cm for each group was compared and was subjected to statistical analysis. The counts of each area (CA1 and CA3) was subjected to One-way ANOVA analysis to find out any statistically significant differences in mean data between the treatment groups in that area. For the analysis, all values were presented as mean ± SD. Significance was set at $P < 0.05$ for all tests.

RESULTS

Changes in Serum AChE

Sham-treated group of mice treated with aluminum followed by normal saline showed a mean serum AChE of 4.79U/mL, with 12.9% reduction, compared to the control group.

HupA post-treatment caused further reduction in the serum AChE. Serum AChE in Hup A treated groups ranged between 2.53 to 3.72 U/mL (Figure 1).

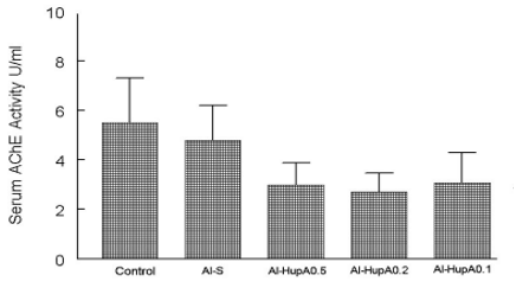

Figure 1: Serum acetylcholinesterase (AChE) activity reduced following HupA treatment aluminum treated mice. Mean ± SEM (n=5). No significant difference among groups

Changes in Brain AChE

Sham treated group of mice treated with aluminum followed by normal saline showed an increase in mean brain AChE by 21.2% compared to the control group. The aluminum-huperzine A treatment groups showed a reduction in the mean brain AChE compared to the aluminum-saline sham treated group. Most significant reduction in the brain AChE was produced by post-treatment with HupA 0.1mg/Kg of body weight (Kruskal-Wallis test, $p<0.05$). The AChE activity was reduced by 49.9% compared to the Al-sal treated group (Figure 2).

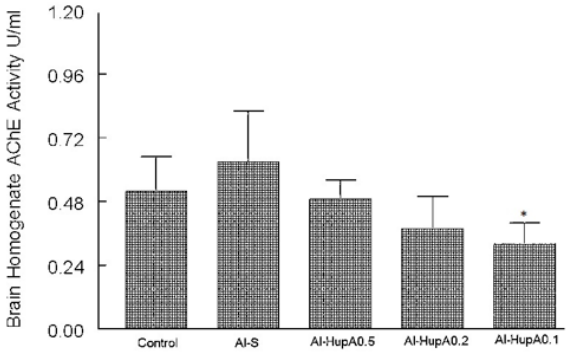

Figure 2: Brain acetylcholinesterase (AChE) activity reduced following HupA in aluminum treated mice. Mean ± SEM (n=5). *p < 0.05, compared with Al-S group

Qualitative Histological Changes in the Hippocampus

Compared to the control group, group receiving aluminum and post-treatment with normal saline showed pyknosis of pyramidal neurons in CA1 area of the hippocampus. HupA post-treatment (0.2 mg/Kg and 0.5 mg/Kg) showed normal-looking pyramidal neurons. Few pyknosed neurons were observed in HupA 0.1 mg/Kg treated groups (Figure 3). CA3 area of the hippocampus showed pyknosed neurons in aluminum-saline treated group like the CA1 area. HupA post-treatment (0.1 mg/Kg, 0.2 mg/Kg and 0.5 mg/Kg) showed in general, pyramidal neurons with clear nucleus and nucleolus in CA3 area of the hippocampus. HupA 0.1 mg/Kg and HupA 0.2 mg/Kg treated groups showed only a few neurons with pyknosed nuclei (Figure 4).

Quantitative Changes in the Histomorphometry of the Hippocampus

Absolute neuronal count per sq.cm of the section of stratum pyramidalis of CA1 and CA3 hippocampal area was compared between control group, aluminum treated group (Al-S) and Huperzine A treated group (Al-HupA). Statistically significant difference between the mean neuronal counts per sq. cm of the section in stratum pyramidalis of the hippocampus between control group, aluminum-saline treated group (Al-S) and aluminum-huperzine treated groups (Al-HupA 0.1 mg/kg, Al-HupA 0.2mg/Kg, Al-HupA 0.5 mg/Kg) was observed as determined by one-way ANOVA [CA1 area, $F(4, 103) = 17.18$, $p = 0.000$; CA3 area, $F(4, 196) = 25.03$, $p = 0.000$]. Mean neuronal counts in stratum pyramidalis of CA1 hippocampal area was reduced by 28.5% in Al-S group compared to control group. Post-hoc Bonferroni test showed that mean neuronal count in CA1 hippocampal area was significantly lower in Al-S group compared to control group ($p<0.001$). Huperzine post-treatment following aluminum exposure increased mean neuronal counts significantly in CA1 hippocampal area by 45 to 47% compared to Al-S group(Al-HupA 0.1 by 47%, Al-HupA 0.2 by 45.7% and Al-HupA 0.5 by 47.2%) ($p<0.001$ in all three groups, post-hoc Bonferroni) (Figure 5). Mean neuronal count in stratum pyramidalis of CA3 hippocampal area was reduced by 39.5% in Al-S group compared to control group ($p<0.001$). Huperzine post-treatment increased mean neuronal count in CA3 hippocampal area by 68.6% and 58.5% in Al-HupA 0.1 mg/Kg and Al-HupA 0.5 mg/Kg post-treatment groups compared to Al-S group ($p<0.001$ in both groups, post-hoc Bonferroni). Huperzine A 0.2 mg/Kg post-treatment group (Al-HupA 0.2) increased mean neuronal count in CA3 hippocampal area by 77.4% compared to Al-S group ($p<0.001$) (Figure 6). No significant difference was observed in the mean neuronal counts between the Huperzine-post treatment groups (p 1.000). No significant difference was observed in the mean neuronal counts between the control group and Huperzine-post treated groups (p 1.000).

DISCUSSION

Changes in the serum and brain AChE

Aluminum/saline treated group demonstrated a 12.9% suppression of mean AChE activity when compared to the control saline/saline treated group. This group received 30 mg/kg aluminum chloride intraperitoneally daily for 14 days. Subsequently aluminum was withdrawn in this group and the mice received normal saline. This result serves to strengthen prior claims about the toxicity of aluminum, since the suppressive effect was persistent even after 14 days of aluminum withdrawal. Post-treatment with three different doses of Huperzine reduced the serum AChE activity. How-

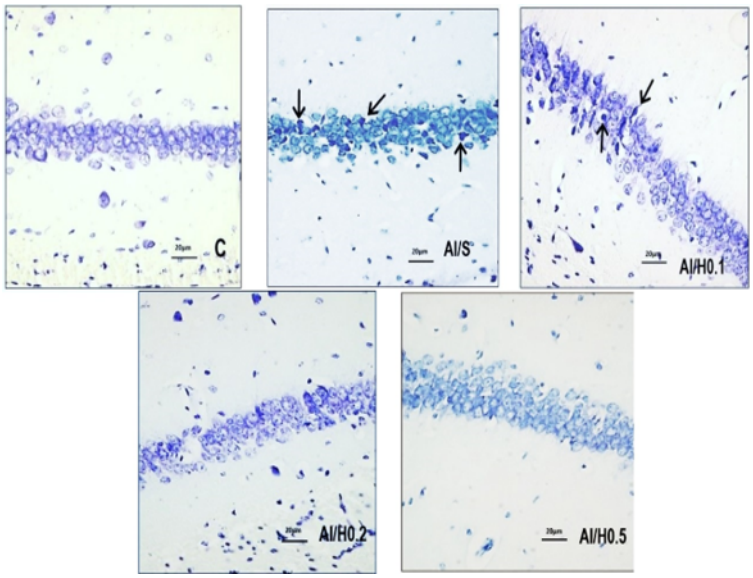

Figure 3: Photomicrograph of CA1 hippocampus. Pyknosis (arrows) in Al/S group. HupA 0.2mg and 0.5mg/Kg treatment: no evidence of pyknosis (Thionin stain, $\times$400, 8 μ)

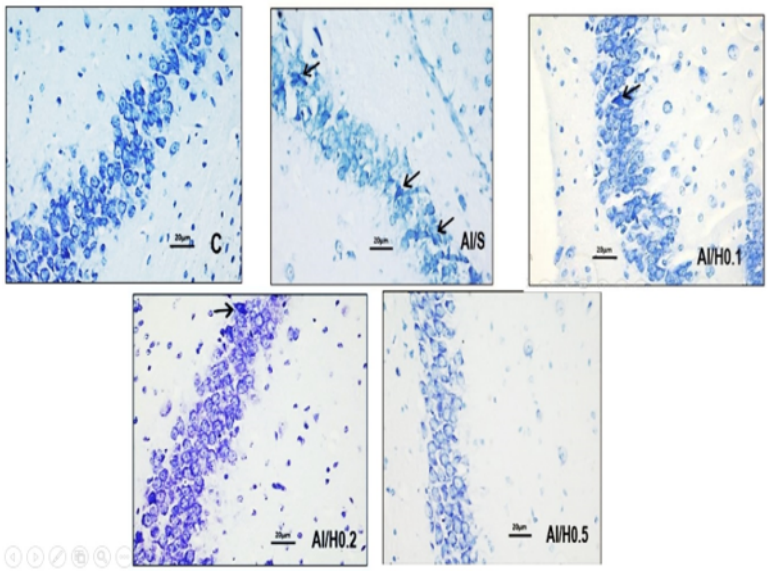

Figure 4: Photomicrograph of CA3 hippocampus. Pyknosis (arrows) in Al/s group. HupA 0.2mg/kg and 0.5mg/Kg treatment: increase in layers of neurons (Thionin stain, $\times$400, 8 μ)

ever, the reduction was not found statistically significant. Brain AChE activity was increased in the Al-S group after 14 days of withdrawal of aluminum treatment. Reduction in the levels of serum AChE activity might be caused by the attachment of aluminum to the SH-groups of the AChE enzyme at the active sites, preventing its function in certain chemical reactions (Zaman *et al.*, 1993). It has been reported that following acute and subacute exposure, aluminum accumulates in all regions of animal brain with maximum accumulation in the hippocampus (Yellamma *et al.*, 2010). A study using zebrafish model, found that in vitro exposure to aluminum chloride varying from 50 to 250 μM increased the brain AChE activity and reduced the locomotor activity. It was suggested that the direct effect of the metal on the enzyme, without any other changes in the biological systems might have caused the increase in brain AChE activity (Senger *et al.*, 2011). In a study using intra-peritoneal administration of aluminum gluconate and aluminum chloride, biphasic activity was noted on brain AChE. At low aluminum concentrations, the AChE activity was slightly increased, whereas at higher concentrations it was inhibited (Gulya *et al.*, 1990).

The comparatively shorter duration of aluminum chloride administered for 14 days followed by its withdrawal for next 14 days, reduced aluminum

concentration which increased the brain AChE activity. This study showed that post-aluminum administration of synthetic Huperzine 0.1 mg/Kg body weight (Al-HupA 0.1) for 14 days reduced serum and brain AChE activity compared to the control group.

A comparable study found that the inhibition of AChE activity was inversely related to levels of acetylcholine measured in the hippocampus (Tang *et al.*, 1994). In AD, elevated level of serum AChE and reduced level of brain AChE deregulate balance of cholinergic system by causing increased level of butyrylcholinesterase (BuChE).

The imbalance and change in the AChE/BuChE ratio cause cholinergic deficit in the brain (Mushtaq *et al.*, 2014). Increased acetylcholine activity brought by huperzine can potentially reverse the cholinergic deficit caused by AD.

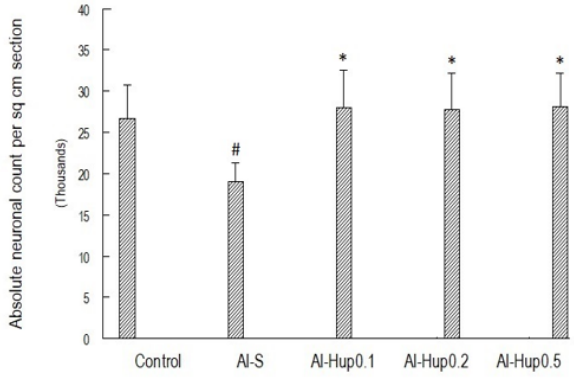

Figure 5: Mean neuronal count in CA1 hippocampus. Increased count in HupA groups. Mean ± SEM (n=5). #p< 0.001, compared to control group. *p < 0.001, compared to Al/S group

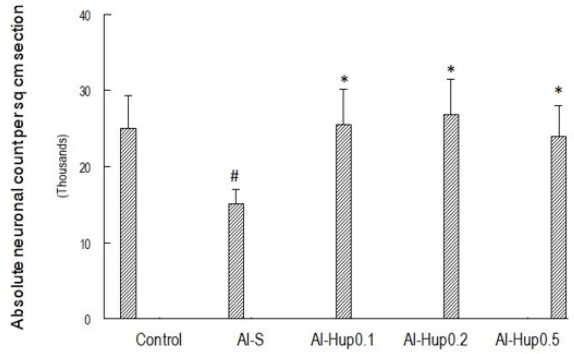

Figure 6: Mean neuronal countin CA3 hippocampus. Increased count in HupA groups. Mean ± SEM (n=5). #p< 0.001, compared to control group. *p < 0.001, compared to Al/S group

Qualitative and Quantitative Changes in the Hippocampal Neurons

Qualitative changes in the hippocampal pyramidal neurons and quantitative changes in the neuronal density were used as indicators of the effectiveness of Huperzine A in treating aluminum toxicity. Two weeks' after intraperitoneal administration of aluminum for 14 days at a dose of 30mg/Kg bodyweight, the neuronal count in CA1 and CA3 areas were significantly reduced by 28.5% and 39.5% respectively compared to control group ($p<0.001$). Several hypotheses have been given to explain how aluminum promotes biological oxidations (Exley, 2004; Kong *et al.*, 1992). Iron-induced and non-iron-induced lipid peroxidation, oxidation of NADH and non-iron-mediated formation of hydroxyl radical have been facilitated by aluminum (Gutteridge *et al.*, 1985; Verstraeten and Oteiza, 2000). Long-term administration of aluminum reduced neuronal count in both CA1 and CA3 areas of hippocampus with significant reduction in CA3 area (Sethi *et al.*, 2008; Miu *et al.*, 2003). Aluminum has greater attraction to bind with negative charge of oxygen donor ligands. It helps aluminum to make strong bonding with phosphate groups of DNA and RNA, affecting DNA topology. Aggregation of cytoskeleton proteins of neurofilament is facilitated by strong binding of aluminum with phosphorylated amino acids (Maya *et al.*, 2016; Méndez-Álvarez *et al.*, 2002). Post-treatment with three different doses of Huperzine A increased mean neuronal count significantly compared to aluminum-saline (Al-S) treated group. This study found significant and comparatively greater reduction in mean neuronal density in CA3 hippocampal area following aluminum treatment. Both qualitative and quantitative studies found that 0.2mg/Kg dosage of synthetic Huperzine A given intraperitoneally, was able to increase mean neuronal count significantly compared to 0.1mg/Kg and 0.5mg/Kg of Huperzine A. Increase in the level of acetylcholine in the hippocampus was observed after intraperitoneal injection of 0.5 μmol/Kg of natural extract of Huperzine A. Huperzine A was found to have similar potency on increasing acetylcholine in hippocampus as compared to the Donezepil and Rivastigmine (Liang and Tang, 2006). A systematic review which included 20 randomized clinical trials, observed that Huperzine A has beneficial effects on improvement in cognitive functions in patients with Alzheimer's disease (Yang *et al.*, 2013). A study compared effect of natural and synthetic Huperzine A and found that synthetic racemic mixture produced reduced biological activity compared to the natural product (Hanin *et al.*, 1993).

In the present study, aluminum exposed mice

showed reduction in serum AChE, increase in brain AChE and reduction in mean neuronal count in hippocampus, two weeks after withdrawal of aluminum. Synthetic huperzine A in the dosage of 0.1mg/Kg reduced brain AChE significantly, whereas maximal increase in neuronal count in CA3 area of hippocampus was observed with dosage of 0.2 mg/Kg of huperzine A. The limitation of the study was the lack of comparison of the effects with the natural forms of huperzine A. Lack of study of behavioural changes was also a limitation towards the extrapolation of aluminum-induced neuronal toxicity in hippocampus to the possible cognitive deficit of Alzheimer's disease. Our present data suggests that Huperzine A can be used as a therapeutic agent in aluminum induced toxicity involving hippocampus.

CONCLUSION

Hippocampal neurodegeneration persists even after withdrawal of aluminum treatment. Both CA1 and CA3 pyramidal neurons in hippocampus are affected in aluminum toxicity and Huperzine A can be used effectively in aluminum-induced neurodegeneration.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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