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ORIGINAL ARTICLE

Amelioration of Cognitive Deficits by *Spirulina platensis* in L-methionine-Induced Rat Model of Vascular Dementia

Pian Wang, Yan Wang, Qingbin Zhang, Hongbo Zhang, Zheng Li, Xiaoying Liu, Lavleen Kaur[1], Manish Kumar[1]

Department of Neurology, Chengdu Fifth Peoples' Hospital, Chengdu, China, [1]Department of Pharmacology, Swift School of Pharmacy, Rajpura, Punjab, India

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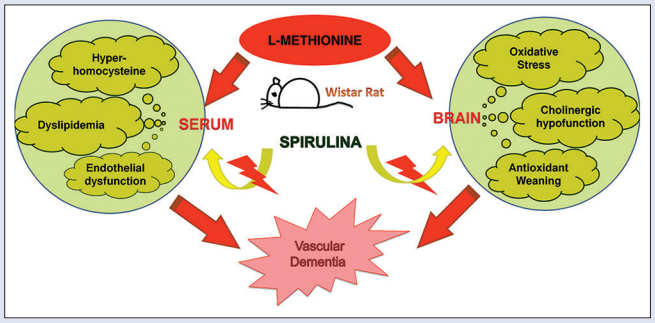
ABSTRACT

Background: Vascular dementia (VaD) is an age-associated highly prevalent brain disorder characterized by progressive cognitive insufficiency. Nutritional deprivation-associated rise in homocysteine (HCy), dyslipidemia, endothelial debility, and redox imbalance is a key facet of VaD. *Spirulina platensis* (spirulina) is a high-nutritional-value cyanobacterium appreciated by modern as well as diverse civilizations of antiquity (e.g., Mexican-Aztec, African-Kanambous). The present study aims to investigate the therapeutic effects of spirulina (*S. platensis*) in VaD. **Materials and Methods:** L-methionine (1.7 g/kg) was given orally to simulate VaD in rats. Spirulina was administered (0.5, 1, and 2 g/kg; *p.o.*) for 4 weeks daily. The neurobehavioral assessments and biochemical analysis in the whole brain (thiobarbituric acid-reactive substances [TBARS], glutathione [GSH], and acetylcholinesterase [AChE]) and serum (cholesterol, HCy, and nitrite) of rats were conducted. **Results:** Cognitive impairment in rats by L-methionine was attributable to statistically significant ($P < 0.05$) elevation in HCy, cholesterol levels, oxidative stress, AChE activity, and a decline of endothelial-derived nitric oxide. Spirulina attenuated ($P < 0.05$) the L-methionine-triggered cognitive insufficiency in rats. TBARS levels and AChE activity were reduced and GSH content was increased by spirulina in the brain of L-methionine-treated rats. Spirulina-attenuated L-methionine triggered increase in blood HCy and total cholesterol levels in rats. In aortic-ring tests, acetylcholine-induced endothelium-dependent relaxation was abolished by L-methionine, which showed endothelial mutilation and was potently attenuated by chronic treatment with spirulina. **Conclusion:** In L-methionine model of VaD, therapeutic intervention by spirulina (*S. platensis*) imparts appreciable relief in cognitive deficits.

Key words: Dementia, endothelial dysfunction, homocysteine, L-methionine, oxidative stress, spirulina

SUMMARY

- Spirulina is a highly nutritious cyanobacterium, is used as a dietary supplement, and has established a place among popular nutraceuticals. Nutritional deficiency (B_6, B_{12}, and folic acid), hyper-homocysteinemia, oxidative stress, and vascular aberrations are hallmarks of vascular dementia (VaD) pathology. L-methionine culminated vascular and brain abnormalities that witnessed a decrease in the cognitive performance of rats. Chronic administration of spirulina markedly attenuated the L-methionine-triggered cognitive impairment in rats that indicates the therapeutic potential of spirulina in VaD.



Abbreviations used: AD: Alzheimer's disease, ADDTC: State of California's Alzheimer's Disease Diagnostic and Treatment Centers, CADASIL: Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy, ChAT: Choline acetyl transferase, DSM-IV: Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, EDRF: Endothelial-derived relaxing factor, eNOS: Endothelial nitric oxide synthase, FDG-PET: Fluorodeoxyglucose-positron emission tomography, HCy: Homocysteine, HHCy: Hyper-homocyteinemia, HMG-CoA reductase: 3-hydroxy-3-methyl-glutaryl-CoA reductase, ICD-10: International Statistical Classification of Diseases and Related Health Problems 10th Revision, L-Meth: L-methionine, L-NAME: N(ω)-nitro-L-arginine methyl ester, L-NIO: N5-(1-Iminoethyl)-L-ornithine, L-NMMA: N (G)-monomethyl L-arginine, NMDA: N-methyl-D-aspartate, nNOS: Neuronal nitric oxide synthase, NO: Nitric oxide, PUFA: Polyunsaturated fatty acid, VaD: Vascular dementia.

Correspondence:

Dr. Yan Wang,
Department of Neurology, Chengdu Fifth Peoples' Hospital, Chengdu 611130, China.
E-mail: 17340085006@sina.cn
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INTRODUCTION

Nutritional factors-associated decline in cognitive performance in old age has gained much attention in recent years.[1] Substantial amount of data supports manifestation of the myriad of neurologic and psychiatric abnormalities in vitamin deficiency states. In earlier studies, dietary consumption of multivitamins (e.g., B, C, folic acid, carotene, and essential nutrients) highlighted the therapeutic efficacy of nutritional intervention strategies in restricting the progressive cognitive decline.[2] Vascular dementia (VaD) is a nutritional deficiency-associated[3,4] cerebrovascular disease that primarily affects population over 65 years of age.[5] Chronic cerebral angiopathy (e.g., amyloid-β) provides impetus to neurodegenerative disorders and predisposes the central nervous system to neurotoxins owing to the loss of blood–brain barrier integrity.[6] Cerebral hypoperfusion, oxidative stress, hyperhomocysteinemia (HHCy), and dysfunctions of

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neurovascular unit manifest mild cognitive impairment at early stages and full-blown dementia in advanced stages in patients of VaD.[7,8]

The neurovascular coupling involves a complex association between neuron and blood perfusion whereby neuronal stimulation leads to increase in cerebral blood flow in harmony with cerebral metabolism. It is achieved through multiple mechanisms (e.g., metabolic, myogenic, and autonomic); however, nitric oxide (NO) (endothelial-derived relaxing factor [EDRF]) remains the chief vasodilator molecule *in vivo*.[9] Endothelial damage in VaD disturbs the NO-based vascular smooth muscle relaxation that impairs the blood flow in brain regions notwithstanding the metabolic demands. Chronic cerebral hypoperfusion accentuates redox imbalance, peroxidative changes in biomolecules, and synaptic dysfunction. Several risk factors associated with VaD include thromboembolic events, cardiovascular abnormalities, dyslipidemia, diabetes, smoking, obesity, and higher homocysteine (HCy) levels. At present, the treatment strategies in current regimens are mainly symptomatic or modify the risk factors (e.g., donepezil, memantine, piracetam, citicoline, pentoxifylline, vasodilators, ergot derivatives, and anti-thrombotics) and aimed to avoid onset or delay the progression of the disease.[4] There is a need of an effective drug molecule that not only arrests the progression of disease, but also reverses the pathology of VaD.

Spirulina (*Spirulina platensis*) is a multicellular, blue-green, free-floating, filamentous cyanobacterium (class: Cyanophyta) that flourishes mostly in alkaline water bodies in subtropical and tropical areas including America, Mexico, Asia, and Central Africa. Spirulina is a whole food supplement and highly rated among nutraceuticals and health products.[10] It is the richest source of digestible vegetable protein (60%). It possesses a wide spectrum of nutrients including B-vitamins (B_6 and B_{12}), β-carotene, tocopherols, phenolic acids, essential fatty acids (e.g., γ-linoleic acid), phycocyanin, and minerals (e.g., Fe, Ca, Mg, Mn, Zn, K, and Se).[11] Animal studies have shown that spirulina imparts potent antiviral,[12,13] anticancer,[14,15] DNA repair,[16] antitumor,[17] hepatoprotective,[18] hypocholesterolemic,[19] erythropoietic,[20] immunomodulatory,[21] hypoglycemic,[22,23] and general health-improving effects. Several animal and *in-vitro* studies exhibited the neuroprotective activities of spirulina against Fe-,[24] 6-hydroxydopamine-,[25] and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-[26] triggered neurotoxicity. Dietary spirulina attenuates the microglial activation, brain inflammatory cascade, ischemic brain damage, cerebral infarct size, and neuron apoptosis (e.g., caspase-3 activity) in adult rats.[27] Spirulina possesses potent antioxidant and anti-inflammatory activities.[28] Administration of spirulina during pregnancy results in rise in the birth weight of offspring.[29] The cluster of biological activities and chemical constituents of spirulina may give relief in VaD and also enhance the memory. However, the use of spirulina in cerebrovascular-origin cognitive impairment has not been explored in enough details so far. In the current research, we aim to examine the effects of spirulina in L-methionine-elicited VaD in rats.

MATERIALS AND METHODS

Drugs and chemicals used

Spirulina (*S. platensis*) (Cosmic Nutracos Solutions Pvt Ltd, Solan, Himachal Pradesh, India), L-methionine, thiobarbituric acid, sulphosalicylic acid (Loba Chemie Pvt Ltd, Mumbai, Maharashtra, India), acetylcholine (Ach), 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB), dithiothreitol, phosphotungstic acid, *N*-1-Napthyl ethylene diamine dihydrochloride, sulphanilamide, sodium nitroprusside (SNP) (Himedia Labs, Mumbai), *n*-butanol, Folin–Ciocalteu reagent (Merck, Mumbai, India); acetylthiocholine iodide (AcTh), bovine serum albumin (BSA), and sodium dodecyl sulfate (SRL, Mumbai) were used. All the other chemicals were of analytical grade. Physiological salt solution (PSS) (Krebs–Henseleit solution) for vascular reactivity test was freshly prepared using double-distilled water (NaCl, 119 mM; KCl, 4.7 mM; $NaHCO_3$, 25 mM; $MgSO_4$, 1.0 mM; glucose, 11.1 mM; KH_2PO_4, 1.2 mM; and $CaCl_2$, 2.5 mM). L-Methionine (1.7 g/kg) was prepared in warm distilled water (45°C) by constant stirring.[30] The doses of spirulina (0.5, 1, and 2 g/kg body weight)[31,32] were prepared using 0.5% w/v carboxymethylcellulose (CMC) vehicle suitable for oral administration. Piracetam (NACETAM®) from Neon Laboratories Pvt Ltd, Noida, Uttar Pradesh, India, was used as a standard drug administered orally at dose 500 mg/kg/day.[33]

Animals used and drug administrations

Rats (Wistar) of both sexes (weight range 200–250 g) were purchased from the "Central Animal Facility," All India Institute of Medical Sciences, New Delhi, India, after approvals from Institutional Animal Ethics Committee (IAEC) vide research protocol no. SSP/IAEC/18/17. The animals were maintained at the "Animal House Facility (AHF)" of the establishment maintaining the customary laboratory environment, duly following the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forests and Climate Change, GOI (New Delhi). The animals were provided free admittance to water and standard rodent pellet diet (Ashirwad Industries, Mohali, Punjab, India). All the behavioral tests were performed from 0800 to 1800 h under optimal conditions. The rats were familiarized to laboratory setting for 15 days before the initiation of experiments. The "AHF" attendants were blinded to different treatments. After acclimatization, 54 rats were randomly divided into nine groups ($n = 6$) in a single-blind fashion. Naive rats were not subjected to any treatment; vehicle control group was given 0.5% CMC and distilled water (10 ml/kg, *p.o.*); spirulina (2) group was given spirulina (2 g/kg, *p.o.*); piracetam group was given piracetam (500 mg/kg, *p.o.*); L-Meth group was injected L-methionine (1.7 g/kg, *p. o.*); L-Meth+spirulina (0.5) was given L-methionine and spirulina (0.5 g/kg, *p.o.*); L-Meth+spirulina (1) group was given L-methionine and spirulina (1 g/kg, *p.o.*); L-Meth+spirulina (2) group was treated with L-methionine and spirulina (2 g/kg, *p.o.*); L-Meth+piracetam group received L-methionine and piracetam (500 mg/kg, *p.o.*). All the treatments were performed from the 1st day to 28th day daily [Figure 1].

Ambulatory performance

A digital actophotometer (INCO, Ambala, Haryana, India) was utilized to evaluate the locomotor performance of rats on day 1 and day 22. The rats were habituated in the apparatus for 5 min and then observed for a phase of 10 min. The result was shown as counts/10 min.[34]

Morris water maze test

In this protocol, place-condition prototype was used to evaluate the spatial (long term) memory of animals using Morris water maze (MWM), which uses a fixed underwater platform with randomization of initial location of every rat in every subsequent trial.[35] With each trial, the animal learns to use allocentric and egocentric signs to flee on the hidden platform, hence a relative position of water maze in comparison to supplementary items in the laboratory helping as major distal visual cue was not troubled in the whole experiment. An iron-made round reservoir (diameter 2 m and height 60 cm) was poured up to 30 cm with water (25 ± 1°C) and separated into four identical quadrants utilizing two threads, fastened exactly perpendicular to each other on the perimeter of the round tank. The quadrants were designated clockwise (Q1, Q2, Q3, and Q4). The test was conducted in three phases, namely, habituation, acquisition (days

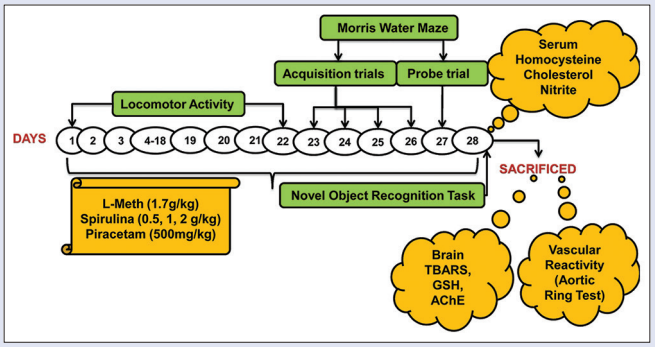

Figure 1: Experimental design

23–26), and retrieval phases. The habituation phase was conducted 24 h before the initiation of training trials. Each rat was allowed 120 s to discover the maze devoid of the platform. In the acquisition period, a metal dais (11 cm^2 arena) was positioned at Q4 of the tank, 2 cm underneath the plane of water. The dais was masked by the addition of a nontoxic colorant in the water. Each rat was tenderly released in front of the wall of the tank with site shifting from Q1 to Q4 (day 23), Q2 to Q1 (day 24), Q3 to Q2 (day 25), and Q4 to Q3 (day 26) for every examination. After tracing of camouflaged platform (120 s), an intertrial break of 30 s was provided on the dais. The animals incapable to trace the dais were guided gently on the platform. Escape latency time (ELT) is the mean time utilized by the animal to find the veiled dais in the water-filled tank. ELT of the 26th day versus ELT of the 23rd day was designated as an indicator of spatial learning. Maintenance of memory was probed 24 h post acquisition assessment. In the retrieval phase, each animal was released into the maze (devoid of platform) at 180° from the original position of the platform and given 120 s for spatial navigation. The average time used by the rats in the four quadrants was observed. The average time taken by the animal in residing the target quadrant (TSTQ) probing for the concealed dais was taken as an indicator of retrieval memory.[34]

Novel object recognition task

In this paradigm, the impulsive probing activities of small laboratory animals is used to figure out recognition or working-type memory, which renders this test non-aversive and non-rewarding.[36,37] A top-open box (80 cm × 40 cm × 60 cm) made of plywood was positioned in a noise-attenuated area, which provided uniform illumination (60 W light-emitting diode-tube). Each rat was permitted to survey the vacant arena of the box for 5 min for 3 consecutive days before the training trial. On day 28, two alike objects were positioned in two randomly selected contrary corners of the apparatus, maintaining a distance of 10 cm from the edges. The 10-cm height wooden objects (cylinder, pyramid, and cube), in triplicate, were painted with different colors (red, gray, and brown) and were of enough weight to make immovable by the animals. The animal was set loose at the center of the box arena for exploration (5 min) of the two identical objects (A1 and A2) that consisted of directing the muzzle at an object (≤2 cm distance) or sensing the object with nose (T_1). After an intertrial interval of 60 min (posttraining trial), recognition memory of rats was tested using one replica of the familiar object (A3) and one new (B) object (T_2). All permutations and positions of the objects were offset to lessen probable prejudice caused by penchant for particular positions or objects. The open box and wooden objects were carefully wiped with 20% ethyl alcohol after every test to limit the olfactory signs. The total exploration time (ET) in the training trial ($T_1 = ET_{A1} + ET_{A2}$) and in retrieval trial ($T_2 = ET_{A3} + ET_B$) was observed. The capability of the animals to differentiate amid the well-known and new objects through retrieval trial depicts the recognition memory, expressed as discrimination index ($DI = ET_B - ET_{A3}/ET_{A3} + ET_B$).[34]

Preparation of test samples for biochemical determinations

After completion of behavioral tasks, the blood samples from the retro-orbital venipuncture were pooled in Eppendorf tubes and subsequently permitted to coagulate for 15 min at room temperature for partitioning of serum. The coagulate was dislocated using a glass stirrer and centrifuged for 15 min at 3000 rpm. Estimation of HCy, nitrite, and cholesterol was done using separated serum. Afterward, the rats were euthanized by cervical dislocation technique; the complete brain was harvested and instantaneously positioned within crushed-ice pursued by soaking in ice-cold isotonic normal saline solution (0.9% w/v sodium chloride) in order to eliminate extraneous blood and tissue residues. Thereafter, the brain tissue was homogenized using a phosphate buffer (50 mM; pH 7.4) at temp 4°C by employing a tissue homogenizer (Remi Motors Ltd., Mumbai) to prepare 10% w/v brain homogenate. Triton X-100 (1% v/v) was added in the buffer to facilitate the extraction of enzymes in the solution. The homogenate was centrifuged at 15,000 rpm (duration 20 min; temp 4°C) in a high-speed cooling centrifuge machine (Remi Instrument Ltd, Mumbai), and the sediment was separated from the supernatant for the evaluation of acetylcholinesterase (AChE) activity and glutathione (GSH) levels.

Estimation of vascular endothelial activity utilizing isolated aortic ring

The descending thoracic aorta was isolated immediately after sacrificing the rats and thoroughly cleaned using a PSS. An aortic ring (3–5 mm width) was positioned (using stainless steel [SS] loops and silk sutures) in the tissue bath chamber previously filled with PSS (37.8°C), supplied with carbogen (95% oxygen and 5% carbon dioxide). One SS loop was coupled to isometric force displacement transducer that was additionally adjoined to a student-physiograph (INCO, Ambala). The tissue was provided passive stretch tension (2.5 g) to achieve optimal responsive length, pursued by calibration for 90 min along with rinsing with PSS after 20-min duration each time. The aortic ring was primed with potassium chloride (80 mM) to ensure its liveliness and get better contractile response. The aortic loop was applied with phenylephrine (3×10^{-6} M) up to the point when steady-state contractility is achieved. Collective dose-dependent activities of acetylcholine (10^{-8}–10^{-4} M) and SNP (10^{-8}–10^{-4} M) were observed with undamaged or abraded endothelium, respectively, at 30-min time intervals. The inner surface of the aortic loop was abraded tenderly, using a dampened filter paper for 30 s to shed-off endothelial cells. The failure of acetylcholine 10^{-6} M-triggered relaxation established the nonexistence of endothelial lining.[38,39]

Evaluation of total serum cholesterol

The total serum cholesterol was determined spectrophotometrically (CyberLab Analytical Instruments, Mumbai, India) at (λ_{max}) 540 nm by cholesterol oxidase/peroxidase-aminophenazone (CHOD/PAP) technique by employing a commercially accessible kit (Nicholas India Pvt Ltd, Ahmedabad, Gujarat, India).[40] Rats with serum total cholesterol levels of >175 mg/dl were considered to have hyperlipidemia.

Estimation of serum homocysteine

The methionine *α, γ*-lyase catalyzes the production of hydrogen sulphide (H_2S) that reacts with chromophore whose absorbance is noted at (λ_{max}) 660 nm. A volume of 1 ml of dithiothreitol (reducing agent) was thoroughly mixed with 9 ml of Tris buffer (solution A). The reducing agent was used to free the bound HCy. A volume of 2 ml of this solution was thoroughly mixed with 1 ml of methionine *α,γ*-lyase and transferred to solution A. A volume of 20 µl of the sample was mixed with solution A (200 µl) and incubated at room temperature for 5 min. Afterward, the assay mixture was mixed with an oxidant and again incubated for 5 min at room temperature. The absorbance was noted at 660 nm wavelength, and the result was expressed as µM HCy.[41]

Estimation of serum nitrite

Briefly, a mixture of serum or standard sample (0.1 ml), carbonate buffer (0.4 ml; pH 9), and copper–cadmium alloy (150 mg) was incubated at room temperature for 1 h and thereafter, NaOH (0.1 ml; 0.35 M) and $ZnSO_4$ solutions (0.4 ml; 120 mM) were mixed. After 10 min, the mixture was centrifuged for 10 min at 4000 rpm. To the supernatant (0.1 ml), Greiss reagent (0.5 ml; an equal ratio of 1% sulphanilamide in 3 M HCl and 0.1% *N*-1-Napthyl ethylene diamine dihydrochloride in water) was mixed, and the assay mixture was incubated at room temperature in the dark for 10 min. The absorbance was calculated at a wavelength (λ_{max}) of 548 nm. The concentration of nitrite ("*n*" µmol/mg of brain protein) was quantified utilizing a customary curve of sodium nitrite (10–100 µM).[42]

Estimation of brain acetylcholinesterase activity

The reaction mixture consisted of the supernatant (0.05 ml), 1.585 M AcTh (0.1 ml), 0.01 M DTNB (0.1 ml), and 0.1 M sodium–potassium phosphate buffer (3 ml, pH 8). An alteration in absorbance (*Δ*A) was calculated for 2 min after every 30 s at a wavelength (λ_{max}) of 412 nm employing a double-beam spectrophotometer (CyberLab Analytical Instruments, India). AChE activity was quantified by using a molar extinction-coefficient of the chromophore $\varepsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$ at λ_{max} = 412 nm and reported as micromole of AcTh hydrolyzed/min/mg brain protein.[43]

Estimation of thiobarbituric acid-reactive substances

Quantification of thiobarbituric acid-reactive substances (TBARS) through a pink-colored malondialdehyde (MDA)- Thiobarbituric acid $(TBA)_2$ adduct estimates the lipid peroxidation substance, MDA. The tubes containing assay mixture (4 ml) of brain homogenate (0.1 ml), sodium dodecyl sulfate (0.2 ml; 8.1%), 20% glacial acetic acid (1.5 ml; pH 3.5), TBA (1.5 ml; 0.8%), and distilled water (0.7 ml) were vigorously mixed, heated in a water bath (1 h; temp 95°C), and cooled beneath tap water. The tubes were dynamically mixed with *n*-butanol and pyridine mixture (15:1 ratio; volume 5 ml) and then centrifuged for 10 min at 4000 rpm. The absorbance of superficial organic layer (2 ml; *n*-butanol phase) was quantified spectrophotometrically at a λ_{max} = 532 nm wavelength. TBARS ("*n*" nanomole per mg brain protein) was quantified using $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.[44]

Quantification of reduced glutathione

The supernatant (1 ml) of the brain homogenate was protein precipitated with 4% sulphosalicylic acid (1 ml) and then cold digested for 1 h at 4°C temperature. The assay mixture was centrifuged (speed 2000 rpm; time 10 min; and temperature 4°C) after 5 min of cold digestion. The resultant supernatant (0.1 ml) was added with sodium–potassium phosphate buffer (0.3 M; 2.7 ml; and pH 8) and DTNB (0.1 mM; 0.2 ml; and pH 8). By using a spectrophotometer, the absorbance was observed at a wavelength of λ_{max} = 412 nm. Glutathione (GSH) (micromole GSH/mg brain protein) was calculated using $\varepsilon = 1.36 \times 10^4\ M^{-1}\ cm^{-1}$ of the chromophore.[45]

Determination of protein in the brain

Briefly, the 0.15-ml supernatant of the brain homogenate was made up to 1 ml and afterward, Lowry's reagent (5 ml) was added. The assay mixture was vortexed thoroughly, reserved at room temperature for 15 min, and then added with 0.5-ml Folin–Ciocalteu reagent. The tube contents were shaken dynamically and incubated at room temperature for 30 min. BSA 0.2–2.4 mg/ml was utilized to plot a customary curve, absorbance was noted spectrophotometerically at λ_{max} = 750 nm, and protein concentration was reported as mg/ml of the supernatant.[46]

Data analysis

The results were analyzed for intergroup variation using one-way ANOVA followed by Tukey's *post hoc* test. A two-way ANOVA was followed by the Bonferroni's *post hoc* test, and GRAPHPAD-PRISM-5 software (GraphPad Software, Inc., California, USA) was utilized. All the results were reported as mean ± standard error of the mean, and $P < 0.05$ was considered statistically significant.

RESULTS

Ambulation of rats was unaltered by drug treatments

Among different groups, no significant variation in the mean ambulatory activity in response to diverse drug treatments was observed [Figure 2].

Spirulina enhanced spatial memory of L-methionine-treated rats

In MWM test, the ELT of different groups showed no significant difference on day 23, however noteworthy intergroup variation surfaced on day 24 during the acquisition trials. Naïve rats and control group rats depicted marked ($P < 0.001$) reduction in day 26 ELT in relation to that of day 23, attributed to trial-based acquisition of spatial learning. Chronic administration of L-methionine appreciably ($P < 0.001$) increased the ELT in contrast to treatment with vehicle only. Administration of spirulina (0.5 and 1 g/kg) in L-Meth-treated rats decreased the ELT ($P < 0.05$) in contrast to rats that received L-methionine alone. Spirulina (2 g/kg) considerably lowered the ELT ($P < 0.01$)

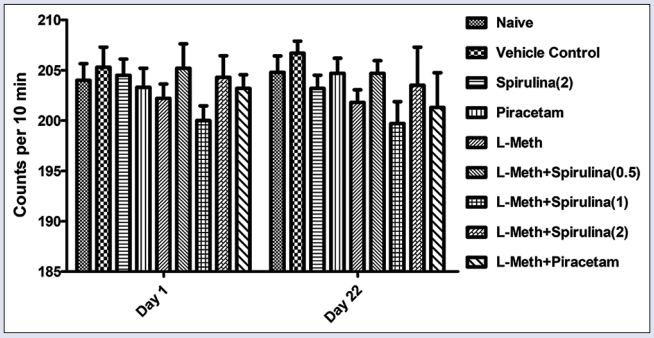

Figure 2: Drug treatments showed no significant alteration in the locomotor activity of rats. Values are expressed as mean ± standard error of the mean ($n = 6$). The data were analyzed using two-way ANOVA followed by Bonferroni's *post hoc* test

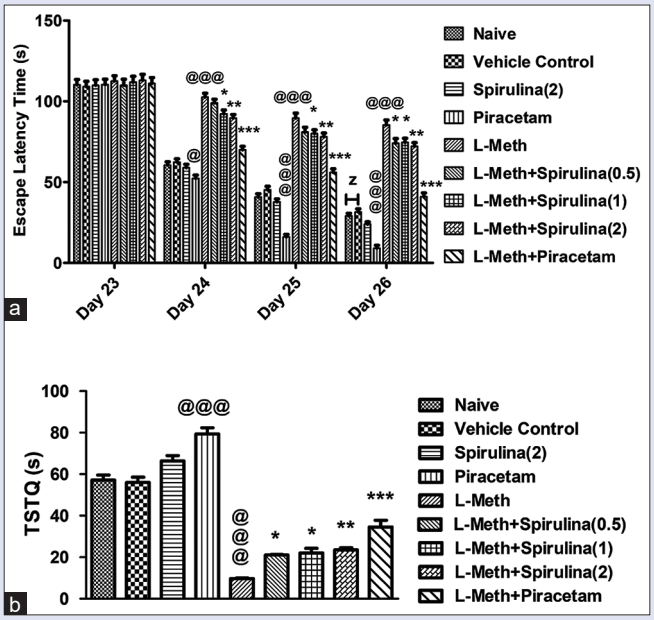

Figure 3: Spirulina increased the spatial memory of L-Meth-treated rats in Morris water maze test. Statistical analysis of (a) escape latency time (s) was achieved using two-way ANOVA followed by Bonferroni's *post hoc* test and (b) time spent in target quadrant (s) was achieved using one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean ± standard error of the mean ($n = 6$). Statistical significance at $^{z}P < 0.001$ day 26 versus day 23 ELT, $^{@}P < 0.05$, $^{@@}P < 0.01$, and $^{@@@}P < 0.001$ versus vehicle control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus L-Meth group

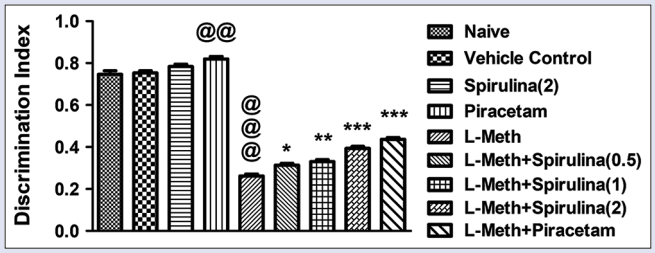

Figure 4: Spirulina improved the working memory of L-Meth-treated rats in novel object recognition task. Statistical analysis of discrimination index was achieved using one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean ± standard error of the mean ($n = 6$). Statistical significance at $^{@@}P < 0.01$, $^{@@@}P < 0.001$ versus vehicle control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus L-Meth group

in L-Meth-treated rats [Figure 3a]. In probe trial, administration of L-methionine considerably ($P < 0.001$) decreased the TSTQ when juxtaposed to rats that received vehicle alone. Spirulina (0.5 and 1 g/kg) abrogated ($P < 0.05$) the L-Meth-induced decline in the TSTQ with respect to treatment with L-methionine alone. Spirulina (2 mg/kg) markedly increased the TSTQ ($P < 0.01$) of L-Meth-treated rats with respect to rats that were subjected to L-methionine alone [Figure 3b]. Treatment with piracetam (nootropics) markedly ($P < 0.001$) declined the ELT and elevated the TSTQ of rats in comparison to controls.

Spirulina ameliorated the working memory of L-methionine-treated rats

Long-term administration of L-methionine statistically significantly ($P < 0.001$) lowered the DI in comparison to vehicle-alone treatment. Spirulina (0.5 g/kg) increased ($P < 0.05$) the DI in L-Meth-treated rats in comparison to rats that received L-methionine alone. L-Meth+spirulina (1) group showed considerable increase ($P < 0.01$) in DI when juxtaposed to L-methionine group. L-methionine+spirulina (2) group depicted a marked elevation ($P < 0.001$) in DI with respect to L-methionine group. Piracetam-treated groups depicted noteworthy ($P < 0.001$) rise in DI when compared to groups subjected to vehicle or L-Meth-alone treatments [Figure 4].

Spirulina ameliorated endothelium-dependent relaxation in L-methionine-treated rats

Acetylcholine and SNP produced dose-dependent endothelium-dependent and sovereign relaxation in phenylephrine precontracted aortic loops. L-methionine group expressed conspicuous ($P < 0.001$) reduction in acetylcholine-triggered endothelium-dependent relaxation. However, long-term administration of spirulina (1 and 2 mg/kg) to L-Meth-treated rats statistically significantly ($P < 0.05$) enhanced ACh-triggered endothelial-dependent relaxation when juxtaposed to the rats that were subjected to L-methionine alone. Standard nootropics (piracetam) appreciably ($P < 0.05$) attenuated the inhibitory consequence of L-methionine on ACh-triggered endothelial-dependent relaxation [Figure 5a]. L-methionine administration had no statistically significant ($P > 0.05$) effect on SNP-induced endothelium-independent relaxation [Figure 5b].

Spirulina lowered homocysteine levels in the serum of rats

Oral administration of spirulina (2 g/kg) alone reduced ($P < 0.05$) the serum HCy levels with respect to treatment with vehicle only. L-methionine amplified ($P < 0.001$) the serum HCy levels when compared with vehicle-alone treatment. Spirulina (0.5 and 1 g/kg) attenuated ($P < 0.05$) the L-meth-induced ascend in the serum HCy content with respect to rats that received L-methionine alone. L-methionine+spirulina (2) group showed marked ($P < 0.01$) reduction in HCy content in comparison to L-methionine group. Treatment with piracetam conspicuously declined the serum HCy content with respect to separate administrations of vehicle or L-methionine alone [Figure 6a].

Spirulina elevated serum nitrite content in L-methionine-treated rats

L-methionine diminished ($P < 0.001$) the serum nitrite contents when compared to treatment with vehicle alone. Treatment of L-Meth-administered rats with spirulina (1 mg/kg) augmented ($P < 0.05$) the serum nitrite content in comparison to rats that received L-methionine alone. Spirulina (2 g/kg) markedly ($P < 0.01$) attenuated the L-Meth-ensued lowering of serum nitrite levels. L-Meth+piracetam group exhibited marked ($P < 0.01$) rise in the serum nitrite content in comparison to L-methionine group [Figure 6b]. However, oral administration of spirulina (2 g/kg) or standard drug (piracetam) *per se* in rats moderately ($P > 0.05$) enhanced the serum nitrite levels in comparison to rats that received vehicle-only treatment.

Spirulina declined serum total cholesterol content in L-methionine-treated rats

L-methionine group exhibited increase ($P < 0.001$) in the serum cholesterol levels when juxtaposed with the vehicle control group. Spirulina (0.5 g/kg) attenuated ($P < 0.05$) the L-Meth-triggered increase in total cholesterol content with respect to L-Meth-alone treatment.

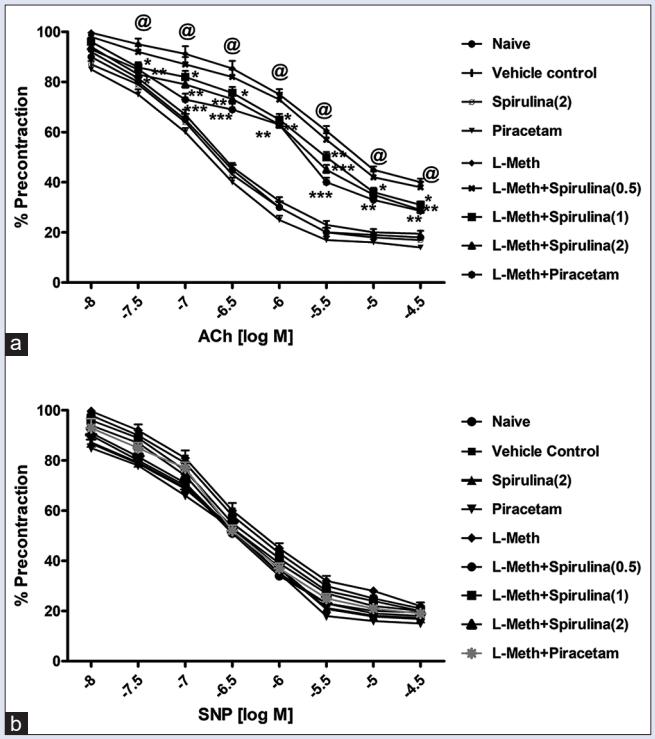

Figure 5: Spirulina improved the relaxation of aortic ring in L-methionine-treated rats. Statistical analysis of (a) endothelium-dependent and (b) endothelium-independent relaxation was achieved using two-way ANOVA followed by Newman–Keul's *post hoc* test. Values are expressed as mean ± standard error of the mean ($n = 6$). Statistical significance at $^{@}P < 0.001$ versus vehicle control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus L-Meth group

L-Meth+spirulina (1) group exhibited a substantial decrease ($P < 0.01$) in total serum cholesterol levels with respect to L-Meth-alone group. L-Meth+spirulina (2) group showed marked ($P < 0.001$) reduction in total cholesterol in comparison to L-Meth-alone group. Administration of piracetam conspicuously declined the serum cholesterol content with respect to treatment with vehicle or L-methionine [Figure 6c].

Spirulina attenuated L-methionine-triggered elevation in the brain acetylcholinesterase activity

L-methionine markedly increased ($P < 0.001$) the brain AChE activity with respect to vehicle-only treatment in rats. Spirulina (1 g/kg) statistically significantly attenuated ($P < 0.05$) the L-Meth-triggered increase in AChE activity when compared with the administration of L-methionine alone. Spirulina (2 g/kg) substantially declined ($P < 0.01$) the AChE activity in the brain of L-Meth-treated rats. Piracetam-treated groups showed marked decline in the brain AChE activity when compared to vehicle- or L-methionine alone-treated groups [Figure 6d].

Spirulina enhanced GSH level in the brain of L-methionine-treated rats

Spirulina (2 g/kg) treatment caused an increase ($P < 0.05$) in the brain GSH levels when compared with administration of the vehicle. L-methionine declined ($P < 0.001$) the brain GSH levels when compared with vehicle-only treatment. Treatment of L-Meth-administered rats with spirulina (0.5 g/kg) augmented ($P < 0.05$) the GSH content when compared to rats that received L-methionine alone. L-Meth+spirulina (1) group exhibited marked elevation ($P < 0.01$) in brain GSH levels with respect to L-Meth-alone group. L-Meth+spirulina (2) group showed a conspicuous elevation ($P < 0.001$) of brain GSH content. Chronic administration of piracetam markedly amplified the GSH content in rats when compared with rats that received L-methionine or vehicle alone [Figure 6e].

Spirulina mitigated L-methionine-induced rise in the brain thiobarbituric acid-reactive substances content

Oral administration of spirulina (2 g/kg) *per se* reduced ($P < 0.05$) the brain TBARS contents in comparison to treatment with vehicle alone. L-methionine magnified ($P < 0.001$) the brain TBARS contents when compared with vehicle-only treatment. Treatment of L-Meth-administered rats with spirulina (0.5 and 1 g/kg) reduced ($P < 0.05$) the TBARS content when compared to rats that received L-methionine alone. L-Meth+spirulina (2) group displayed marked ($P < 0.01$) decrease in TBARS levels with respect to L-methionine group. Treatment with piracetam markedly ($P < 0.001$) declined the TBARS content in comparison to separate administrations of vehicle or L-methionine alone [Figure 6f].

DISCUSSION

At the present rate, the worldwide burden of dementia will progress up to 75 million total cases by 2030. Although Alzheimer's disease (AD) has been the foremost basis of dementia, VaD has levied considerable socioeconomic implications globally over the past few decades being the second frequent sort of dementia subsequent to AD. This is also substantiated by the fact that ~50% cases of AD consist of VaD pathology.[5] Even though VaD is largely sporadic, genetic basis of vascular cognitive impairment has also been recognized (e.g., CADASIL) further aided by international diagnostic guidelines.[47] Nonetheless, nutritional deficiency-related cerebrovascular abnormalities, oxidative stress in the brain, and HHCy are chief contributors to the etiopathogenesis of VaD.[48] In several earlier studies, L-Meth-induced endothelial dysfunction, HHCy, dyslipidemia, and nutritional and redox impairments are related with vascular cognitive impairments.[38] Spirulina is a nutrient-rich microalgae that own biological activities, highlighting its potential in the therapeutics of brain disorders.[27] In the present protocol, we aimed to investigate the therapeutic potential of spirulina (*S. platensis*) in the management of VaD using L-methionine-induced VaD model in rats.

Serum analysis showed that chronic administration of L-methionine markedly enhanced the blood HCy and total cholesterol content in rats. Furthermore, L-methionine appreciably attenuated Ach-induced endothelium-dependent relaxation, further substantiated by a decrease in serum nitrite levels. These results depicted L-Meth-triggered damage to the endothelial cells and NO physiology thereof. In earlier studies, high total cholesterol during the later stages of life has been associated with cognitive deficits of AD type and vascular origin.[49] Dietary ingestion of L-methionine conspicuously elevates the blood and hepatic output of cholesterol in rodents.[50] Increase in cognitive prowess by statins in experimental models of dementia (e.g., streptozotocin, ischemic brain injury, and L-Meth) through Rho-ROCK and Akt-endothelial NO synthase (eNOS) pathways supports the association of cholesterol and NO in dementia.[51] Mild-to-moderate plasma HHCy is identified as another independent major risk factor for VaD. HCy is a thiol-containing excitatory amino acid by-product of folate and methionine metabolism. Nutritional deficiency or gene-associated HHCy significantly predisposes brain to free radicals, N-methyl-D-aspartate (NMDA)-excitotoxicity,

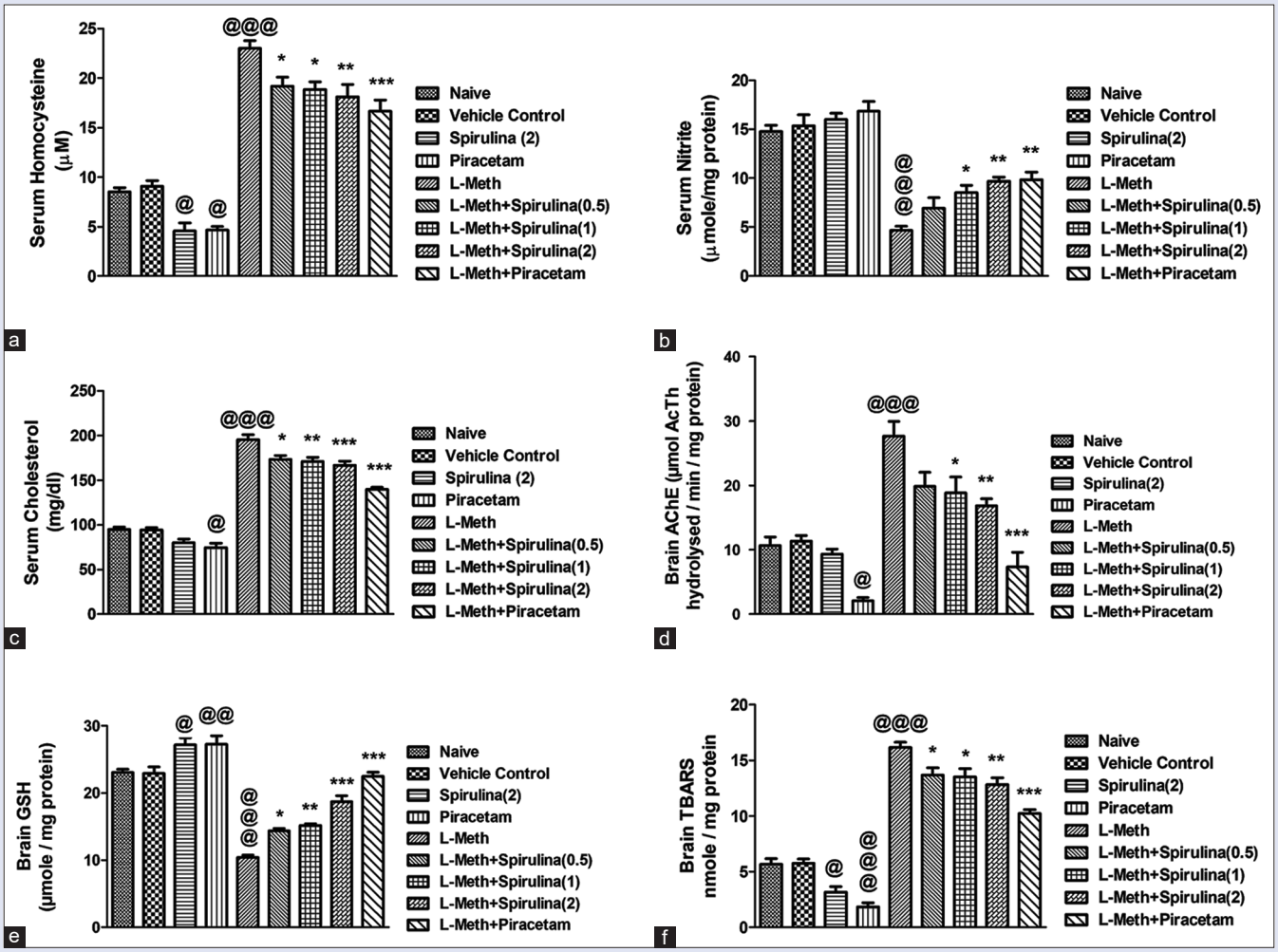

Figure 6: Spirulina attenuated L-Meth-triggered derangement of biochemical parameters. Statistical analysis of (a) serum homocysteine level, (b) serum nitrite content, (c) serum cholesterol levels, (d) brain acetylcholinesterase activity, (e) brain glutathione content, and (f) thiobarbituric acid-reactive substances level was accomplished using one-way ANOVA followed by Tukey's *post hoc* test. Values are expressed as mean ± standard error of the mean ($n = 6$). Statistical significance at $^{@}P < 0.05$, $^{@@}P < 0.01$, and $^{@@@}P < 0.001$ versus vehicle control group, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus L-Meth group

and cholinergic[52] and EDRF (NO) impotency.[53] In a number of studies, neurorestoration and cognitive improvement through reduction in HCy[54,55] has been linked with eNOS expression.[56] Growing evidence suggests that supplementation of vitamins (B_1, B_6, and folic acid) mitigates HHCy and associated metabolic or brain abnormalities (e.g., VaD).[57]

In the present study, chronic administration of spirulina abolished the L-Meth-induced rise in blood HCy and total cholesterol. Interestingly, spirulina *per se* also declined the blood HCy content in rats. A significant increase in ACh-induced endothelium-dependent relaxation by spirulina in aortic loops of rats that were previously pretreated with L-methionine exhibited restoration of endothelial functions. Spirulina also arrested the decline of nitrite content in L-Meth-treated rats. Many studies have outlined the vital roles of NO in the neurobiology of learning and memory. Glutamatergic activation of NMDARs in neurons gives rise to NO that operates as a retrograde messenger in the facilitation of synaptic plasticity and long-term potentiation (LTP) *via* cyclic guanosine monophosphate (cGMP). In experimental models of dementia, NO donors (e.g., SNP and molsidomine) bestowed cognitive improvement,[58] whereas contrasting results were observed in response to treatment with NO antagonists (e.g., L-NAME, L-NMMA, and L-NIO) as well as NMDAR blockers (e.g., MK-801 and AP5).[59] In comparison to neuronal NO synthase,[60] eNOS-dependent NO biosynthesis in vessels has been ascribed pivotal for LTP.[61] Gene knockout studies revealed significant memory loss in eNOS-deficient ($eNOS^{-/-}$) mice.[62] Piracetam is a nootropic drug used in the therapy of cognitive impairment in VaD. The present findings revealed piracetam-induced significant reduction in blood HCy and total cholesterol in L-Meth-treated rats and in piracetam *per se*-treated rats. The decline of nitrite content in response to L-methionine was effectively negated by piracetam in rats.

Vascular abnormalities (e.g., HCy, cholesterol, and NO) critically accentuate the redox imbalance.[30] MDA, 5-HNE, isoprostanes, and acrolein are some of the secondary products of free radical-induced lipid peroxidation, with MDA being the most mutagenic product that forms neurotoxic adducts with the acetaldehyde, advanced glycation end products (AGEs), and DNA-crosslinks. Furthermore, aging accelerates the formation of these aldehydes that further aggravate oxidative damage.[63] Measurement of TBARS correlates with MDA levels is a well-recognized biomarker of oxidative burden. In this

study, chronic administration of L-methionine enhanced the TBARS and abridged the GSH content in the brain of animals. GSH remains the most valuable endogenous antioxidant in the brain to combat oxidative stress and lipid peroxidation. It is a tripeptide required for the regulation of the redox homeostasis in neurons, hence deficits in GSH weaken the resist of brain toward oxidants.[64] The findings of the current study supported spirulina-induced increase in GSH level in the brain of L-Meth-treated rats. Furthermore, in harmony with earlier studies,[10] elevations of brain GSH content and decline in lipid peroxidative changes in the brain of spirulina *per se*-administered rats indicated the antioxidant property of spirulina. The standard drug (piracetam) used in this protocol reduced the TBARS content and magnified the GSH levels in the brain of rats.

Cholinergic agonists have shown promising outcomes in patients of VaD, although symptomatically only. Neurodegenerative changes in the basal prosencephalon (including nucleus basalis of Meynert) and cholinergic projections are the characteristic hallmarks in VaD pathology.[65] In hypoxia-based animal models, decrease in muscarinic receptors, cholineacetyltransferase activity, and Ach level in the brain is commensurate with vascular cognitive impairment. Furthermore, in previous studies, cholinergic hypofunction is associated with the deregulation of endothelial function, eNOS activity, and cerebral blood flow.[66] In the present study, L-methionine markedly augmented the AChE activity in the brain of rats, which was efficiently offset by long-term treatment with spirulina. Piracetam also attenuated the rise in AChE activity in the brain rats.

The findings of behavioral studies were aptly supported by the biochemical analysis measurements. The diverse groups depicted no significant disparity in mean ambulatory activity that excluded the interference of ambulation of rats on the outcome behavioral determinations. In this protocol, MWM test was used to assess the spatial long-term memory andnovel object recognition task (NORT) to evaluate the working memory of rats. Chronic administration of L-methionine simulated the vascular cognitive impairment in rats, which was shown by increase in ELT during acquisition trials, decrease in TSTQ during probe trial in MWM, and decline of DI in probe trials of NORT. Treatment of L-Meth-administered rats with spirulina exhibited marked improvement in locating the hidden platform during acquisition trials (ELT) and enhanced the TSTQ. Spirulina augmented the performance of rats to discriminate between novel object and familiar object (DI) in NORT. However, administration of spirulina manifested only moderate ($P > 0.05$) increase in memory functions in normal rats. Piracetam also elevated the cognitive abilities of L-Meth-treated and normal rats as well. The present findings corroborated that spirulina effectively attenuated the L-Meth-triggered cognitive impairment in rats [Figure 7].

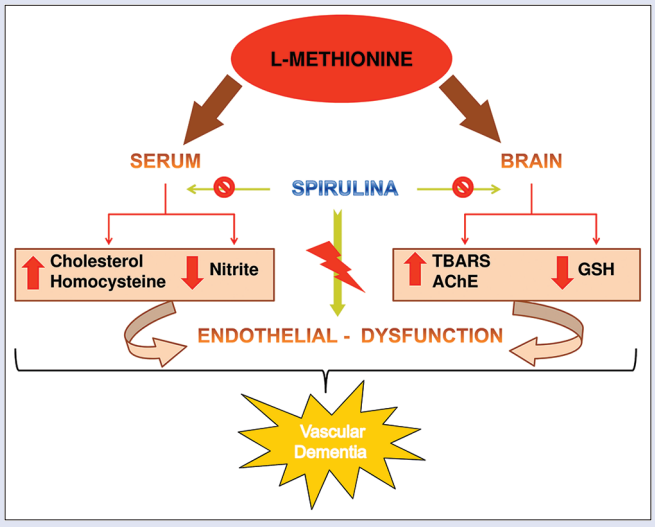

Figure 7: Putative mechanisms of the therapeutic effects of spirulina in vascular dementia

CONCLUSION

In the current research, oral administration of L-methionine deteriorated the memory functions in rats. Endothelial debility, HHCy, oxidative stress, and cholinergic impairments were noted in L-methionine-treated rats. Treatment with spirulina restored the cognitive abilities in L-Meth-treated rats. It is very pertinent to state that spirulina is the most accomplished source of nutrients that gives promising therapeutic effects to manage cognitive abnormalities in VaD.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Otsuka M, Yamaguchi K, Ueki A. Similarities and differences between Alzheimer's disease and vascular dementia from the viewpoint of nutrition. Ann N Y Acad Sci 2002;977:155-61.
2. Selhub J, Bagley LC, Miller J, Rosenberg IH. B vitamins, homocysteine, and neurocognitive function in the elderly. Am J Clin Nutr 2000;71:614S-20S.
3. Troen AM, Shea-Budgell M, Shukitt-Hale B, Smith DE, Selhub J, Rosenberg IH. B-vitamin deficiency causes hyperhomocysteinemia and vascular cognitive impairment in mice. Proc Natl Acad Sci U S A 2008;105:12474-9.
4. Perez L, Heim L, Sherzai A, Jaceldo-Siegl K, Sherzai A. Nutrition and vascular dementia. J Nutr Health Aging 2012;16:319-24.
5. Nyenhuis DL, Gorelick PB. Diagnosis and management of vascular cognitive impairment. Curr Atheroscler Rep 2007;9:326-32.
6. Zlokovic BV. Neurovascular pathways to neurodegeneration in Alzheimer's disease and other disorders. Nat Rev Neurosci 2011;12:723-38.
7. Iadecola C. The pathobiology of vascular dementia. Neuron 2013;80:844-66.
8. Series H, Esiri M. Vascular dementia: A pragmatic review. Adv Psychiatr Treat 2012;18:372-80.
9. Phillips AA, Chan FH, Zheng MM, Krassioukov AV, Ainslie PN. Neurovascular coupling in humans: Physiology, methodological advances and clinical implications. J Cereb Blood Flow Metab 2016;36:647-64.
10. Khan Z, Bhadouria P, Bisen PS. Nutritional and therapeutic potential of spirulina. Curr Pharm Biotechnol 2005;6:373-9.
11. Wu Q, Liu L, Miron A, Klímová B, Wan D, Kuča K. The antioxidant, immunomodulatory, and anti-inflammatory activities of spirulina: An overview. Arch Toxicol 2016;90:1817-40.
12. Mader J, Gallo A, Schommartz T, Handke W, Nagel CH, Günther P, *et al.* Calcium spirulan derived from *Spirulina platensis* inhibits herpes simplex virus 1 attachment to human keratinocytes and protects against herpes labialis. J Allergy Clin Immunol 2016;137:197-203.
13. Chen YH, Chang GK, Kuo SM, Huang SY, Hu IC, Lo YL, *et al.* Well-tolerated spirulina extract inhibits influenza virus replication and reduces virus-induced mortality. Sci Rep 2016;6:24253.
14. Grawish ME. Effects of *Spirulina platensis* extract on Syrian hamster cheek pouch mucosa painted with 7,12-dimethylbenz[a]anthracene. Oral Oncol 2008;44:956-62.
15. Li B, Gao MH, Zhang XC, Chu XM. Molecular immune mechanism of C-phycocyanin from *Spirulina platensis* induces apoptosis in HeLa cells *in vitro*. Biotechnol Appl Biochem 2006;43:155-64.

16. Ibrahem MD, Ibrahim MA. The potential effects of *Spirulina platensis* (*Arthrospira platensis*) on tissue protection of Nile tilapia (*Oreochromis niloticus*) through estimation of P53 level. J Adv Res 2014;5:133-6.
17. Okuyama H, Tominaga A, Fukuoka S, Taguchi T, Kusumoto Y, Ono S. Spirulina lipopolysaccharides inhibit tumor growth in a toll-like receptor 4-dependent manner by altering the cytokine milieu from interleukin-17/interleukin-23 to interferon-γ. Oncol Rep 2017;37:684-94.
18. Ferreira-Hermosillo A, Torres-Duran PV, Juarez-Oropeza MA. Hepatoprotective effects of *Spirulina maxima* in patients with non-alcoholic fatty liver disease: A case series. J Med Case Rep 2010;4:103.
19. Nagaoka S, Shimizu K, Kaneko H, Shibayama F, Morikawa K, Kanamaru Y, *et al*. A novel protein C-phycocyanin plays a crucial role in the hypocholesterolemic action of *Spirulina platensis* concentrate in rats. J Nutr 2005;135:2425-30.
20. Nasirian F, Mesbahzadeh B, Maleki SA, Mogharnasi M, Kor NM. The effects of oral supplementation of *Spirulina platensis* microalgae on hematological parameters in streptozotocin-induced diabetic rats. Am J Transl Res 2017;9:5238-44.
21. Finamore A, Palmery M, Bensehaila S, Peluso I. Antioxidant, Immunomodulating, and microbial-modulating activities of the sustainable and ecofriendly spirulina. Oxid Med Cell Longev 2017;2017. doi: 10.1155/2017/3247528.
22. Nasirian F, Dadkhah M, Moradi-Kor N, Obeidavi Z. Effects of *Spirulina platensis* microalgae on antioxidant and anti-inflammatory factors in diabetic rats. Diabetes Metab Syndr Obes 2018;11:375-80.
23. Gargouri M, Hamed H, Akrouti A, Dauvergne X, Magné C, El Feki A. Effects of *Spirulina platensis* on lipid peroxidation, antioxidant defenses, and tissue damage in kidney of alloxan-induced diabetic rats. Appl Physiol Nutr Metab 2018;43:345-54.
24. Bermejo-Bescós P, Piñero-Estrada E, Villar del Fresno AM. Neuroprotection by *Spirulina platensis* protean extract and phycocyanin against iron-induced toxicity in SH-SY5Y neuroblastoma cells. Toxicol *In vitro* 2008;22:1496-502.
25. Strömberg I, Gemma C, Vila J, Bickford PC. Blueberry- and spirulina-enriched diets enhance striatal dopamine recovery and induce a rapid, transient microglia activation after injury of the rat nigrostriatal dopamine system. Exp Neurol 2005;196:298-307.
26. Chamorro G, Pérez-Albiter M, Serrano-García N, Mares-Sámano JJ, Rojas P. *Spirulina maxima* pretreatment partially protects against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine neurotoxicity. Nutr Neurosci 2006;9:207-12.
27. Wang Y, Chang CF, Chou J, Chen HL, Deng X, Harvey BK, *et al*. Dietary supplementation with blueberries, spinach, or spirulina reduces ischemic brain damage. Exp Neurol 2005;193:75-84.
28. Deng R, Chow TJ. Hypolipidemic, antioxidant, and antiinflammatory activities of microalgae spirulina. Cardiovasc Ther 2010;28:e33-45.
29. Kapoor R, Mehta U. Effect of supplementation of blue green alga (spirulina) on outcome of pregnancy in rats. Plant Foods Hum Nutr 1993;43:29-35.
30. Koladiya RU, Jaggi AS, Singh N, Sharma BK. Ameliorative role of atorvastatin and pitavastatin in L-methionine induced vascular dementia in rats. BMC Pharmacol 2008;8:14.
31. El-Tantawy WH. Antioxidant effects of spirulina supplement against lead acetate-induced hepatic injury in rats. J Tradit Complement Med 2016;6:327-31.
32. Soni RA, Sudhakar K, Rana RS. Spirulina-from growth to nutritional product: A review. Trends Food Sci Technol 2017;69:157-71.
33. Vasudevan M, Parle M. Memory-enhancing activity of *Thespesia populnea* in rats. Pharm Biol 2007;45:267-73.
34. Kumar M, Bansal N. Fasudil hydrochloride ameliorates memory deficits in rat model of streptozotocin-induced Alzheimer's disease: Involvement of PI3-kinase, eNOS and NFκB. Behav Brain Res 2018;351:4-16.
35. Morris R. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47-60.
36. Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data. Behav Brain Res 1988;31:47-59.
37. Abada YS, Nguyen HP, Schreiber R, Ellenbroek B. Assessment of motor function, sensory motor gating and recognition memory in a novel BACHD transgenic rat model for Huntington disease. PLoS One 2013;8:e68584.
38. Mangat GS, Jaggi AS, Singh N. Ameliorative effect of a selective endothelin ETA receptor antagonist in rat model of L-methionine-induced vascular dementia. Korean J Physiol Pharmacol 2014;18:201-9.
39. Shah DI, Singh M. Inhibition of protein tyrosin phosphatase improves vascular endothelial dysfunction. Vascul Pharmacol 2006;44:177-82.
40. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. Clin Chem 1974;20:470-5.
41. Zhao LP, You T, Chan SP, Chen JC, Xu WT. Adropin is associated with hyperhomocysteine and coronary atherosclerosis. Exp Ther Med 2016;11:1065-70.
42. Sastry KV, Moudgal RP, Mohan J, Tyagi JS, Rao GS. Spectrophotometric determination of serum nitrite and nitrate by copper-cadmium alloy. Anal Biochem 2002;306:79-82.
43. Ellman GL, Courtney KD, Andres V Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
44. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351-8.
45. Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82:70-7.
46. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265-75.
47. Korczyn AD. What is new in vascular dementia? BMC Med 2016;14:175.
48. Jellinger KA. Pathology and pathophysiology of vascular cognitive impairment. A critical update. Panminerva Med 2004;46:217-26.
49. Solomon A, Kivipelto M, Wolozin B, Zhou J, Whitmer RA. Midlife serum cholesterol and increased risk of Alzheimer's and vascular dementia three decades later. Dement Geriatr Cogn Disord 2009;28:75-80.
50. Hirche F, Schröder A, Knoth B, Stangl GI, Eder K. Effect of dietary methionine on plasma and liver cholesterol concentrations in rats and expression of hepatic genes involved in cholesterol metabolism. Br J Nutr 2006;95:879-88.
51. Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, *et al*. The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals. Nat Med 2000;6:1004-10.
52. Stefanello FM, Monteiro SC, Matté C, Scherer EB, Netto CA, Wyse AT. Hypermethioninemia increases cerebral acetylcholinesterase activity and impairs memory in rats. Neurochem Res 2007;32:1868-74.
53. Hainsworth AH, Yeo NE, Weekman EM, Wilcock DM. Homocysteine, hyperhomocysteinemia and vascular contributions to cognitive impairment and dementia (VCID). Biochim Biophys Acta 2016;1862:1008-17.
54. Streck EL, Bavaresco CS, Netto CA, Wyse AT. Chronic hyperhomocysteinemia provokes a memory deficit in rats in the Morris water maze task. Behav Brain Res 2004;153:377-81.
55. Baydas G, Ozer M, Yasar A, Tuzcu M, Koz ST. Melatonin improves learning and memory performances impaired by hyperhomocysteinemia in rats. Brain Res 2005;1046:187-94.
56. Meye C, Schumann J, Wagner A, Gross P. Effects of homocysteine on the levels of caveolin-1 and eNOS in caveolae of human coronary artery endothelial cells. Atherosclerosis 2007;190:256-63.
57. Kamat PK, Vacek JC, Kalani A, Tyagi N. Homocysteine induced cerebrovascular dysfunction: A link to Alzheimer's disease etiology. Open Neurol J 2015;9:9-14.
58. Gulati P, Singh N, Muthuraman A. Pharmacologic evidence for role of endothelial nitric oxide synthase in neuroprotective mechanism of ischemic postconditioning in mice. J Surg Res 2014;188:349-60.
59. Rickard NS, Gibbs ME, Ng KT. Inhibition of the endothelial isoform of nitric oxide synthase impairs long-term memory formation in the chick. Learn Mem 1999;6:458-66.
60. Kantor DB, Lanzrein M, Stary SJ, Sandoval GM, Smith WB, Sullivan BM, *et al*. A role for endothelial NO synthase in LTP revealed by adenovirus-mediated inhibition and rescue. Science 1996;274:1744-8.
61. Huang EP. Synaptic plasticity: A role for nitric oxide in LTP. Curr Biol 1997;7:R141-3.
62. Austin SA, Santhanam AV, Hinton DJ, Choi DS, Katusic ZS. Endothelial nitric oxide deficiency promotes Alzheimer's disease pathology. J Neurochem 2013;127:691-700.
63. Ayala A, Muñoz MF, Argüelles S. Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev 2014;2014:360438.
64. Dringen R, Hirrlinger J. Glutathione pathways in the brain. Biol Chem 2003;384:505-16.
65. Engelhardt E, Moreira DM, Laks J. Vascular dementia and the cholinergic pathways. Dement Neuropsychol 2007;1:2-9.
66. Wang J, Zhang HY, Tang XC. Cholinergic deficiency involved in vascular dementia: Possible mechanism and strategy of treatment. Acta Pharmacol Sin 2009;30:879-88.