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ORIGINAL ARTICLE

Effect of piracetam, vincamine, vinpocetine, and donepezil on oxidative stress and neurodegeneration induced by aluminum chloride in rats

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Abstract In this study, we investigated the effects of the memory-enhancing drugs piracetam, vincamine, and vinpocetine or the cholinesterase inhibitor donepezil on the development of oxidative stress, inflammation, and brain damage induced in rat brain by aluminum chloride ($AlCl_3$). Saline (control), piracetam (100 or 300 mg/kg), vincamine (10 or 20 mg/kg), vinpocetine (10 or 20 mg/kg), piracetam 100 mg/kg plus vincamine 10 mg/kg, piracetam 100 mg/kg plus vinpocetine 10 mg/kg, or donepezil 5 mg/kg were administered once daily intraperitoneally for 45 days along with $AlCl_3$ (10 mg/kg, intraperitoneally). Malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide, acetylcholinesterase (AChE), butrylcholinesterase (BChE), paraoxonase (PON1) activities, and prostaglandin E_2 (PGE_2) concentrations were measured in brain. Histopathology and caspase-3 immunohistochemistry (an apoptotic marker) were also performed. Results indicated that (1) compared to controls, injection of $AlCl_3$ significantly increased brain lipid peroxidation (MDA) and nitric oxide concentrations together with decreased GSH concentrations. PON1 activity in brain was significantly decreased, while AChE and BChE activities were significantly increased compared to control animals. Cortical atrophy, neuronal shrinkage, red neurons, surrounded by vacuolations with cytoplasmic neurofibrillary tangles, intense caspase-3 expression in degenerated neurons, and amyloid deposition were observed; (2) in $AlCl_3$-treated rats, (i) lipid peroxidation was significantly decreased by the lower doses of piracetam, vincamine, and vinpocetine as well as by piracetam plus either vincamine or vinpocetine; (ii) nitric oxide was significantly decreased by the lower doses of piracetam, and vinpocetine, by both doses of vincamine, and by piracetam plus either vincamine or vinpocetine; (iii) nitric oxide also showed significant decrease after treatment with donepezil; (iv) both GSH and PON1 activity showed significant increase following the administration of the test drugs; (v) PGE_2 significantly increased by the higher dose of piracetam, vincamine, vinpocetine, and piracetam plus either vincamine or vinpocetine; (vi) AChE and BChE activities decreased after treatment with the lower dose of piracetam, vinpocetine, and piracetam plus either vincamine or vinpocetine; (vii) AChE activity decreased following 20 mg/kg vincamine, and BChE activity decreased following 10 mg/kg vincamine; (viii) AChE but not BChE activity decreased after donepezil; (ix) on histopathology, the low dose of singly used drugs and donepezil had the best improvement in neuronal look, cortical thickness, and degree of vascular congestion. Rats treated with 10 mg/kg vinpocetine showed decreased capsase-3 immunoreactivity in brain and regenerating neurons. These results suggest that while the low therapeutic doses of the nootropic drugs piracetam, vincamine, and vinpocetine display antioxidant and neuroptotective effects, their high doses are likely to have prooxidant and proinflammatory properties.

Keywords Aluminum chloride · Donepezil · Oxidative stress · Piracetam · Vincamine · Vinpocetine

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Introduction

The pyrrolidine derivative piracetam, the *Vinca minor* alkaloid vincamine, and its synthetic derivative vinpocetine have widely been used for decades to treat memory dysfunction and cognitive impairment that occurs during normal aging, cerebrovascular disorders, or Alzheimer's disease (Winblad 2005; Heckman et al. 2015). Piracetam (2-oxo-1-pyrrolidine-acetamide) was the first of the so-called "nootropic" drugs, a term that was introduced by Giurgea (1973) to denote this class of medications which protect memory processes against disruption by chemical or physical agents. In rodent experiments, the drug prevented memory changes caused by scopolamine (Chopin and Briley 1992) or morphine (Aksu et al. 1998). Clinically, it has been used in the treatment of aphasia following stroke (Kessler et al. 2000) and in cognitive impairment in the elderly (Waegemans et al. 2002). In stroke patients, the drug led to better recovery of cognitive impairment evaluated by event-related potentials compared to treatment with acetylsalicylic acid (Stahlhut et al. 2014). Piracetam also improved cognitive performance of patients undergoing coronary bypass surgery (Fang et al. 2014). The drug improved mitochondrial function and ATP production in models of amyloid beta (Aβ)-induced impairment of mitochondrial function (Kurz et al. 2010) or in PC12 cells subjected to oxidative stress (Keil et al. 2006). Piracetam resulted in increased cerebral blood flow in stroke (Kessler et al. 2000) and increased membrane fluidity of hippocampal membranes from Alzheimer's disease patients and elderly controls (Eckert et al. 1999).

Vincamine, an extract of periwinkle, and its synthetic derivative vinpocetine (vinpocetine-ethyl apovincaminate) are both used in the treatment of cognitive decline due to cerebrovascular insufficiency (Fischhof et al. 1996; Hadjiev 2003). These agents increase cerebral blood flow and regional glucose uptake (Szilágyi et al. 2005; Jovanović et al. 2013). Vinpocetine is an inhibitor of cyclic GMP phosphodiesterase (Ahn et al. 1989), a potent ligand of peripheral benzodiazepine binding sites (Gulyás et al. 2005) and a blocker of NaV1.8 sodium channel activity (Zhou et al. 2003). Both vinpocetine and its main metabolite cis-apovincaminic acid (cAVA) exert neuroprotective effects (Nyakas et al. 2009; Tárnok et al. 2008). Vinpocetine in addition exerts an anti-inflammatory action inhibiting tumor necrosis factor-alpha (TNF-α)-induced nuclear factor-kappa B (NF-κB) activation, monocyte adhesion and chemotaxis, and the induction of proinflammatory mediators (Jeon et al. 2010). The drug reduces atherosclerotic lesion formation, oxidized low-density lipoprotein (LDL) uptake, and foam cell formation (Cai et al. 2013). These actions are likely to benefit patients with cerebrovascular insufficiency by preventing the progression of atherosclerosis.

There is an increasingly recognizable role for oxidative stress and neuroinflammation in the etiology of various brain disorders (Roth et al. 2005; Danielson and Andersen 2008; Sajdel-Sulkowska et al. 2008; Frank-Cannon et al. 2009; Abdel-Salam et al. 2015). Oxidative stress describes a state of ongoing oxidative damage to the cell that is caused by overwhelming reactive oxygen species or deficient protective mechanisms. Reactive oxygen metabolites are produced by the cell metabolic processes. Sources include the mitochondrial respiratory chain, the neutrophil enzymes NADPH oxidase and myeloperoxidase, and the enzyme xanthine oxidase. Within the cell, the ability of reactive oxygen metabolites to inflict damage to cell membranes, proteins, and DNA is counterbalanced by a number of molecules such as ascorbic acid, α-tocopherol, carotenes, the tripetide glutathione, and anti-oxidant enzymes, e.g., superoxide dismutases, catalases, and glutathione peroxidases (Sies 1991; Halliwell and Gutteridge 1989; McCord 2000). Under basal conditions, this balance is not so tight and a certain degree of oxidative stress is present. High levels of oxidative stress are associated with tissue damage and disease development (Halliwell 2001). Oxidative stress has been implicated in the pathogenesis of several neurodegenerative disorders, stroke, and aging and in the accompanying cognitive decline (Perry et al. 2007; Warner et al. 2004). Several factors are likely to account for the susceptibility of the brain tissue to oxidative stress. These include a high rate of oxygen utilization, rich content of polyunsaturated fatty acids, the presence of the redox-active transition metal ions Cu^{++} and Fe^{++}, and decreased anti-oxidant enzymes (Halliwell 1992).

Aluminum, the most abundant metal in the Earth's crust, has been implicated in accelerating brain aging and in the development of Alzheimer's disease (Drago et al. 2007; Yumoto et al. 2009; Bondy 2014). The metal is widely used in building, construction, containers, packaging, electrical conductors, and many equipments. Aluminum can cross the blood–brain barrier and accumulate in the cortex, cingulate bundles, corpus callosum, hippocampus, and cerebellum (Xu et al. 1992; Struys-Ponsar et al. 1997; Yuan et al. 2012). It is found in glia, astrocytes, and neuronal cells (Golub et al. 1999; Lévesque et al. 2000). The neurotoxicity of aluminum involves enhancing lipid peroxidation (Esparza et al. 2003; Pallavi et al. 2008), altering axonal transport, and phosphorylation levels of neurofilaments (Exley 2004). Aluminum causes progressive memory deterioration, oxidative stress, hyperphosphorylated tau, and aggregation of β-amyloid, the major component of senile plaques (Walton 2007, Walton and Wang 2009). The latter are the main neuropathological features of Alzheimer's disease (Castellani et al. 2006). The aluminum-based rodent model for Alzheimer's disease can thus be used to study the pathogenetic mechanisms and the possible therapeutic interventions for Alzheimer's disease (Kawahara 2005).

In the present study, the memory-enhancing drugs—piracetam, vincamine, and vinpocetine—used either singly or in combination were evaluated for their ability to reduce brain

oxidative stress, inflammation, and neuronal damage evoked by systemic injection of aluminum chloride into rats. In addition, the effects of these drugs were compared to those of the acetylcholiesterase inhibitor donepezil, a commonly used drug for Alzheimer's disease (Wilkinson et al. 2004).

Materials and methods

Animals

Adult male Sprague–Dawley rats, weighing 120–130 g (age 10–11 weeks), were used. Animals were obtained from the breeding colony maintained at the animal house of National Research Center (NRC, Cairo, Egypt). All animals were housed under conventional laboratory conditions throughout the period of experimentation at room temperature of 25± 2 °C, 60–70 % humidity, and 12-h light/dark cycle, fed standard laboratory pellets (20 % proteins, 5 % fats, 1 % multivitamins), and allowed free access to tap water. Animals were allowed at least 1 week of acclimatization before analyses. All animals received human care and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985). Equal groups of seven rats each were used in all experiments.

Drugs and chemicals

Donepezil hydrochloride (Pfizer Egypt, Cairo, A.R.E.), piracetam (Pharco Pharmaceuticals, Alexandria, A.R.E.), vinpocetine (Amriya Pharmaceutical industries, Alexandria, A.R.E.), and vincamine (GlaxoSmithKline Pharmaceutical Co., Salam city, Cairo, A.R.E.) were used. Aluminum chloride ($AlCl_3$) was obtained from Sigma (USA.) All drugs were dissolved in saline and freshly prepared immediately before use. Anti-caspase-3 antibody kit was obtained from Abcam plc. (Cambridge Science Park, Cambridge CB4 0FL, UK).

Experimental design

Rats were randomly assigned into 11 groups, with 7 rats each. Group 1 received the vehicle (saline). Group 2 received $AlCl_3$ at the dose of 10 mg/kg. Groups 3 and 4 received $AlCl_3$ in combination with piracetam (100 or 300 mg/kg). Groups 5 and 6 received $AlCl_3$ in combination with vincamine (10 or 20 mg/kg). Groups 7 and 8 received $AlCl_3$ in combination with vinpocetine (10 or 20 mg/kg). Group 9 received $AlCl_3$ in combination with piracetam (100 mg/kg) plus vincamine (10 mg/kg). Group 10 received $AlCl_3$ in combination with piracetam (100 mg/kg) plus vinpocetine (10 mg/kg). Group 11 received $AlCl_3$ in combination with donepezil (5 mg/kg). Drugs were administered intraperitoneally daily for 45 days. Rats were euthanized 24 h following the last treatment by cervical dislocation under light ether anaesthesia, and the brain of each rat was rapidly excised, washed with ice-cold saline solution (0.9 % NaCl), immediately frozen on dry ice-cold glass plate, and stored at −80 °C for further determination of biochemical parameters. For biochemical studies, the brain was homogenized in ice-cold phosphate buffer to obtain 10 % homogenate. For histopathology and immunopathology studies, the brain of two to three rats, randomly selected from each group, was removed, rapidly rinsed in ice-cold saline solution, and placed in 10 % buffered formalin.

Biochemical studies

Determination of lipid peroxidation

Lipid peroxidation was assayed by measuring the level of malondialdehyde (MDA) in the brain homogenates. Malondialdehyde was determined by measuring thiobarbituric reactive species using the method of Ruiz-Larrea et al. (1994). In this assay, the thiobarbituric-acid-reactive substances react with thiobarbituric acid to produce a pink-colored complex having peak absorbance at 532 nm.

Determination of reduced glutathione

Reduced glutathione (GSH) was determined in brain tissue by Ellman's method (1959). The procedure is based on the reduction of Ellman's reagent by –SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid; the nitromercaptobenzoic acid anion has an intense yellow color which can be determined spectrophotometrically. A mixture was directly prepared in a cuvette: 2.25 ml of 0.1 M K-phosphate buffer, pH 8.0; 0.2 ml of the sample; and 25 μl of Ellman's reagent (10 mM 5,5′-dithio-bis-2-nitrobenzoic acid in methanol). After 1 min, the assay absorbance was measured at 412 nm and the GSH concentration was calculated by comparison with a standard curve.

Determination of butyrylcholinesterase activity

Butyrylcholinesterase activity in brain supernatant was determined by colorimetric method using butrylcholinesterase diagnostic kit (CHRONOLAB, Barcelona, Spain). The principle of the method is that cholinesterase hydrolyzes butrylcholinesterase to butyrate and thiocholine. The latter reacts with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) forming 5-mercapto-2-nitrobenzoic acid (5-MNBA). The rate of 5-MNBA formation, measured by spectrophotometer, is proportional to the enzymatic activity of cholinesterase in the sample.

Determination of acetylcholinesterase activity

The determination of cholinesterase activity in the brain was a modification of the method of Ellman et al. (1961) as described by Gorun et al. (1978). The principle of the method is the measurement of thiocholine produced when acetylcholine is hydrolyzed. The color was read immediately at 412 nm.

Determination of nitric oxide

Nitric oxide was determined in the brain homogenate according to the method described by Miranda et al. (2001).

Determination of paraoxonase activity

Arylesterase activity of paraoxonase was measured spectrophotometrically in supernatants using phenylacetate as a substrate (Higashino et al. 1972; Watson et al. 1995).

Determination of prostaglandin E2 concentration

Prostaglandin E_2 concentration in rat brain supernatant was assayed by ELISA technique using prostaglandin E_2 assay kit purchased from Glory Science Co., Ltd., TX, USA, according to the instructions provided. The parameter prostaglandin E_2 (PGE_2) immunoassay is a 3.5-h forward sequential competitive enzyme immunoassay designed to measure PGE_2 in cell culture supernates.

Histopathology and immunopathology studies

For histopathological study, the brain was removed, placed in 10 % buffered formalin, dehydrated in graded alcohol, and embedded in paraffin. The paraffin blocks were serially sectioned at 5-μm thickness. Afterward, sections were mounted on glass slides and counter-stained with hematoxylin and eosin (Hx & E) for evaluation qualitatively under light microscope. Images were captured and processed using Adobe Photoshop version 8.0. For immunopathology, paraffin sections from each case were processed using caspase-3 antibodies. Caspase-3-stained sections were examined at high power for immunohistochemical expression and assessment of apoptosis and were divided into those that were negative (no immunoreactivity in any cells) and positive (membrane and/or cytoplasm immunoreactivity present).

Statistical analysis

Results are expressed as means±SEM. Comparisons between means of different groups were carried out using one-way analysis of variance (ANOVA) test followed by Duncan multiple comparisons test. The level of significance was set at $p<0.05$. SPSS software (SAS Institute Inc., Cary, NC) and Graph pad Prism software (Inc., San Diego, USA, version 5) were used to carry out all statistical tests.

Results

Oxidative stress

Compared with the saline-treated group, $AlCl_3$ injection resulted in a significant increase in the level of MDA by 35.2 %, a decrease in the level of GSH by 72.9 %, and an increase in the level of nitric oxide by 74.21 % (Fig. 1a–c).

It was noted that treatment with the lower doses of piracetam, vincamine, or vinpocetine resulted in significant decrease in brain lipid peroxidation by 26.2 %, 33.5 %, and 23.6 %, respectively. In contrast, the higher dose of these agents failed to significantly alter brain MDA. A decrease in brain MDA was observed after treatment with 10 or 20 mg/kg vinpocetine (by 23.6 % and 18.1 %, respectively), low-dose piracetam plus vincamine (by 18.3 %), or low-dose piracetam plus vinpocetine (by 21.7 %). These figures, however, did not reach statistical significance. Meanwhile, no significant change in brain MDA was observed after treatment with donepezil (Fig. 1a).

The administration of the nootropic drugs piracetam, vincamine, vinpocetine, piracetam plus vincamine, piracetam plus vinpocetine, or donepezil 5 mg/kg almost reversed or restored the depleted brain glutathione (Fig. 1b).

Moreover, significant decrease in brain nitric oxide was observed after treatment with 100 mg/kg piracetam (by 45.8 %), 10 and 20 mg/kg vincamine (by 17.4 % and 17.2 %, respectively), 10 mg/kg vinpocetine (by 27.6 %), piracetam plus vincamine (by 16.8 %), piracetam plus vinpocetine (by 24.6 %), and donepezil (by 23.6 %). Thus, the high dose of piracetam or vinpocetine failed to significantly alter the raised brain nitric oxide in $AlCl_3$-treatred rats (Fig. 1c).

Paraoxonase 1 activity

$AlCl_3$ resulted in significantly decreased brain paraoxonase (PON1) activity by 54.6 %. Compared with the $AlCl_3$ control group, a significant decrease in brain PON1 activity was observed after treatment with piracetam (by 21 % and 75 %), vincamine (by 187.6 % and 98.8 %), vinpocetine (by 42.3 and 80 %), piracetam plus vincamine (by 58.8 %), piracetam plus vinpocetine (by 75.7 %), and donepezil (by 95.2 %). Brain PON1 activity was almost restored to normal value by treatment with vincamine (Fig. 1d).

Acetylcholinesterase activity

In $AlCl_3$-treated rats, brain acetylcholinesterase (AChE) activity was significantly raised by 50.6 % compared with the saline group. Brain AChE activity showed significant decrease after

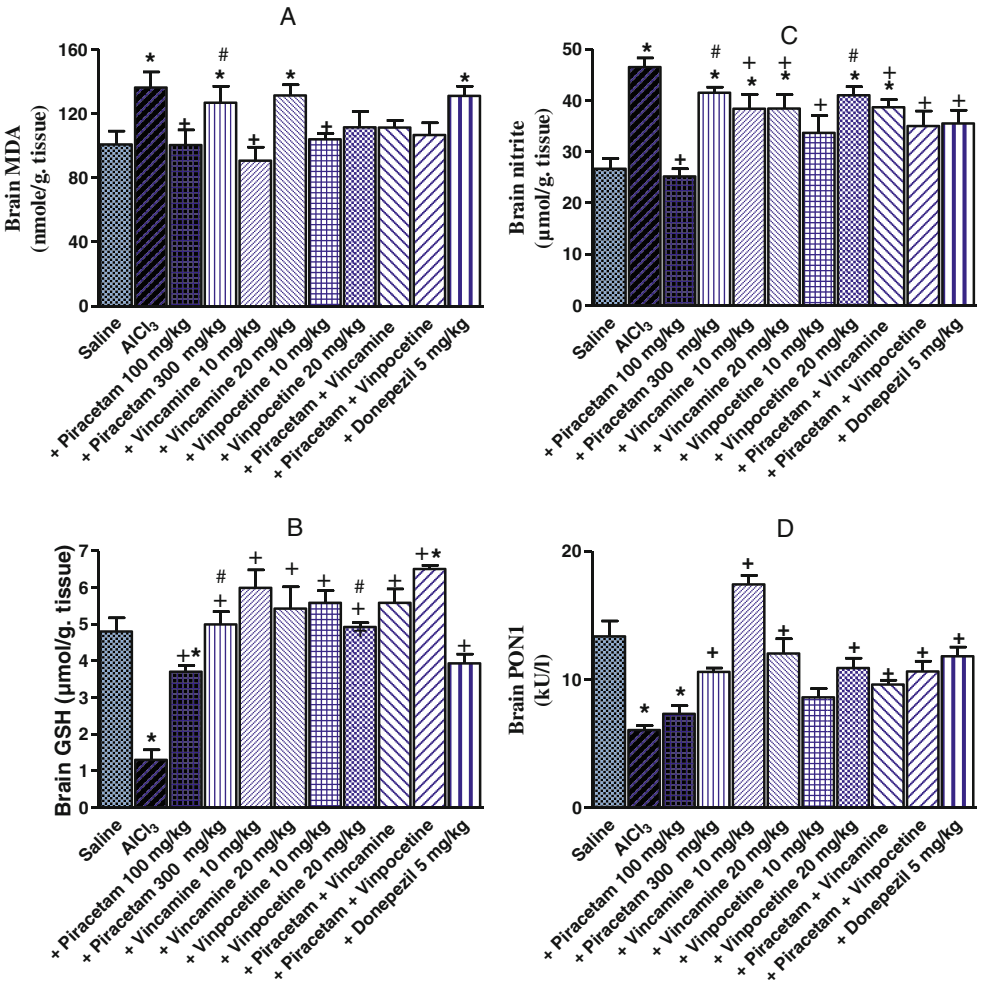

Fig. 1 **a–d** Effect of different nootropic drugs on brain malondialdehyde (MDA), reduced glutathione (GSH), nitric oxide, and paraoxonase activity. *$p<0.05$ versus corresponding saline group, $^{+}p<0.05$ versus $AlCl_3$ control group, $^{\#}p<0.05$ versus lower dose of the same drug

treatment with 100 mg/kg piracetam (by 29.8 %), 20 mg/kg vincamine (by 39.1 %), vinpocetine (by 25.6 % and 38.9 %), piracetam plus vincamine (by 74.3 %), piracetam plus vinpocetine (by 61.5 %), and donepezil (by 59.1 %; Fig. 2).

Butrylcholinesterase activity

In rats treated with $AlCl_3$, butrylcholinesterase (BChE) activity in brain increased by 58.8 % compared with the saline group. BChE activity showed a significant decrease after treatment with 100 mg/kg piracetam (by 32.8 %), 10 mg/kg vincamine (by 42.8 %), vinpocetine (by 37.7 and 22.3 %), piracetam plus vincamine (by 37.9 %), piracetam plus vinpocetine (by 38.1 %), and donepezil (by 42.1 %). The administration of donepezil had no significant effect on BChE activity in $AlCl_3$-treated rats (Fig. 3).

Prostaglandin E_2

The administration of $AlCl_3$ did not led to a significant change in brain PGE_2 compared with the saline group (17.8 % increase, $p>0.05$). In $AlCl_3$-treated rats, however, brain PGE_2 concentration increased by the high dose of piracetam (by 46.4 %), vincamine (by 39 %), vinpocetine (by 57.6 %),

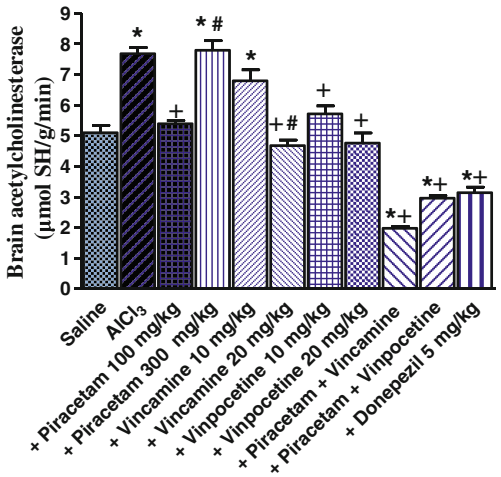

Fig. 2 Effect of different nootropic drugs on brain acetylcholinesterase (AChE) activity. *$p<0.05$ versus corresponding saline group, $^{+}p<0.05$ versus $AlCl_3$ control group, $^{\#}p<0.05$ versus lower dose of the same drug

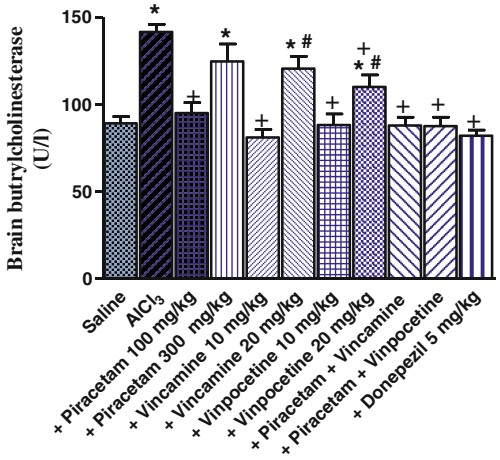

Fig. 3 Effect of different nootropic drugs on brain butrylcholinesterase (BChE) activity. *$p<0.05$ versus corresponding saline group, $^{+}p<0.05$ versus $AlCl_3$ control group, $^{\#}p<0.05$ versus lower dose of the same drug

piracetam plus vincamine (by 27.1 %), and piracetam plus vinpocetine (by 21.3 %)(Fig. 4).

Histopathological results

Microscopic examination of the brain tissue from normal rats stained with Hx & E revealed regular normal-looking neurons with vesicular nuclei and prominent nucleoli with relatively pale-stained faint nuclear chromatin. The surrounding glial cells were evenly distributed and had small nuclei with densely stained condensed chromatin. The cerebral cortex was of average thickness, all indicative of normal cerebral tissue (Fig. 5a, b).

Rats that received only $AlCl_3$ showed atrophy of the cortex, shrinkage of the neuronal cell bodies, pyknosis of their nuclei, disappearance of the nucleoli, and loss of Nissl substance, with intense eosinophilia of the cytoplasm (red neurons),

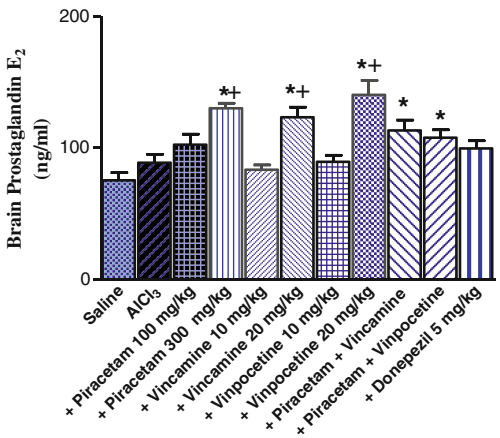

Fig. 4 Effect of different nootropic drugs on brain prostaglandin E_2 (PGE_2) activity. *$p<0.05$ versus corresponding saline group, $^{+}p<0.05$ versus $AlCl_3$ control group

surrounded by vacuolations with cytoplasmic neurofibrillary tangles and associated with vascular congestion in the cortex and choroid plexus (Fig. 6a). Rats treated with $AlCl_3$ along with piracetam (100 mg/kg) showed near average cortical thickness with mildly degenerated neurons. No neurofibrillary tangles were detected but there was still little vascular congestion (Fig. 6b). With the higher dose of the drug (300 mg/kg), cortical thickness was almost like the saline control with degenerated neurons surrounded by vacuolations. No neurofibrillary tangles were seen. Vascular congestion was still detected (Fig. 6c). In the group treated with $AlCl_3$ along with donepezil at 5 mg/kg, there was average cortical thickness, mildly degenerated neurons, but no neurofibrillary tangles. Little vascular congestion was observed (Fig. 6d).

With vincamine treatment at 10 mg/kg, neuronal degeneration was evident but to a lesser extent than in the $AlCl_3$ control group. No neurofibrillary tangles were detected, but vascular congestion was seen in both choroid plexus and brain substance (Fig. 7a). Rats treated with $AlCl_3$ and vincamine at 20 mg/kg showed cortical neuronal degeneration with surrounding vacuolations. Average cortical thickness, scattered neurofibrillary tangles, and associated vascular congestion were observed (Fig. 7b).

Following vinpocetine treatment at 10 mg/kg, mild neuronal degeneration with occasional peri-neuronal vacuoles was seen. The cortical thickness was average. No neurofibrillary tangles were detected but mild congestion was seen (Fig. 7c). With vinpocetine treatment at 20 mg/kg, degenerative changes were seen in most of neurons with surrounding vacuolations but with normal cortical thickness. No neurofibrillary tangles were seen but mild vascular congestion was evident (Fig. 7d).

Brain sections of rats treated with piracetam (100 mg/kg) and either vincamine (10 mg/kg) or vinpocetine (10 mg/kg) showed cortical neuronal degeneration with surrounding vacuolations. Average cortical thickness, scattered neurofibrillary tangles, associated vascular congestion, and brain edema were seen (Fig. 8a, b).

Overall, the groups treated with low doses of singly used drugs and the donepezil-treated group had the best improvement in neuronal look, cortical thickness, and degree of vascular congestion, while the combined drugs in addition to evident previously mentioned parameters showed brain edema.

Caspase-3 immunoreactivity

Figure 9 shows brain sections stained with caspase-3 antibody from saline control rat (Fig. 9a, b) and after $AlCl_3$, where degenerated neurons for caspase-3 were seen (Fig. 9c–e). Following treatment with vincamine at 10 mg/kg, degenerated neurons for caspase-3 were observed but with some regenerating neurons (Fig. 9f).

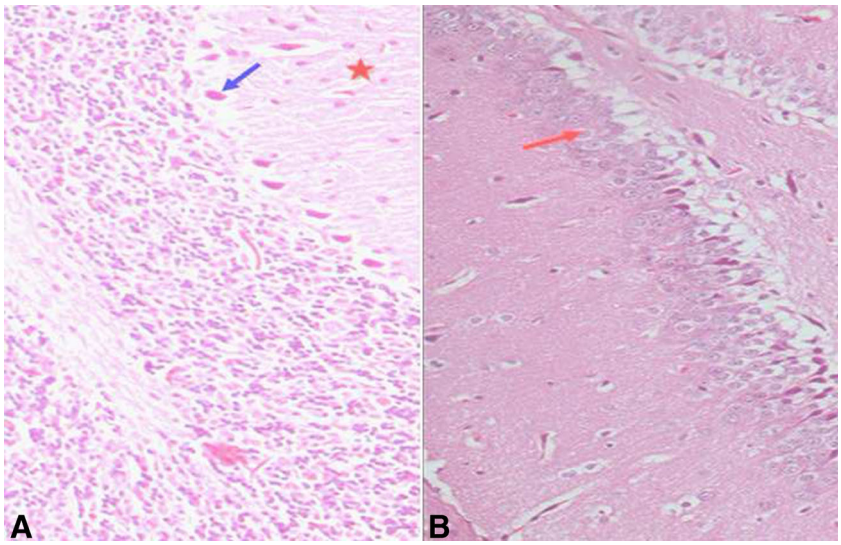

Fig. 5 Hx & E stained sections showing the structure of the normal cerebral cortex (**a**, **b**): the innermost granular layer (*red arrow*) composed of many small cells; Purkinje cell layer, formed of large flask-shaped cells (*blue arrow*); and the outermost molecular layer that contains a few small nerve cells and many unmyelinated nerve fibers (*red star*) (Hx & E ×400)

Congo red staining

Sections from $AlCl_3$ control rats stained with Congo red stain showed amyloid deposition in degenerated ganglionic cells (Fig. 10a, b). Figure 10c shows amyloid deposition in neurons after treatment with vincamine 20 mg/kg. Figure 10d–f shows amyloid deposition in neurons after treatment with piracetam 100 mg/kg+vincamine 10 mg/kg.

Discussion

In this study, the repeated daily administration of $AlCl_3$ for 45 days in rats was associated with increased brain oxidative stress. Malondialdehyde, a marker of lipid peroxidation, was increased (Gutteridge 1995), suggesting free radical attack on membrane lipids. Meanwhile, reduced glutathione, a major brain anti-oxidant and free radical scavenger (Bains and Shaw 1997; Dringen 2000), showed a marked decrease, possibly due to increased consumption by the increased free radicals. The intracellular tripeptide glutathione (glycyl–glutamic acid–cysteine) is the brain's major redox buffer, and reduced levels have been detected in the brain from patients with neurodegenerative diseases such as Parkinson's disease (Sofic et al. 1992; Sian et al. 1994) and Alzheimer's disease (Ramassamy et al. 2000). The study also indicated increased nitric oxide levels in brains of $AlCl_3$-treated rats. Nitric oxide is an important biological molecule involved in neurotransmission and vasodilatation. It is synthesized in vivo from L-arginine by the action of nitric oxide synthases (NOS), a family with four isoforms: endothelial, neuronal, inducible, and mitochondrial (Guix et al. 2005). It is nitric oxide which is

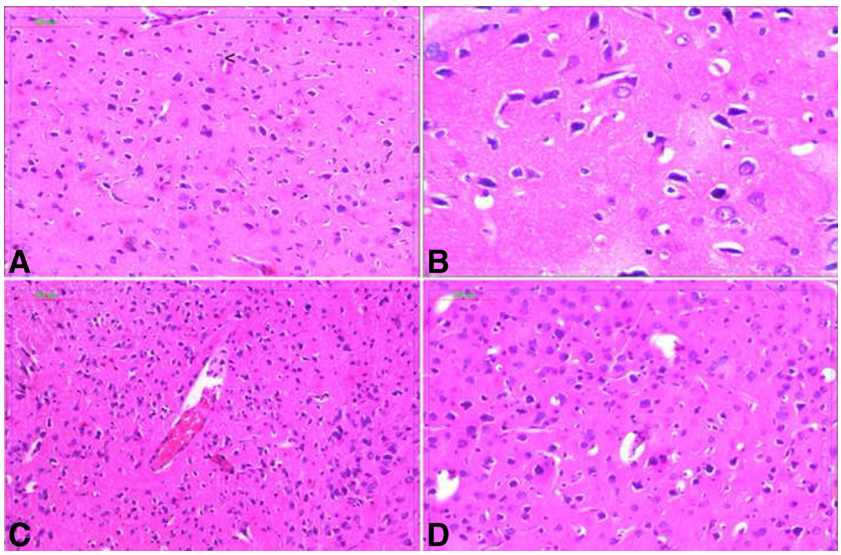

Fig. 6 Hx & E stained sections of brain tissue of **a** $AlCl_3$ (10 mg/kg)-treated rat showing wide neuronal degeneration and vacuolations with neurofibrillary tangles (*arrowhead*) (Hx & E ×100); **b** $AlCl_3$+piracetam (100 mg/kg) showing neuronal degeneration with surrounding vacuolations (note some healthy neurons) (Hx & E ×400); **c** $AlCl_3$+piracetam (300 mg/kg) showing congestion in the brain substance (Hx & E ×100); **d** $AlCl_3$+donepezil (5 mg/kg) showing neuronal regeneration (Hx & E ×200)

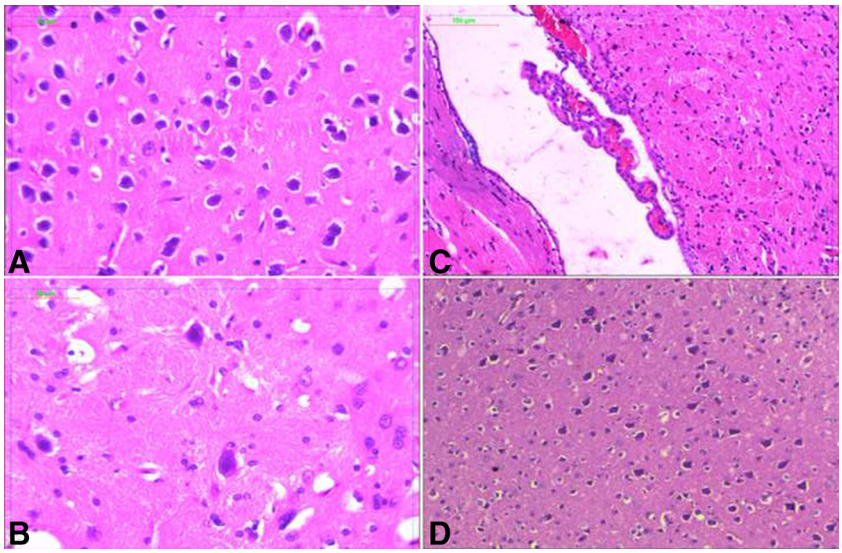

Fig. 7 Hx & E stained sections of brain tissue of **a** $AlCl_3$+ vincamine (10 mg/kg) showing regenerating neurons. Note the restoration of nuclear roundness, appearance of nucleolus, and the open chromatin pattern (Hx & E ×400); **b** $AlCl_3$+vincamine (20 mg/kg) showing neuronal degeneration (Hx & E ×400); **c** $AlCl_3$+vinpocetine (10 mg/kg) showing congested choroid plexus (Hx & E ×100); **d** $AlCl_3$+ vinpocetine (20 mg/kg) showing neuronal degeneration (Hx & E ×200)

generated in excess and for prolonged time by the inducible form of nitric oxide (iNOS) expressed following immunological or inflammatory stimulation in the immune and glial cells that evokes tissue damage (Guix et al. 2005; Förstermann and Sessa 2012). Nitric oxide neurotoxicity can be mediated via oxidative and nitrative stresses. The most important is the reaction of nitric oxide with superoxide ($O2•^-$) which results in the formation of peroxynitrite ($ONOO^-$), a highly reactive radical capable of damaging cellular macromolecules (lipids, proteins, DNA) (Boje 2004). The present findings are consistent with other studies showing elevated levels of oxidative stress (Yuan et al. 2012) and decreased reduced glutathione and glutathione reductase activity (Jacewicz et al. 2009) in rat brain following repeated administration of $AlCl_3$. Although Al has a fixed oxidation number and therefore cannot undergo redox reactions, the metal can induce oxidative damage through multiple mechanisms, e.g., by altering iron homeostasis and stimulating iron-initiated free-radical-induced tissue damage (Gutteridge et al. 1985).

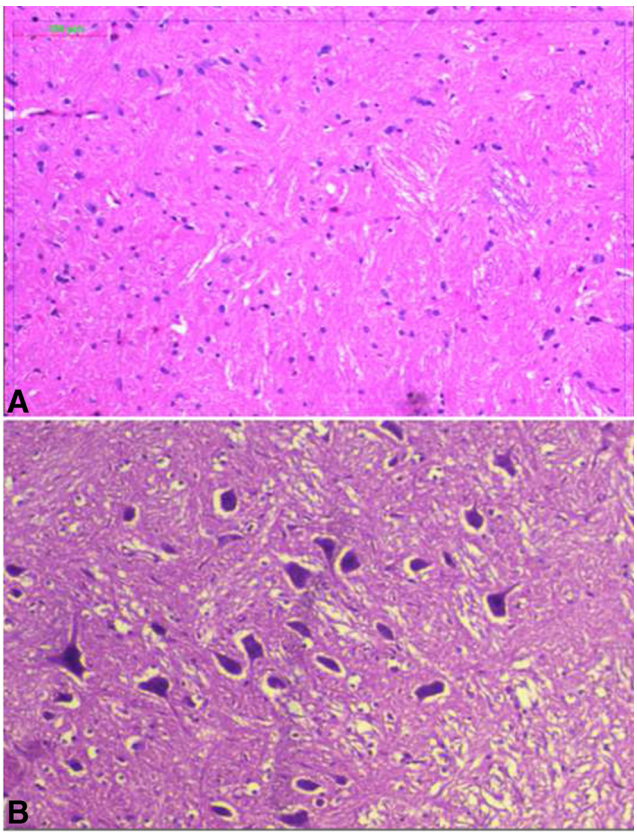

Fig. 8 Hx & E stained sections of brain tissue of $AlCl_3$ along with piracetam (100 mg/kg) and either **a** vincamine (10 mg/kg) showing brain edema (Hx & E ×100) or **b** vinpocetine (10 mg/kg) showing neuronal degeneration, vacuolations, and brain edema (Hx & E ×200)

The present results also indicated decreased paraoxonase 1 (PON1) activity in the brain of $AlCl_3$-treated rats. The paraoxonase family of enzymes is involved mainly with the detoxification of organophosphates and nerve agents (La Du 1992). PON1 has an anti-oxidant role (Watson et al. 1995; Rajkovic et al. 2011; Furlong 2008) and has recently been of much interest in view of a possible association with neurodegenerative disorders, e.g., Alzheimer's disease and other dementias (Menini and Gugliucci 2013). In these patients, the level of the enzyme activity decreased in their sera (Wehr et al. 2009). A markedly decreased PON1 activity was also observed in an experimental model of multiple sclerosis (Abdel-Salam et al. 2012). The decline in PON1 activity in the present study is consistent with earlier observations using the same model (Abdel-Salam et al. 2014) and suggests inactivation of the enzyme by the increased level of oxidative stress and/or direct inhibition by $AlCl_3$.

Several nootropic drugs, e.g., piracetam, vincamine, and vinpocetine, are being used to improve cognitive functions

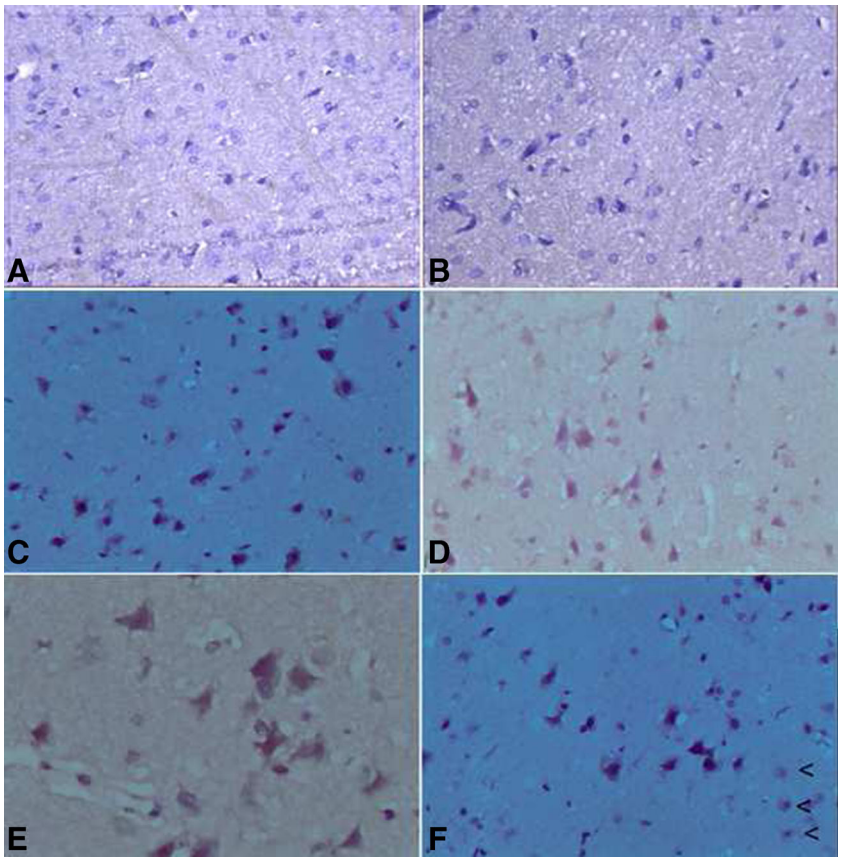

Fig. 9 Brain sections stained with caspase-3 antibody from **a**, **b** saline control rat showing cytoplasmic staining neurons for caspase-3 (Hx & E ×100); **c–e** $AlCl_3$ control showing cytoplasmic staining of degenerated neurons for caspase-3 (Hx & E ×100, 200, 400); **f** $AlCl_3$+vincamine (10 mg/kg) showing cytoplasmic staining of degenerated neurons for caspase-3. Note some regenerating neurons (*black arrows*) (Hx & E ×100)

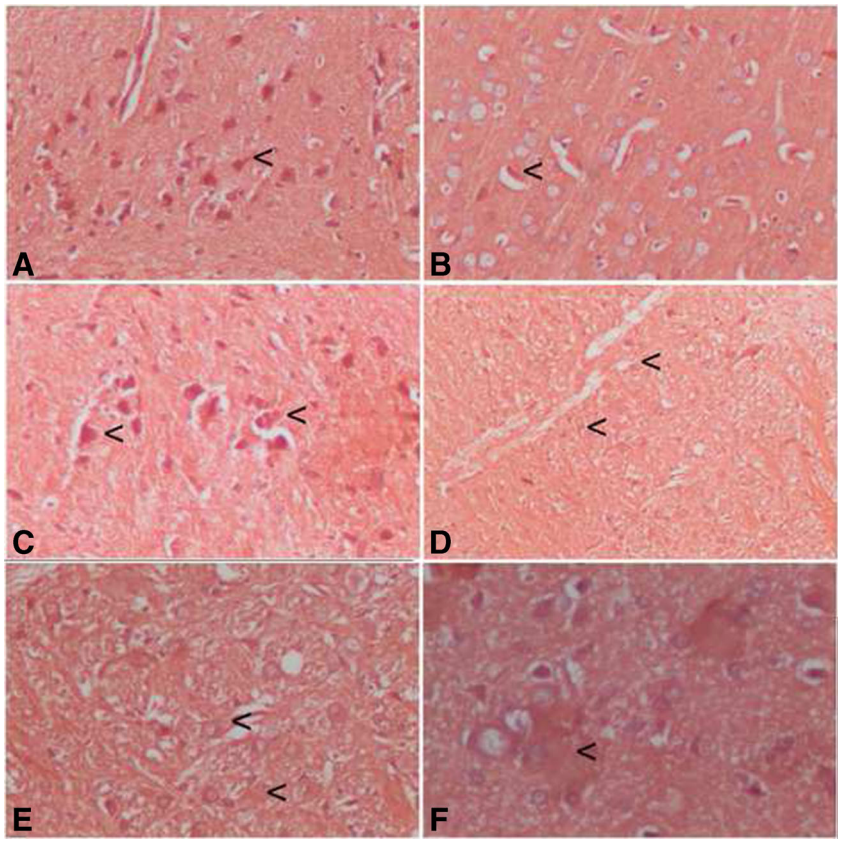

Fig. 10 Brain sections stained with Congo red from **a** $AlCl_3$ control rats showing amyloid deposition in degenerated ganglionic cells (*arrowhead*) (×200); **b** $AlCl_3$ control rats showing neurofibrillary tangles and amyloid deposition (*arrowhead*) (×200); **c** $AlCl_3$+vincamine 20 mg/kg-treated group showing amyloid deposition in neurons (*arrowhead*) (×100); **d**, **e** $AlCl_3$+piracetam 100 mg/kg+vincamine 10 mg/kg-treated rats showing amyloid deposition in neurons (*arrowhead*) (×100 and ×200); **f** $AlCl_3$+piracetam 100 mg/kg+vincamine 10 mg/kg-treated rats showing homogenous structureless amyloid deposition (*arrowhead*) (×400)

in the elderly with chronic cerebral hypo-perfusion or in those with devastating Alzheimer's disease (Waegemans et al. 2002; Hadjiev 2003). In this study, these agents were evaluated for their ability to modulate the biochemical changes and neuronal injury caused by $AlCl_3$. It was noted that treatment with the low doses of piracetam, vincamine, and vinpocetine resulted in significant decrease in brain lipid peroxidation and nitric oxide content, suggesting an anti-oxidant effect. In contrast, the higher dose of the agents were not effective in decreasing lipid peroxidation or brain nitric oxide although they lessened the decline reduced glutathione and PON1 activity induced by $AlCl_3$. These findings suggest that the nootropic drugs piracetam, vincamine, and vinpocetine at high doses might increase oxidative stress.

Studies have reported anti-oxidant effects for piracetam and vinpocetine. In vitro, vinpocetine demonstrated hydroxyl radical scavenging capability near to that of vitamin E in the Fenton reaction (Oláh et al. 1990). The drug also showed marked anti-oxidant activity against free radicals generated by phenazine methosulphate. Piracetam showed anti-oxidant capacity only at concentrations 10 times higher than the therapeutic concentrations (Horvath et al. 2002). In rabbits with spinal cord ischemia/reperfusion injury, the drug displayed neuroprotective and anti-oxidant properties (decreasing malondialdehyde and xanthine oxidase level, while increasing glutathione peroxidase activity) (Kalkan et al. 2011). In rat primary cortical cells, subjected to oxygen and glucose deprivation, piracetam reduced neuronal damage and exerted anti-oxidant effects (He et al. 2014). Moreover, either piracetam (1 mM) or vinpocetine (0.1 μM) protected astrocytes against hypoxic injury in culture, by increasing intracellular ATP, and inhibiting caspase-3 activity. The higher concentration of vinpocetine (10 μM) was, however, detrimental in hypoxic conditions (Gabryel et al. 2002).

One possible explanation for the observed effects of the high doses of the nootropic drugs might be their ability to increase brain dopamine and/or dopaminergic neurotransmission (Rago et al. 1981; Stancheva et al. 1991; Budygin et al. 1996; Trejo et al. 2001). This might increase free radical generation due to increased dopamine metabolism, by monoamine oxidase, and/or dopamine autoxidation into reactive dopamine quinines with consequent attack on thiol compounds such as GSH and cysteinyl residues on proteins (LaVoie and Hastings 1999; Hermida-Ameijeiras et al. 2004). It is also possible that these drugs act as free radicals, and this explains sparing of reduced glutathione and PON1 activity. The present findings confirm previous observations in a demyelination model caused by intracerebral injection of ethidium bromide in rats. In that study, the increase in brain MDA decreased by treatment with vinpocetine at 1.5 mg/kg or piracetam at 150 mg/kg but increased by the higher dose of vinpocetine or piracetam (6 and 300 mg/kg, respectively) (Abdel-Salam et al. 2012).

Neuroinflammation is an important contributor in the pathogenesis of neurodegenerative diseases (Frank-Cannon et al. 2009). The arachidonic acid metabolite prostaglandin E_2 (PGE_2) is involved in the inflammatory responses following brain injury, bacterial or viral infections (Matsumura and Kobayashi 2004; Vasilache et al. 2015), and high levels have been found in neurodegenerative disorders such as Alzheimer's disease (Hoozemans et al. 2001), prion's disease (Pocchiari and Levi 2000), and autism (Brigandi et al. 2015). Moreover, selective inhibition of the EP_2 receptor or EP_2 receptor deletion was associated with decreased neuronal injury and improved functional recovery in experimental models of brain damage (Rojas et al. 2015; Leclerc et al. 2015). In this study, increased levels of PGE_2 have been demonstrated in $AlCl_3$-treated rats. Other researchers have indicated increased levels of PGE_2, and other arachidonic acid metabolites in rat hippocampus following chronic aluminum gluconate administration. This occurred along with hippocampal neuronal injury and deficits in learning and memory (Wang et al. 2015). We also observed increased brain PGE_2 concentrations after treatment with the high dose of piracetam, vincamine, and vinpocetine, as well as by the combined administration of the lower doses of piracetam plus either vincamine or vinpocetine. This finding is intriguing and might suggest an enhancing effect on the aluminum-induced brain inflammation by the high doses of the nootropic agents. In their study, Bhattacharya et al. (1989) reported significant increase in rat brain PGE_2 and PGF_2 alpha after treatment with 100 mg/kg piracetam.

Impaired central cholinergic neurotransmission is a key component of memory decline in disorders like Alzheimer's disease (Coyle et al. 1983; Francis et al. 1999) and possibly in normal aging (Bartus et al. 1982). Consequently, boosting cholinergic activity through increasing available acetylcholine (ACh) with the use of centrally acting cholinesterase inhibitors like donepezil is a cornerstone of therapy and results in clinically measurable benefit in cognitive function, and behavior (Sugimoto et al. 2002; Wilkinson et al. 2004). In mammals, two cholinesterases exist in brain, predominantly acetylcholinesterase (AChE) (80 %), and butyrylcholinesterase (BChE) or pseudocholinesterase. Inhibiting AChE enhances cholinergic neurotransmission and increases the availability of acetylcholine. BChE found in neurons and glial cells is considered to play a minor role in regulating brain ACh levels (Greig et al. 2001; Giacobini 2001). There is, however, evidence that inhibition of BChE might find utility in treating advanced Alzheimer's disease (Greig et al. 2001; Giacobini 2004). In the present study, AChE and BChE activities in brain increased following the repeated administration of $AlCl_3$. Several studies reported alterations in AChE activity in rats chronically treated with aluminum. Mice treated orally with $AlCl_3$ or aluminum lactate showed increased AChE activity in their brain homogenates (Zatta et al. 2002). Mice

given $AlCl_3$ (10 mg/kg/day) for 90 days also showed significant elevation of AChE activity in cerebral cortex and hippocampus and memory impairment (Yang et al. 2013). Kaizer et al. (2008) found increased AChE activity in striatum, and hypothalamus of rats following chronic treatment with $AlCl_3$ (50 mg/kg/day). AChE activity in cerebellum, hippocampus, and cerebral cortex was, however, decreased by Al. Stevanović et al. (2009) reported a significant rise in AChE activity in the rat brain and serum following treatment with $AlCl_3$. Gulya et al. (1990) suggested that the cholinotoxic effects of Al are likely the result of neurodegenerative effects. The authors found significant decrease in choline acetyltransferase activities but no change in AChE activity in the parietal cortex, the hippocampus, and the striatum of rats after chronic Al treatment. In vitro, low concentrations of Al slightly increased while high concentrations inhibited AChE activity.

In the present study, AChE and BChE activities showed significant and marked decrease after treatment with the lower dose of piracetam, vinpocetine, and piracetam plus either vincamine or vinpocetine. AChE activity decreased following 20 mg/kg vincamine, and BChE activity after decreased following 10 mg/kg vincamine. AChE but not BChE activity decreased after donepezil. The latter is an effective and selective inhibitor for AChE (Sugimoto et al. 2002). When given to rats, donepezil (2.5 mg/kg, p.o.), inhibited whole-brain AChE activity and increased the synaptic ACh concentration in the hippocampus (Kosasa et al. 1999). It is likely, however, that the observed changes in AChE and BChE activities in brain of $AlCl_3$-treated rats following the lower doses of piracetam, vinpocetine, piracetam reflect neuroprotection.

Moreover, histopathological examination and caspase-3 immunolocalization in brain have shown that $AlCl_3$ resulted in neuronal degeneration and induced apoptosis associated with the increase of caspase-3 expression in degenerated neurons. In the present study, the biochemical findings of decreased oxidative stress with the low doses of piracetam, vincamine, or vinpocetine in contrast to the effect of the high doses of these drugs in increasing brain PGE_2 appear to correlate with the histopathological observations, where reduced neuronal degeneration and improved cortical thickness were seen only in the brain of $AlCl_3$-treated rats after treatment with the low dose of singly used drugs.

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