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ORIGINAL ARTICLE

Neuroprotective effects of zafirlukast, piracetam and their combination on L-Methionine-induced vascular dementia in rats

Ahmed M. Fayez[a], Ahmed S. Elnoby[b,c], Nada H. Bahnasawy[c], Omar Hassan[a,c]*

[a]Pharmacology Department, October University for Modern Science and Arts, 11787 6 October City, Egypt
[b]Clinical Pharmacy Department, Children's Cancer Hospital Egypt 57357, Cairo, Egypt
[c]Faculty of Pharmacy, October University for Modern Science and Arts, 11787 6 October City, Egypt

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*Correspondence and reprints:
omhassan@msa.eun.eg

ABSTRACT

Vascular dementia is considered a vascular cognitive impairment disease caused by neuronal degeneration in the brain. Several studies have supported the hypothesis that oxidative stress and endothelial dysfunction are the main pathogenic factors in vascular dementia. This current study aims to determine the possible neuroprotective effects of zafirlukast, piracetam and the combination of piracetam and zafirlukast on L-methionine-induced vascular dementia in rats. Male Wistar albino rats were divided into five groups. Group I was the normal control, and group II received L-methionine (1700 mg/kg, P.O.) for 32 days. The remaining groups received zafirlukast (20 mg/kg, P.O.), piracetam (600 mg/kg, P.O.) or their combination (zafirlukast 20 mg/kg + piracetam 600 mg/kg, P.O.) for 32 days after L-methionine administration. Afterwards, the cognitive and memory performances of the rats were investigated using the novel object recognition (NOR) test; rats were then sacrificed for histopathological and biochemical analyses. L-methionine-induced vascular dementia altered rats' behaviours and the brain contents of different neurotransmitters and acetylcholinesterase activity while increasing levels of oxidative stress and causing notable histopathological alterations in brain tissues. The treatment of vascular dementia with zafirlukast and the combination improved neurochemical, behavioural and histological alterations to a comparable level to those of piracetam. Thus, zafirlukast, piracetam and the combination of both drugs can be considered as potential therapeutic strategies for the treatment of vascular dementia induced by L-methionine. To the best of our knowledge, this study is the first to explore the neuroprotective effects of zafirlukast and piracetam on L-methionine-induced vascular dementia.

INTRODUCTION

Vascular dementia is a cerebrovascular disease caused by an impairment in the blood supply to the brain that results in subsequent lesions in different parts of the brain [1]. Vascular dementia is usually characterized by cognitive impairments, such as deficits in attention and executive function [2]. A persistent shortage in cerebral blood flow leads to ischaemia of the brain tissue and reduced levels of oxygen and nutrients, resulting in cell death [3]. Oxidative stress is postulated to play a critical role in vascular dementia [4]. Indeed, free radicals such as free oxygen species cause neuropathological lesions and damage the brain by reacting with substrates such as lipids, proteins and nucleic acids [5]. In addition, the brain is highly sensitive to

oxidative stress due to its high content of polyunsaturated fatty acids, its high demand for oxygen for its metabolic requirements and its significantly low concentration of antioxidants [6].

Homocysteine (HcY), a nonprotein thiol-containing amino acid, is a metabolite derived from the demethylation of methionine [7]. Hyperhomocysteinaemia (HHcy) has proven to play a key role in the neurological dysfunction in the brain by increasing oxidative stress. HHcy impairs the synthesis of nitric oxide (NO), a vasodilator, by inducing the accumulation of asymmetric dimethylarginine (ADMA), a potent inhibitor of nitric oxide synthase and by inhibiting the expression of dimethylaminohydrolase (DDAH), the main catabolic enzyme of ADMA [8]. Moreover, homocysteine has been reported to be neurotoxic and to exacerbate amyloid beta (Aβ)-induced neuronal damage, as homocysteic acid increases the accumulation of Aβ-42 in neuronal cells [9].

Zafirlukast, a selective and potent cysteinyl leukotriene receptor antagonist, has been used to manage bronchial asthma [10]. It is considered the first member of the leukotriene antagonist family to be used in the prophylaxis of asthma for more than 20 years [11]. It prevents the binding of leukotrienes, which are inflammatory mediators, to their binding sites [12]. Several studies have claimed that lipoxygenase (LOX) and cyclooxygenase (COX) inhibitors may exert desirable neuroprotective actions on several neurodegenerative diseases [13]. The neuroprotective effects of zafirlukast are mediated by its ability to block cysteinyl leukotriene receptors and subsequently inhibit the generation of reactive oxygen species (ROS) and reduce oxidative stress [14].

Piracetam is a cyclic derivative of the neurotransmitter γ-aminobutyric acid (GABA), which acts on cognitive, neural and vascular functions, but the mechanism of action is still unknown [15]. Piracetam has been shown to restore fluidity in brain membranes [16] and in hippocampal membranes in patients with Alzheimer's disease; this restored fluidity has been associated with improvements in avoidance learning [17]. Moreover, piracetam stimulates prostacyclin synthesis, which exerts a vasodilatory effect on blood vessels and inhibits platelet aggregation [15]. Piracetam also increases cerebral blood flow in humans with acute cerebral ischaemia [18]. Furthermore, piracetam has been shown to exert a neuroprotective effect by decreasing levels of lipofuscin, an indicator for neuronal membrane damage [19].

We proposed to use piracetam, which is a neuroprotective agent in vascular dementia, because it works on (i) activation of the adenylate kinase enzyme which is responsible for conversion of ADP into ATP. Consequently, this improves the deficiency of ATP in the brain cells, since one of the early events in dementia is mitochondrial dysfunction and low energy [20]. Moreover, (ii) piracetam increases the expression of acetylcholine receptors in the frontal region of the brain, which consequently enhances acetylcholine level in the brain by nearly 30 to 40% [21]. The co-administration of piracetam with zafirlukast; cysteinyl leukotriene receptor antagonist, can have an additional effect in treatment of vascular dementia, in which both drugs work by opposing the Aβ1–42-induced cognitive deficits, which though to be a result of neuronal inflammation and apoptosis mediated by cysteinyl leukotriene signaling.

The current study aimed to investigate the possible neuroprotective effects of zafirlukast, piracetam and their combination on L-methionine-induced vascular dementia in rats. To our knowledge, this novel study is the first to investigate the neuroprotective effects of zafirlukast and the combination of zafirlukast and piracetam on L-methionine-induced vascular dementia.

MATERIAL AND METHODS

Animals

The present experiment involved 40 adult male Wistar albino rats (weighing between 180 and 200 g) purchased from the National Institute of Ophthalmology, Giza, Egypt. Rats were divided into five groups and allowed to accommodate in the animal house at the Faculty of Pharmacy, October University for Modern Science and Arts, for 1 week before the experiments were initiated. Rats were housed in a room adjusted to a temperature of 25 ± 2 °C, 60–70% humidity and exposed to 12-h light and 12-h dark cycles. Rats were provided a standard rat pellet chow diet purchased from El-Naser Chemical Co., Cairo, Egypt. Our present study was conducted according to the Ethics Committee for Animal Experimentation at the Faculty of Pharmacy, October University of Modern Science and Arts.

Drugs and chemicals

L-methionine was purchased from Sigma-Aldrich (USA). The drug was dissolved in saline and administered orally (1700 mg/kg) for 32 days to induce endothelial dysfunction and vascular dementia [22]. Zafirlukast (20 mg/kg, P.O.)[13,23] and piracetam

(600 mg/kg, P.O.)[24] were purchased from Egyptian Group Pharmaceutical Company (Obour City, Egypt) and Amoun Pharmaceutical Company (Obour City, Egypt), respectively. Biochemical analyses were performed using kits for the acetylcholinesterase (AChE) enzyme, interleukin-6 (IL-6), interleukin-10 (IL-10) and Aβ-42 from CUSABIO Biomedical Company (China). Kits for the endothelial nitric oxide synthase (eNOS) enzyme, acetylcholine (ACh), total cholesterol and reduced glutathione (GSH) were purchased from MyBioSource Company (USA), Shanghai Crystal Day Biotech Company, EIAab Company (China) and Biodiagnostic (Egypt), respectively.

Experimental design

Forty rats were randomly divided into five groups: each group contained eight rats. Group I was the normal control group received normal saline daily for 32 days, group II was treated with L-methionine (1700 mg/kg, P.O.), group III was treated with zafirlukast (20 mg/kg, P.O.), group IV was treated with piracetam (600 mg/kg, P.O.), and group V was treated with the combination of zafirlukast and piracetam. L-Methionine was administered for 32 days to induce vascular dementia. The remaining groups were administered zafirlukast, piracetam or the combination for 32 days after the administration of L-methionine. All groups undergo the same procedures in oral administration of medications and normal saline in normal control group that were given by oral gavage. Three days prior to the end of the experiment, the novel object recognition (NOR) test was performed to assess cognitive performance and memory. Immediately after the NOR test, rats were sacrificed by decapitation and the brains were isolated and frozen. The hippocampus and cortex were carefully isolated from the brain to measure levels of biochemical parameters, such as AChE, eNOS, ACh, Aβ-42, malondialdehyde (MDA), GSH, total cholesterol, IL-6 and IL-10. Two brains from each group were preserved in 10% formalin for the histological examination of cortex and hippocampus.

NOR test

The NOR test was performed to assess the cognitive function and memory of rats on the last 3 days of the experiment [25].

Apparatus

The apparatus used in the NOR test was a wooden black open rectangular box (65 × 45 × 65 cm). The test was performed in a room with a low level of noise and constant illumination. The objects used in the NOR test were two different opaque cubes (familiar objects) and a pink pyramid (novel object) with an opaque cube; the objects were 6-cm high and sufficiently heavy to prevent the rats from moving them. In addition, the objects were placed in opposite corners at a distance of 10 cm from the wall.

Procedures

The NOR test involves three phases: habituation, familiarization and test phases. In the habituation phase, rats were individually placed in the apparatus and allowed to explore the empty open field arena for 3 min. Twenty hours after the habituation trial, two trials were performed (T1 and T2), separated by an inter-trail interval (24 h). During T1 (familiarization phase), rats were placed in the open field and allowed to explore two identical familiar objects (a1 and a2). After the inter-trial interval, the T2 (test phase) was performed with a novel object (b) and a fresh familiar object (a). Objects and the open field were cleaned with 70% ethanol following each trial to minimize the presence of olfactory stimuli. The time the rats spent exploring the objects during T1 and T2 was recorded using a stopwatch, and live videos were recorded to investigate the rats' movements. Exploration was defined as rats directing their noses 2 cm from the object or touching it. The test lasted for 3 min, and contact time (20 s) with the objects was fixed in T1 and T2 to ensure the sensitivity and comparability of the test.

Estimation of the ACh content in the brain

The brain ACh content was determined according to the method developed by Mathew et al. [26] using a specific enzyme-linked immune sorbent assay (ELISA) kit provided by Shanghai Crystal Day Biotech Co. The kit used a double-antibody sandwich ELISA to assay the levels of ACh in brain tissues, and values are expressed as nmol/g tissue.

Estimation of AChE levels in the brain

Acetylcholinesterase levels in the brain were estimated according to the method developed by Den Blaauwen et al. [27] using a rat-specific ELISA kit, and AChE levels were expressed as ng/g tissue.

Estimation of the brain contents of noradrenaline (NA) and dopamine (DA)

Brain contents of NA and DA were measured according to the method developed by Aviles et al. [28] and Kobori et al. [29], respectively, using specific ELISA

kits. NA contents are expressed as nmol/g tissue, and DA contents are expressed as ng/g tissue.

Estimation of eNOS contents in the brain
Brain eNOS contents were quantified according to the method described by Den Blaauwen et al. [30] using a specific ELISA kit provided by MyBioSource. The ELISA kit for eNOS applies the competitive enzyme immunoassay technique utilizing a monoclonal anti-eNOS antibody and an eNOS-HRP conjugate. eNOS levels are expressed as pg/g tissue, and the procedures were performed according to the manufacturer's instructions.

Estimation of brain AF062-42 content
Brain Aβ−42 contents were determined using the method described by Wang et al. [31] and a specific ELISA kit provided by CUSABIO. The assay employs the quantitative sandwich enzyme immunoassay technique. Aβ−42 contents are expressed as pg/g tissue.

Estimation of brain contents of IL-6 and IL-10
The tissue IL-6 content was quantified using the method described by Venihaki et al. [32], and IL-10 contents were determined according to the specified method by Eskdale et al. [33]. Both methods were performed using specific ELISA kits provided by CUSABIO. The assays employ the quantitative sandwich enzyme immunoassay technique. IL-6 and IL-10 levels are expressed as pg/g tissue.

Estimation of the total cholesterol level
The total cholesterol content in the brain was measured using a specific ELISA kit provided by EIAab, according to the method developed by Allain et al. [34]. Values are expressed as mg/g tissue.

Estimation of the GSH level in brain
The GSH content of the brain was quantified as described by Beutler [35] using Ellman's reagent and 5-sulfosalicylic acid for the deproteinization of homogenates.

Estimation of MDA levels in the brain
Malondialdehyde is a marker of lipid peroxidation, and its levels were measured according to the method developed by Armstrong and Browne [36] using a specific ELISA kit. Values are expressed as pmol/g tissue.

Estimation of Homocysteine in brain
HcY has neurotoxic effects and plays a role in cognitive and memory decline. HcY level was measured using Rat Homocysteine (Hcy) ELISA Kit catalogue number MBS703069. Values are expressed as nmol/mL.

Histological examination of cortical and hippocampal tissues

Brains obtained from two rats in each group were randomly selected to perform the histological assessment. Autopsy samples of the brains of rats in different groups were collected and fixed with 10% formaldehyde in saline for 24 h. Samples were washed with tap water, and then, serial dilutions of alcohol (methyl, ethyl and absolute ethyl) were used for dehydration. Specimens were cleared with xylene and embedded in paraffin at 56 degrees in a hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at a 4-micron thickness using a sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized and then stained with haematoxylin and eosin for examination under a light microscope.

Statistical analysis

Data are presented as the mean ± SEM. A one-way analysis of variance (ANOVA) test followed by the Tukey-Kramer multiple comparisons test was used to compare the mean values between the groups. Test values were considered statistically significant at $P < 0.05$.

RESULTS

Cognitive function and recognition memory were assessed on the last 3 days of the experiment using the NOR test. The total time rats spent exploring similar objects in the familiarization phase (T1) was calculated and expressed as a mean ± SEM. Compared to the normal control group (18.000 ± 1.653), rats that received L-methionine showed a significant decrease in the total exploration time (2.333 ± 0.210) (*Table I*). Compared to the L-methionine group, the treatment groups that received zafirlukast, piracetam and their combination showed significant increases in the total exploration time of 10.167 ± 0.477, 11.667 ± 1.085 and 10.000 ± 0.683, respectively. Thus, the results indicated a strong preference for the object location by treatment groups and a low preference by the L-methionine group.

Table I Effect of zafirlukast, piracetam and combination on total time of exploration during familiarization phase (T1).

Groups	Total time of exploration (T1) Mean ± SE
Normal control	18.000 ± 1.653
L-methionine	2.333[a] ± 0.210
Zafirlukast	10.167[b] ± 0.477
Piracetam	11.667[b] ± 1.085
Combination	10.000[b] ± 0.683

Data are presented as the Mean ± SEM.

[a]Significantly different from normal control group at $P < 0.05$.

[b]Significantly different from L-methionine group at $P < 0.05$. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

Moreover, the recognition index (RI) or time spent investigating the novel object relative to total time spent investigating the novel and familiar objects was calculated. In the test phase, one familiar object was replaced with a novel object to assess memory performance. Compared to the normal control group (RI = 0.72 ± 0.023, $P < 0.05$), the L-methionine group (RI = 0.352 ± 0.021, $P < 0.05$) showed no preference for the novel object (*Figure 1*). However, compared to the L-methionine control group, rats treated with zafirlukast, piracetam and the combination showed significant increases in RI of 0.589 ± 0.033 ($P < 0.05$), 0.599 ± 0.025 ($P < 0.05$) and 0.586 ± 0.017 ($P < 0.05$), respectively, indicating a strong preference for the novel object.

Compared to the normal control group, L-methionine administration significantly increased the brain contents of NA by 231.71% and DA by 157.93%. Compared to the L-methionine control group, the zafirlukast treatment significantly decreased the brain contents of NA and DA by 42.85 and 40.06%, respectively. Similarly, compared to the L-methionine control group, piracetam significantly decreased the brain contents of NA and DA by 49.45 and 42.24%, respectively, and the combination group showed significant decreases in NA and DA contents of 51.13 and 43.9%, respectively. Moreover, no significant differences in the brain NA and DA contents were detected between all treatment groups (zafirlukast, piracetam and combination) (*Table II*).

Compared to the normal control group, L-methionine significantly decreased the brain ACh levels and significantly increased the AChE activity in the brain by

Table II Effect of zafirlukast, piracetam and combination on brain catecholamines: noradrenaline (NA) and dopamine (DA).

Groups	NA nmol/g tissue	DA ng/g tissue
Normal control	8.23 ± 0.599	12.66 ± 0.924
L-methionine	27.3[a] ± 1.99	32.54[a] ± 2.37
Zafirlukast	15.6[a,b] ± 1.14	19.41[a,b] ± 1.22
Piracetam	13.8[a,b] ± 1.01	19.27[a,b] ± 1.10
Combination	13.34[a,b] ± 0.898	19.07[a,b] ± 1.02

Data are presented as the Mean ± SEM.

[a]Significantly different from normal control group at $P < 0.05$,

[b]Significantly different from L-methionine group at $P < 0.05$. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

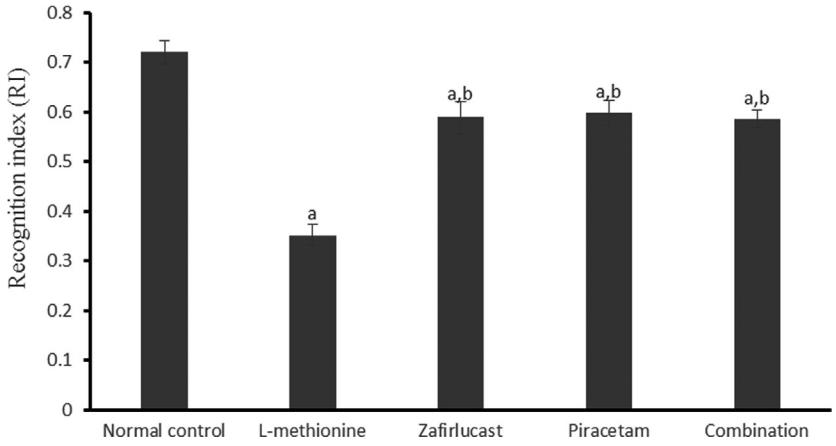

Figure 1 Effect of zafirlukast, piracetam and combination regimen on recognition memory in vascular dementia induced by L-methionine in rats. Novel object recognition test was performed in three consecutive days, and recognition index (RI) was calculated. Data are presented as mean ± SE ($P < 0.05$), where (a) significantly different from normal control group and (b) significantly different from L-methionine group. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test.

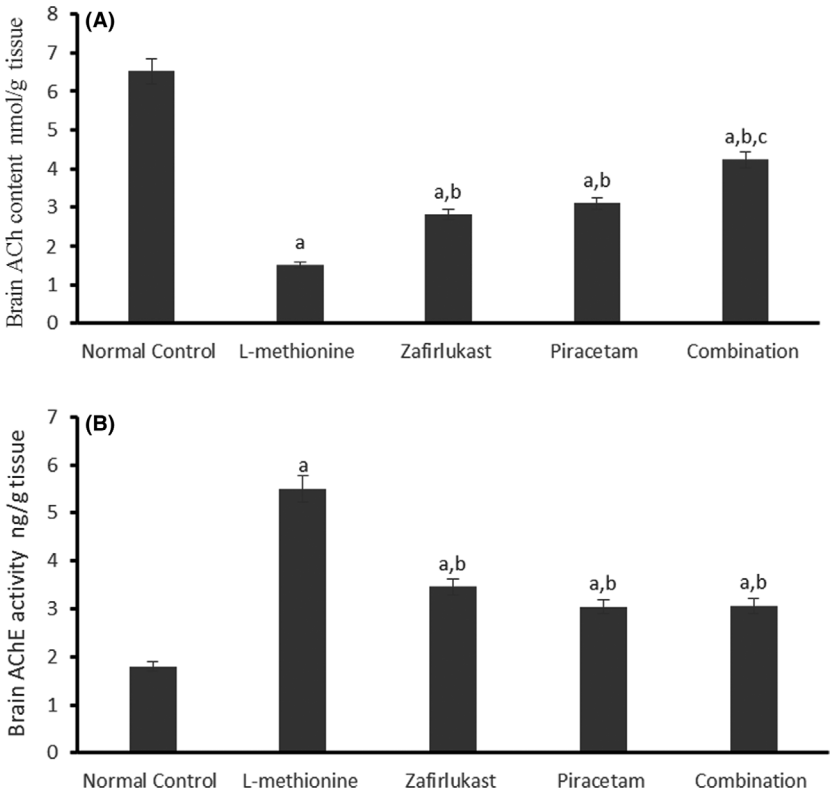

Figure 2 Effect of zafirlukast, piracetam and combination regimen on brain content of acetylcholine (ACh) (A) and acetylcholinesterase (AChE) activity (B) in vascular dementia induced by L-methionine in rats. Data are presented as mean ± SE ($P < 0.05$), where (a) significantly different from normal control group, (b) significantly different from L-methionine group and (c) significantly different from zafirlukast group. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test.

76.87% and 206.4%, respectively. However, compared to the L-methionine control group, zafirlukast treatment significantly increased the brain ACh content by 86.09% and significantly decreased AChE activity by 36.47%. Similarly, compared to the L-methionine control group, the administration of piracetam significantly increased ACh levels and significantly decreased AChE activity by 105.29 and 46.46%, respectively, and the combination group showed a significant increase in ACh levels and a significant decrease in AChE activity of 180.13 and 50.99%, respectively. Furthermore, significant differences in the brain ACh contents were not observed between the zafirlukast and piracetam groups or between the combination and piracetam groups. In contrast, compared to the zafirlukast group, the combination group showed a significant increase of 50.53% in the brain ACh content (*Figure 2*).

Compared to the normal control group, the administration of L-methionine resulted in a significant decrease in the brain eNOS content of 84.87%. However, compared to the L-methionine control group, the administration of zafirlukast resulted in a significant increase of 158.33% in the brain eNOS content. Similarly, compared to the L-methionine control group, piracetam significantly increased the eNOS content by 200.75% and the combination group exhibited a significant increase of 306.06%. Moreover, compared with the piracetam group, the zafirlukast and combination groups did not show a significant difference in the brain eNOS content. Meanwhile, compared to the zafirlukast group, the combination group showed a significant increase in the brain eNOS content of 57.18% (*Figure 3*).

In the present experiment, compared to the normal control group, the administration of L-methionine resulted in a significant decrease of 64.26% in the brain GSH content and a significant increase of 391.08% in the brain MDA content. However, compared to the L-methionine control group, the administration of zafirlukast significantly increased the brain GSH content by 121.69% and decreased the MDA content by 63.42%. Moreover, compared to the L-methionine control group, piracetam administration significantly increased GSH levels by 91.82% and significantly decreased MDA levels by 56.80%. Compared to the L-methionine control group, the combination group exhibited a 122.32% increase in GSH levels and a 64.2% decrease in MDA levels. Furthermore, brain contents of GSH and MDA were not significantly different among the treatment groups (zafirlukast, piracetam and combination) (*Figure 4*).

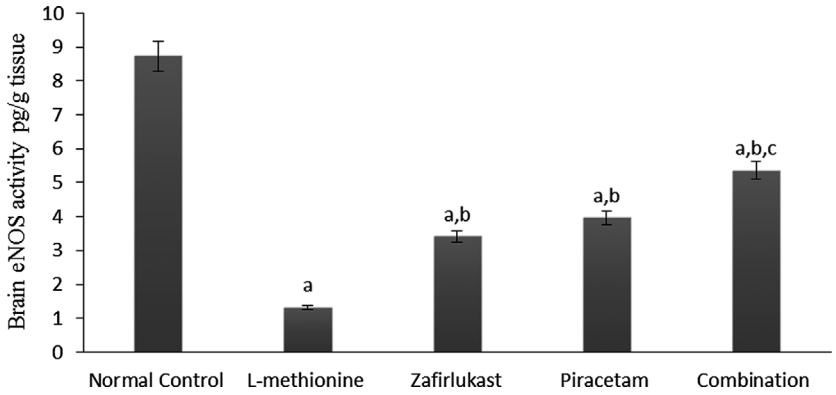

Figure 3 Effect of zafirlukast, piracetam and combination regimen on brain endothelial nitric oxide synthase (eNOS) in vascular dementia induced by L-methionine in rats. Data are presented as mean ± SE ($P < 0.05$), where (a) significantly different from normal control group, (b) significantly different from L-methionine group and (c) significantly different from zafirlukast group. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test.

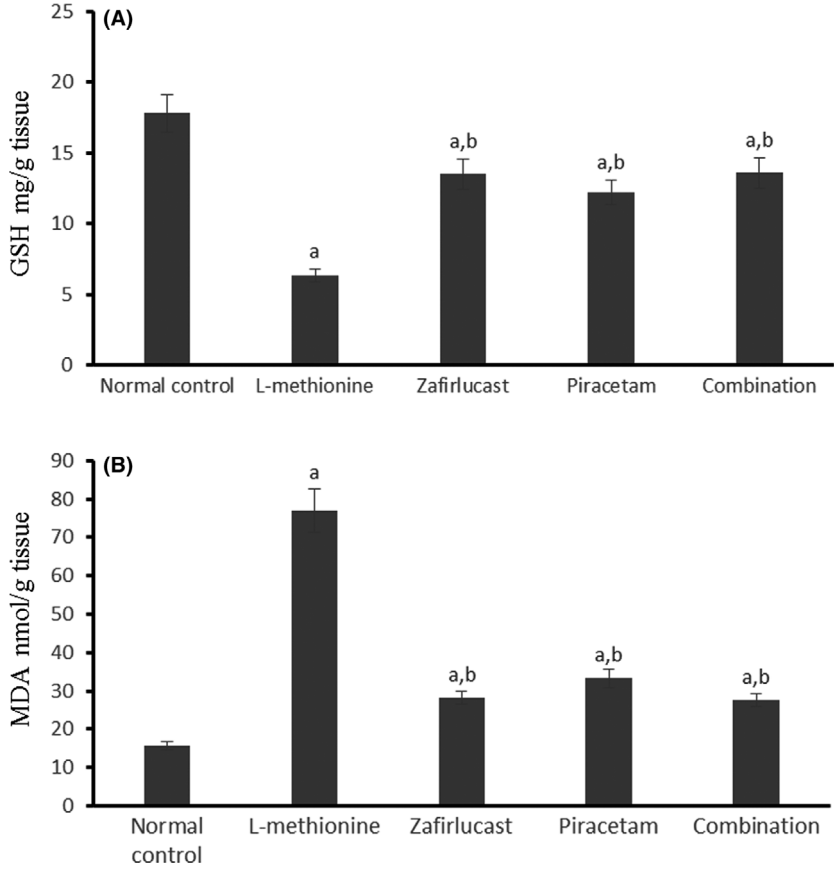

Figure 4 Effect of zafirlukast, piracetam and combination regimen on brain content of glutathione (GSH) (A) and malondialdehyde (MDA) (B) in vascular dementia induced by L-methionine in rats. Data are presented as mean ± SE ($P < 0.05$), where (a) significantly different from normal control group and (b) significantly different from L-methionine group. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test.

Compared to the normal control group, the rats that received L-methionine exhibited a significant increase of 345.23% in the brain Aβ-42 content. However, compared to the L-methionine control group, the administration of zafirlukast, piracetam and the combination significantly decreased Aβ-42 levels by 44.74%, 49.01 and 57.57%, respectively. Moreover, the zafirlukast, piracetam and combination groups did not display significant differences in brain Aβ-42 contents (*Figure* 5).

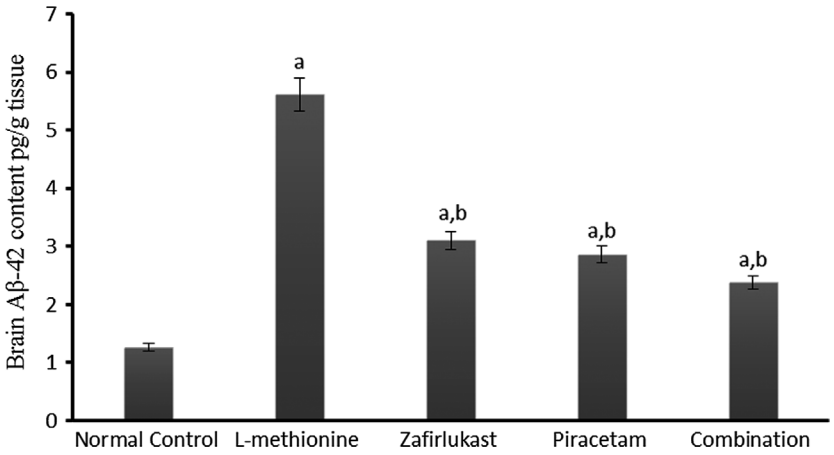

Figure 5 Effect of zafirlukast, piracetam and combination regimen on brain content of amyloid beta-24 (Aβ-42) in vascular dementia induced by L-methionine in rats. Data are presented as mean ± SE ($P < 0.05$), where (a) significantly different from normal control group and (b) significantly different from L-methionine group. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test.

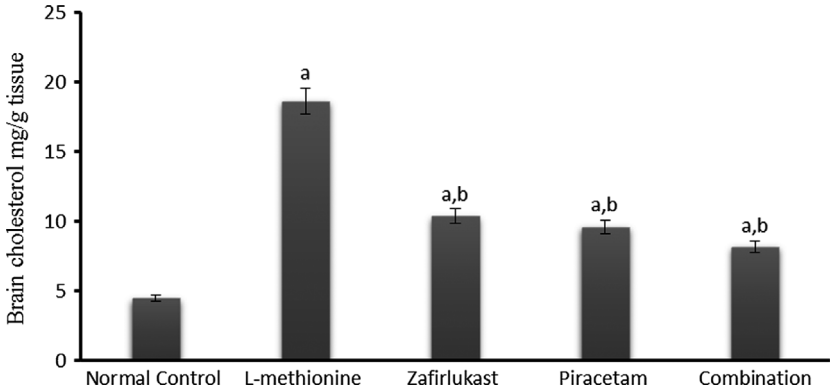

Figure 6 Effect of zafirlukast, piracetam and combination regimen of on brain content of cholesterol in vascular dementia induced by L-methionine in rats. Data are presented as mean ± SE ($P < 0.05$), where (a) significantly different from normal control group and (b) significantly different from L-methionine group. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test.

Compared to the normal control group, the L-methionine treatment significantly increased the brain total cholesterol content by 312.86%. However, compared to the L-methionine control group, treatments with zafirlukast, piracetam and the combination significantly decreased the brain total cholesterol contents by 44.14%, 48.38 and 56.06%, respectively. Moreover, no significant differences were detected between treatment groups (zafirlukast, piracetam and combination) (*Figure 6*).

Compared to the normal control group, L-methionine administration significantly increased the brain IL-6 level by 388.05% and significantly decreased the IL-10 level by 83.87%. In contrast, compared to the L-methionine control group, treatments with zafirlukast, piracetam and the combination significantly decreased the brain IL-6 contents by 35.77, 40.82 and 57.18%, respectively, and significantly increased the brain IL-10 contents by 159.32, 172.03 and 320.33%, respectively. Furthermore, compared to the piracetam group, the zafirlukast and combination groups did not display significant differences in brain IL-6 content. Compared to the zafirlukast group, the combination group showed a significant decrease in the brain IL-6 content of 33.33%. Moreover, compared to the zafirlukast and piracetam groups, the combination group showed a significant increase in the brain IL-10 content of 62.9 and 54.51%. However, no significant difference in the brain IL-10 content was detected between the zafirlukast and the piracetam groups (*Figure 7*).

Homocysteine level significantly increased in L-methionine group as compared to normal control group. Moreover, piracetam, zafirlukast and combination groups showed a significant decrease in HcY as compared to L-methionine group. However, no significant differences were observed among zafirlukast, piracetam and combination groups (*Table III*).

The histopathological investigation of stained brain sections showed serious damage in brain tissues of the L-methionine group compared to those of the normal control group (*Figure 8a-f*); this damage manifested as swelling in the endothelial cells lining the congested blood vessels of the cerebral cortex (*Figure 8d*). Focal

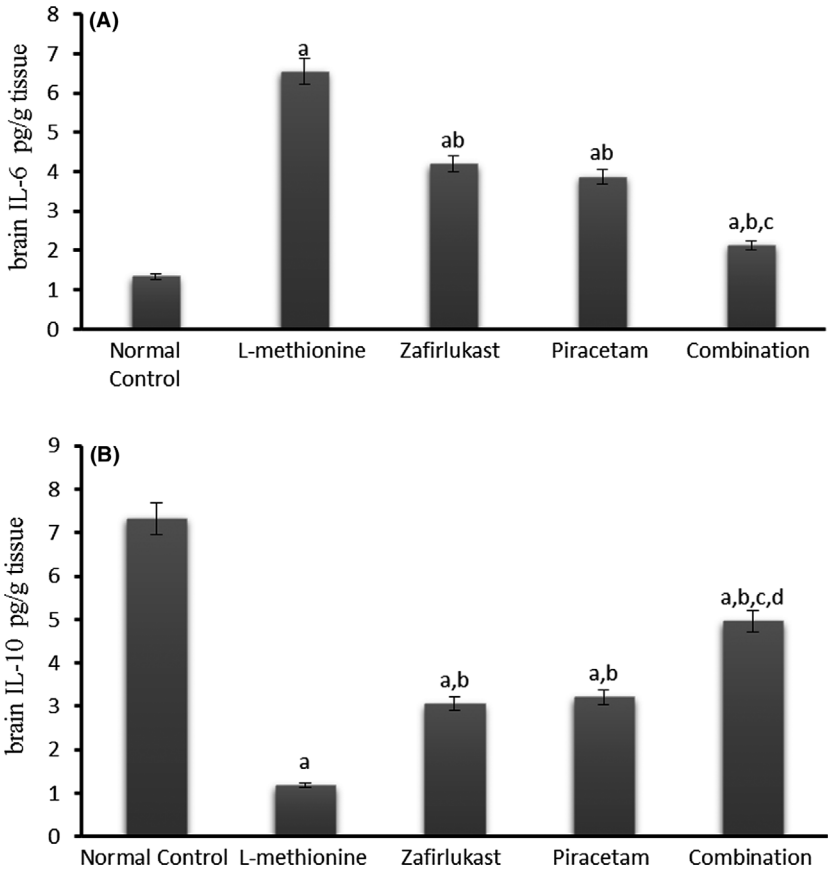

Figure 7 Effect of zafirlukast, piracetam and combination regimen of on brain content of interleukin-6 (IL-6) (A) and interleukin-10 (IL-10) content (B) in vascular dementia induced by L-methionine in rats. Data are presented as mean ± SE ($P < 0.05$), where (a) significantly different from normal control group, (b) significantly different from L-methionine group, (c) significantly different from zafirlukast group and (d) (c) significantly different from piracetam group. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer as a post hoc test.

neuronal degeneration in the subiculum of the hippocampus (*Figure 8*e), nuclear pyknosis and degeneration in the neurons in the fascia dentate and hilus of the hippocampus (*Figure 8f*).

Zafirlukast exhibited protection against L-methionine-induced brain damage, where no histopathological alteration in the neurons of the cerebral cortex (*Figure 9a*), nuclear degeneration and pyknosis in the subiculum showed in (*Figure 9b*), degeneration in some neurons in fascia dentate and hilus of the hippocampus (*Figure 9c*). Furthermore, sections in the piracetam group revealed a protective effect against L-methionine-induced brain damage, where no histopathological alteration in the neurons of the cerebral cortex (*Figure 9d*), mild haemorrhage with few degeneration in the neurons of the subiculum (*Figure* 9e), and no histological alteration in fascia dentate and hilus in the hippocampus (*Figure 9f*). Combination group showing nuclear pyknosis and degeneration in some few neurons in the cerebral cortex (*Figure 9g*), with no histopathological alteration in the neurons of the subiculum, fascia dentate and hilus of the hippocampus (*Figure 9f* and *i*).

Table III Effect of zafirlukast, piracetam and combination on serum homocysteine (HcY).

Groups	HcY(nmol/ml)
Normal control	3.61 ± 0.29
L-methionine	11.32 ± 0.90[a]
Zafirlukast	5.76 ± 0.14[a,b]
Piracetam	6.11 ± 0.31[a,b]
Combination	4.91 ± 0.28[a,b]

Data are presented as the Mean ± SEM.

[a]Significantly different from normal control group at $P < 0.05$.

[b]Significantly different from L-methionine group at $P < 0.05$. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Tukey-Kramer test for multiple comparisons.

DISCUSSION

Homocysteine is a thiol amino acid that is biosynthesized during methionine metabolism [37]. It participates in oxidative stress to induce neurological dysfunction [38] by increasing the production of ROS and oxidative deactivation of nitric oxide [39]. Moreover, homocysteine may block N-methyl-D-aspartate (NMDA)

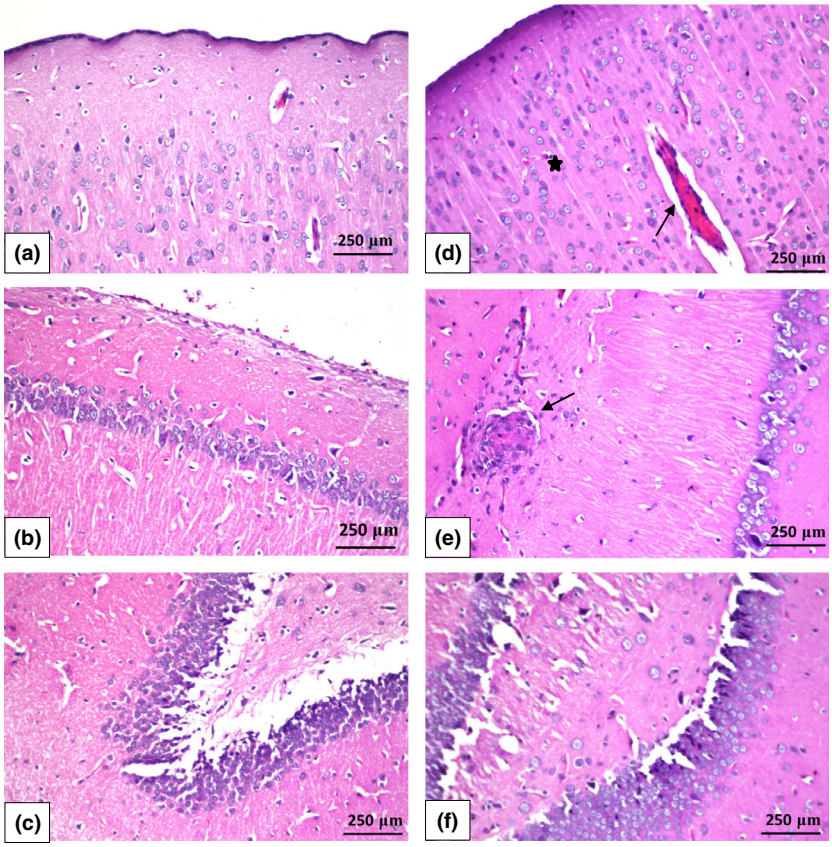

Figure 8 Photomicrograph of stained brain sections of normal control group showing normal histological structure of the neurons in the cerebral cortex (a), subiculum (b) and fascia dentate and hilus of the hippocampus (c). L-methionine group showing swelling in the endothelial cells lining (★) and congested blood vessels in the cerebral cortex (→) (d), focal neuronal degeneration in subiculum of the hippocampus (→) (e), nuclear pyknosis and degeneration in the fascia dentate and hilus of the hippocampus (f) (H & M ×40).

receptors and subsequently cause brain lipid peroxidation [40]. Homocysteine stimulates Aβ deposition in the brain and is involved in neurotoxicity; thus, it has been implicated in dementia, memory impairments, neural dysfunction and cognitive decline [41].

Piracetam has been shown to have a clinically significant role in treating vascular dementia and improves cognitive performance, memory and consciousness [42]. Piracetam normalizes ATP metabolism, enhances phospholipid synthesis and ribosome function and increases glucose implementation [43]. Piracetam has been shown to have antioxidant activities through the blockade of Ca^{2+} entry induced by glutamate in the ischaemic region [44]. Moreover, it reduces cerebral vessel resistance and increases the blood flow [45]. Piracetam exhibits cytoprotective activity and inhibits the apoptosis of astrocytes that have undergone hypoxia and reoxygenation [46]. Moreover, according to the study by HeZet al (2008), piracetam improves learning and memory dysfunction in rats by inhibiting the hypoperfusion-induced decrease in amino acid levels in the brain, while also facilitating synaptic plasticity by decreasing levels of the P53 and BAX proteins; thus, piracetam is a potential treatment for vascular dementia [24]. Piracetam enhances the function and the mobility of mitochondria by enhancing mitochondria membrane fluidity [47]. Piracetam has been shown to exert beneficial effects on pre- and postsynaptic transmission mediated by neurotransmitters, increasing the affinity of choline for its receptor and the densities of cholinergic and NMDA receptors [48].

Zafirlukast has not previously been studied in L-methionine-induced vascular dementia. The observed neuroprotective effects of zafirlukast may be related to its ability to block cysteinyl leukotriene receptors that subsequently inhibit the generation of ROS and reduce oxidative stress [49]. Increased production and accumulation of Aβ in patients with Alzheimer's disease is associated with neuroinflammation and may be related to activation of the LOX and COX pathways [50]. Moreover, arachidonic acid (AA) is a substrate for LOX and COX and is responsible for the production of inflammatory mediators, such as cytokines and leukotrienes [51]. Thus, LOX and COX inhibitors may exert neuroprotective actions [52]. Furthermore, zafirlukast has been reported to exert a significant neuroprotective

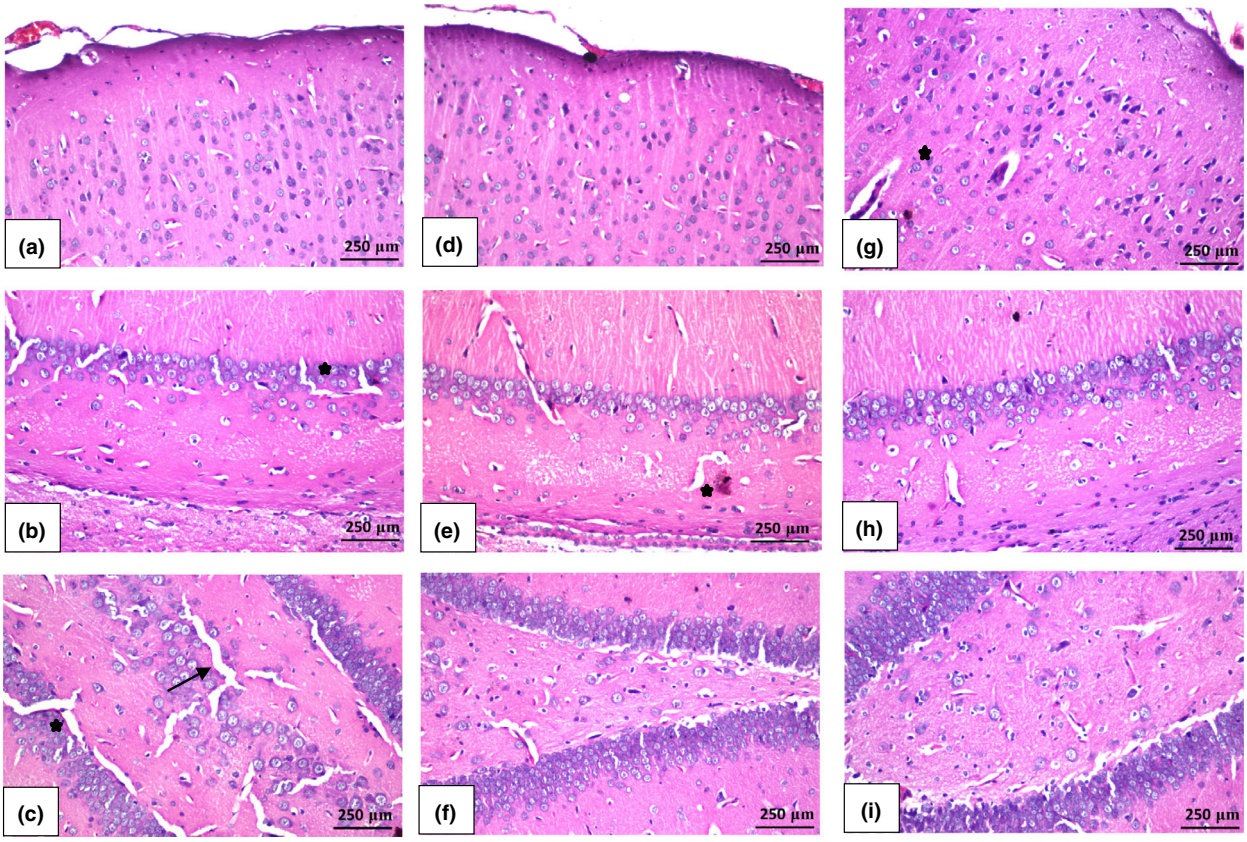

Figure 9 Photomicrograph of stained brain sections of zafirlukast group showing no histopathological alteration in the neurons of the cerebral cortex (a), nuclear degeneration and pyknosis in the subiculum (b), degeneration in some neurons in fascia dentate (*) and hilus (→) of the hippocampus (c). Piracetam group showing no histopathological alteration in the neurons of the cerebral cortex (d), mild hemorrhage with few degeneration in the neurons of the subiculum (★) (e), no histological alteration in fascia dentate and hilus in the hippocampus (f), (H & M ×40). Combination group showing nuclear pyknosis and degeneration in some few neurons (*) in the cerebral cortex (g), and no histopathological alteration in the neurons of the subiculum (h), fascia dentate and hilus of the hippocampus (i), (H & M ×40).

effect that is attributed to the prevention of Aβ−42-induced AD in rats, blockade of LOX metabolite release and reduction in mito-oxidative stress [13].

In the present experiment, administration of L-methionine to rats for 32 days impaired object recognition memory. Thus, the exploration pattern was observed for the L-methionine group, which did not discriminate between the novel and familiar object in T2, showing deficits in performance. However, the normal group was capable of discriminating between objects in T2. Piracetam, zafirlukast and combination treatment groups discriminated between objects, showing improvements in the performance of the rats, and greater total exploration times were recorded for these groups than that for the L-methionine group. This result indicates an improvement in recognition function and memory performance in the treatment groups. Overall, L-methionine was implicated in damaging the hippocampus and cortex, as evidenced by the result of the histological examination. L-Methionine significantly reduced recognition function and memory performance. In contrast, the treatment groups showed mild-to-moderate damage in the hippocampus and cortex and consequently a significant improvement in the memory performance.

In the present study, rats treated with L-methionine for 32 days exhibited pathological and behavioural changes, and these changes were associated with an increase in NA and DA levels in the brain. According to Xu et al. (2012), significantly higher NA levels are detected in patients with advanced Alzheimer's disease than those in patients with mild-to-moderate disease;

thus, an increase in adrenergic activity in the brains of patients with advanced Alzheimer's disease may be associated with cognitive impairment [53]. Moreover, as shown in the study by SvobStrac et al. (2015), cerebrospinal DA levels are increased in patients with vascular dementia [54]. Levels of these neurotransmitters were reduced in groups treated with piracetam, zafirlukast and the combination for 32 days. The effect of piracetam on NA and DA levels may be related to its effect on cerebral vessels and its ability to increase the brain blood flow [45]; piracetam also possesses a cytoprotective activity and reduces apoptosis [46]. The effect of zafirlukast may be linked to its abilities to block LOX metabolite release and reduce mito-oxidative stress [13].

Neurotransmitters have a critical role in learning and memory and contribute to cognitive processes [55]. Stimulation of cholinergic neurons in the hippocampus is associated with memory and recovery of acetylcholine levels in the hippocampus, which is sufficient to reverse memory impairments caused by the disruption of the septohippocampal pathway [56]. Elevated extracellular ACh levels cause vasodilatation, thus increasing the cerebral blood flow and hippocampal blood flow in rats [57]. In the current experiment, L-methionine increased AChE activity and decreased the ACh content in the brain, which may play an important role in VD. The reduction in ACh levels was reversed by piracetam and subsequently improved learning and memory dysfunction. Zafirlukast also elevated ACh content and reduced AChE activity, and this effect may be one of the mechanisms by which zafirlukast improves the brain function of rats.

In the present experiment, L-methionine-induced VD in rats and was associated with an increase in levels of MDA, a lipid peroxidation marker, and with a decrease in brain GSH levels. GSH is the prevalent antioxidant in the brain, boosting defences against oxidative damage by enhancing the antioxidant defence system in the brain [58]. Treatment of the rats with piracetam, zafirlukast and the combination reduced MDA levels and increased GSH contents in the brain.

Nitric oxide is the major enzymatic product of eNOS in brain vessels, which exerts an antithrombotic function and maintains vascular function [59]. NO plays an important role in regulating cerebral blood flow and Aβ production[60]. Moreover, NO is the key factor for maintaining the homoeostatic balance in the brain; thus, an eNOS deficiency might increase the incidence of endothelial dysfunction, and consequently, NO might contribute to neurodegeneration and cardiovascular disease [61]. Moreover, an association between eNOS polymorphisms and the risk of cerebral small vessel disease and silent brain infarction has been reported [62]. In the present study, L-methionine decreased eNOS levels in the brain, resulting in vasoconstriction and a decrease in cerebral blood flow. This effect was not observed in groups receiving piracetam or zafirlukast.

Cytokines, including IL-6 and growth factors, are generated as a result of the proliferation of vascular smooth muscle cells that in turn lead to excessive production of extracellular matrix [63]. Nuclear factor-kappaB (NF-κB) upregulates the expression of IL-6 and other inflammatory factors in an atherosclerotic lesion. NF-κB is the most common eukaryotic transcription factor that has a role in inducing the expression of genes that control cell growth, cell adhesion and inflammatory responses [64]. A complicated series of intracellular signal transduction events induced by external stimuli have a role in regulating the transcription activity of NF-κB. Moreover, NF-κB is important for controlling cell proliferation and transformation [65].

Reactive oxygen metabolites (ROM) are important factors contributing to the activation of NF-κB [66]. Thus, antioxidants block NF-κB activation and inhibit the increased pro-inflammatory cytokine production by reducing ROM levels [58]. In the current study, rats treated with piracetam or zafirlukast exhibited a reduction in the L-methionine-induced increase in IL-6 levels. However, the suppression of the production of IL-10, an inflammatory cytokine, by L-methionine was prevented by treatment with piracetam or zafirlukast.

Amyloid precursor protein-Aβ metabolism may be regulated by lipids, and cholesterol has an essential role in regulating the activity of the enzymes that contribute to Aβ production and the metabolism of the amyloid precursor protein [67]. The reported mechanisms include regulation of the secretase enzyme, promotion of Aβ aggregation, disruption of Aβ transport and reductions in the neurotoxicity of Aβ plaques [68]. Hyperlipidaemia has been reported to be implicated in cognitive impairment, inflammation and cholinergic dysfunction [69]. Hence, hyperlipidaemia may be considered a significant factor in age-related cognitive disorder, vascular dementia and Alzheimer's disease. In the current study, rats treated with piracetam or zafirlukast exhibited a reduction in the L-methionine-induced increase in cholesterol and Aβ-42 levels in the brain.

Homocysteine has been implicated to neurological dysfunction via enhancing production of reactive

oxygen species promoting oxidative stress which increase neurotoxicity of Aβ deposition in the brain [39]. In the present experiment, rats treated with piracetam, zafirlukast and combination of both exhibited a significant decline in HcY level than L-methionine-induced group. However, no significant differences were found between zafirlukast, piracetam and combination groups.

Hence, from this study, each treatment can add a beneficial value when used alone. While in combination, there was no adding value except in the effect on IL-10 (*Figure 7b*) as an anti-inflammatory. Concerning this point, further studies have to be conducted for more investigations concerning their activity and pharmacokinetics.

In conclusion, the incidence of VD has recently been reported to be increasing, because none of the available medications achieve a significant reduction in disease progression. Thus, a substantial investment in medical research is required to develop novel drugs with high clinical efficacy that target the underlying pathological mechanisms to stop the progression of the disease. Indeed, piracetam and zafirlukast achieved improvements in learning and memory in rats with L-methionine-induced VD. Both drugs might slow the progression of the disease, as they exerted neuroprotective, anti-inflammatory and antioxidant effects on rats in the current study.

CONFLICT OF INTEREST

The authors confirm that there are no known conflicts of interest associated with this publication, and there has been no significant financial support for this work that could have influenced its outcome.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Neuroprotective effects of zafirlukast, piracetam and their combination on L-Methionine-induced vascular dementia in rats.