Computers in Biology and Medicine 108 (2019) 305–316

Contents lists available at ScienceDirect

Computers in Biology and Medicine

journal homepage: www.elsevier.com/locate/compbiomed

Glycogen synthase kinase-3 inhibition as a potential pharmacological target for vascular dementia: In silico and in vivo evidence

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ARTICLE INFO

Keywords:
Vascular dementia
Learning and memory
Glycogen synthase kinase
Network pharmacology
Lithium carbonate

ABSTRACT

Vascular dementia is a serious problem as it creates significant disability and dependency in the affected person. Lives of these patients can be improved through the advent of novel drug targets which can be targeted by pharmacological therapies. However, finding a precise and druggable target for vascular dementia is experimentally impossible and challenging task owing to a complex and mostly unknown interplay between the cognitive abilities of the brain with a diversity of vascular diseases. To address this issue, we have systematically analyzed the literature reports by using well-known methods and approaches of bioinformatics (viz. network pharmacology, reverse pharmacology, enrichment analysis of KEGG pathways, biological processes of Gene Ontology and DIAMOnD algorithm). Because glycogen synthase kinase-3 (GSK-3) seems to be one of the most promising targets, therefore, we have tested the capacity of lithium carbonate, a classical inhibitor of GSK-3, for treatment of dementia resulting from mild chronic cerebral hypoperfusion in mice. To this end, our study shows in-vivo validation of predicted target, i.e., pharmacological deactivation of GSK-3 enzyme and its impact on cognitive abilities employing a behavioral test battery, i.e., object recognition task, step-through passive avoidance task, elevated plus maze task and water maze task. In this framework, we observed that lithium carbonate attenuates recognition, emotion, spatial and fear-motivated learning and memory impairments along with attenuation of oxidative stress, cholinergic dysfunction and glutamate-induced excitotoxicity in cerebral cortex and hippocampus. In conclusion, we propose GSK-3 as a promising drug target for vascular dementia in light of experimental results and in-silico predictions.

1. Introduction

Vascular dementia is a heterogeneous syndrome in which underlying cause is a vascular disease in some form, and its ultimate manifestation is a cognitive decline in one or more domains, e.g., learning and memory, language, executive function, and attention. These impairments not only impact individuals who have dementia but also interfere with their families, communities, and societies. Patients with vascular dementia are at significantly high risk of ischemic stroke [1] but no specific medications are available to these patients. For the development of effective medicines discovery of novel, superior and druggable target is warranted. However, the finding of such target is a challenging task because pathophysiology of vascular dementia is mostly unknown and a large volume of clinical and experimental data is being accumulated every year in this research area (approx. 700 PubMed counts for VaD). However, collection, analysis and making sense out of this data became a hurdle.

Past few years have witnessed the successful use of big data in drug discovery. Therefore, we believe that finding diseased target through the lens of bioinformatics can be fruitful for further wet lab validations. To this end, we have collected and analyzed the literature reports by using well-known methods and approaches of bioinformatics (viz. network pharmacology, reverse pharmacology, enrichment analysis of KEGG pathways, biological processes of Gene Ontology & DIAMOnD algorithm). A list of druggable targets related to the treatment of vascular dementia has been predicted based on known gene-vascular dementia associations and known drug targets.

In this list, glycogen synthase kinase-3β (GSK-3β) seems to be a most promising target because it has been implicated in many processes which are relevant to vascular dementia such as thrombosis [2],

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https://doi.org/10.1016/j.compbiomed.2019.03.002
Received 2 December 2018; Received in revised form 13 February 2019; Accepted 2 March 2019

atherosclerosis [3], diabetes mellitus [4], disruption of blood-brain barrier [5], oxidative stress [6], neuroinflammation [7], neuronal death [8], synaptic plasticity [9], mood [10], learning [11] and memory [12].

Apart from this selection, GSK-3α implicated in many neuronal functions [13] and its inhibition reduces the formation of amyloid plaques and neurofibrillary tangles in Alzheimer's disease [14]. Thus, both isoform of the glycogen synthase kinase-3 seems to be relevant with vascular dementia.

Pharmacological inhibition of these enzymes is possible with the use of a drug having well-established clinical safety, i.e., lithium carbonate [15]. Human observational studies show that treatment with lithium may reduce the risk of dementia among patients with bipolar disorder [16] and may facilitate recovery of spatial learning and memory after transient global ischemia [17]. However, the effect of lithium on recognition, emotion, spatial and fear-motivated learning, and memory remain unexplored under mild chronic cerebral hypoperfusion. Mild chronic cerebral hypoperfusion is a well-recognized cause of cerebral ischemia and vascular dementia [18]. It can be induced in mice by occlusion of the right common carotid artery and mimics core feature of vascular dementia, e.g., cerebral hypoxia, neuroinflammation, white matter lesion and cognitive impairment [19].

We hypothesized that lithium carbonate could treat vascular dementia through deactivation of GSK-3 enzyme involving enlisted pathways (Fig. 11) predicted through the lens of bioinformatics. To this end, present work aimed at in-vivo testing of the selected target. i.e., inhibition of GSK-3 pathway and shows capacity of lithium carbonate, a classical inhibitor of GSK-3, in treatment of vascular dementia induced by occlusion of right common carotid artery in Swiss albino mice employing a behavioral test battery i.e. object recognition test, step-through passive avoidance test, elevated plus maze test and Morris water maze test.

2. Material and methods

2.1. Animals

Institutional Animal Ethics Committee has approved this experimental protocol (approval number 107/GO/ReBi/S/99/CPCSEA/2017-57). We procured the Swiss albino mice of either sex from Disease Free Small Animal House, the Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, India. Mice were obtained at 10 weeks of age when individual body weight was ~25 g and were group housed (6 mouse/cage) under controlled temperature (22 ± 3 °C), humidity (50 ± 5%) and light-dark cycle (12 h light: 12 h dark, lights on at 8:00 a.m.) with free access to standard chow and water ad libitum. The experimental design and Fig. 1 represents the number of mice and grouping details. These experiments were carried out in strict accordance with the guidelines and regulations of the Committee for Control and Supervision of Experiments on Animals.

2.2. Experimental design

The experimental design is depicted chronologically (Fig. 1). Eight groups made by random separation of mice, i.e., one naïve, one sham, one negative control, one positive control, three tests, and one vehicle-treated group. Right common carotid artery of 42 out of total 52 mice was occluded, but 35 underwent standard or test treatments followed by behavioral, molecular and histopathological assessments. The description of eight groups are as follows (1) naïve group (n = 7); (2) sham group (n = 7), given surgical incision only; (3) negative control group (n = 7), given surgical incision followed by right common carotid artery occlusion; (4) positive control group (n = 7), given 0.5 mg/kg donepezil dissolved in normal saline, i.p. after right common carotid artery occlusion; (5) low dose lithium carbonate (L-LC) in test group (n = 7), given 20 mg/kg LC in normal saline, i.p. after right common carotid artery occlusion; (6) medium dose lithium carbonate (M-LC) in test group (n = 7), given 40 mg/kg LC in normal saline, i.p. after right common carotid artery occlusion; (7) high dose lithium carbonate (H-LC) in test group (n = 7), given 80 mg/kg LC in normal saline, i.p. after right common carotid artery occlusion; (8) vehicle treated group (n = 7), given normal saline, i.p. after right common carotid artery occlusion. The standard and test treatment were given daily, i.e., before and after the appearance of symptoms whereby the dose of lithium was selected based on published data in mice [20].

2.3. Induction of vascular dementia

2.3.1. Unilateral common carotid artery occlusion (UCCAO)

Unilateral occlusion of a common carotid artery in the mouse is a model of vascular dementia [19]. In this model, mouse develops white matter lesions and cognitive impairment under the chronic hypoperfusion of ipsilateral cerebral hemisphere. Briefly, mice familiarized to food in a petri-dish placed on the floor for 48 h before surgical procedure. The surgical tools were sterilized by autoclaving (121 °C, 15 PSI, for 15 min). The mice anesthetized by intraperitoneal administration of thiopental sodium (50 mg/kg). Ventral neck region shaved, and the exposed skin disinfected by cotton swabs (70% ethanol). Anesthesia confirmed by loss of hind paw pinch withdrawal and blink reflexes. Hereafter, midline neck incision made, and the soft tissues are pulled apart whereby right common carotid artery carefully separated from the vagus nerve and accompanying veins, and permanently ligated by tying a knot of non-absorbable silk thread (6-0) around the artery. The incision sutured and disinfected. These operated mice returned to their home cages where free access to masticated food in a petri-dish placed on the floor for seven days.

2.3.2. Sham operation

The sham operation was identical to that performed on mice underwent right common carotid artery occlusion, except occlusion the artery. Briefly, mice anesthetized by intraperitoneal administration of thiopental sodium (50 mg/kg). Ventral neck region shaved, and the exposed skin disinfected by cotton swabs (70% ethanol). Anesthesia confirmed by loss of hind paw pinch withdrawal and blink reflexes. Hereafter, midline neck incision made, and the soft tissues pulled apart. The area of incision sutured and disinfected. These operated mice returned to their home cages where free access to masticated food in a petri-dish placed on the floor for seven days.

2.3.3. Post-operative inclusion/exclusion criteria

The inclusion of right common carotid artery occluded mice without any harm to the vagus nerve or accompanying veins. Exclusion of operated animals with a motor deficit, assessed by neurological deficit scoring and accelerated rotarod test as motor deficit can interfere with escape action and swimming performance of demented mice in behavioral tasks.

2.4. Functional assessments

The functional assessment was carried out by neurological deficit score and accelerated rotarod test to assess motor deficits if any.

2.4.1. Neurological deficit score

Neurological function assessed as described [21] on the 5-point scale, i.e., no neurological deficit = 0, failure to extend right paw fully = 1, circling to the right = 2, falling to right = 3, did not walk spontaneously and have depressed levels of consciousness = 4.

2.4.2. Accelerated rotarod test

This test carried out as described [22]. Briefly, the individual mouse subjected to a rotating rod (3 cm rod diameter) with speed set to accelerate 0.1 rpm/s from 2 to max 40 rpm over a trial length of 300 s. This test was carried out before the behavioral test, i.e., day 16, 20, 24,

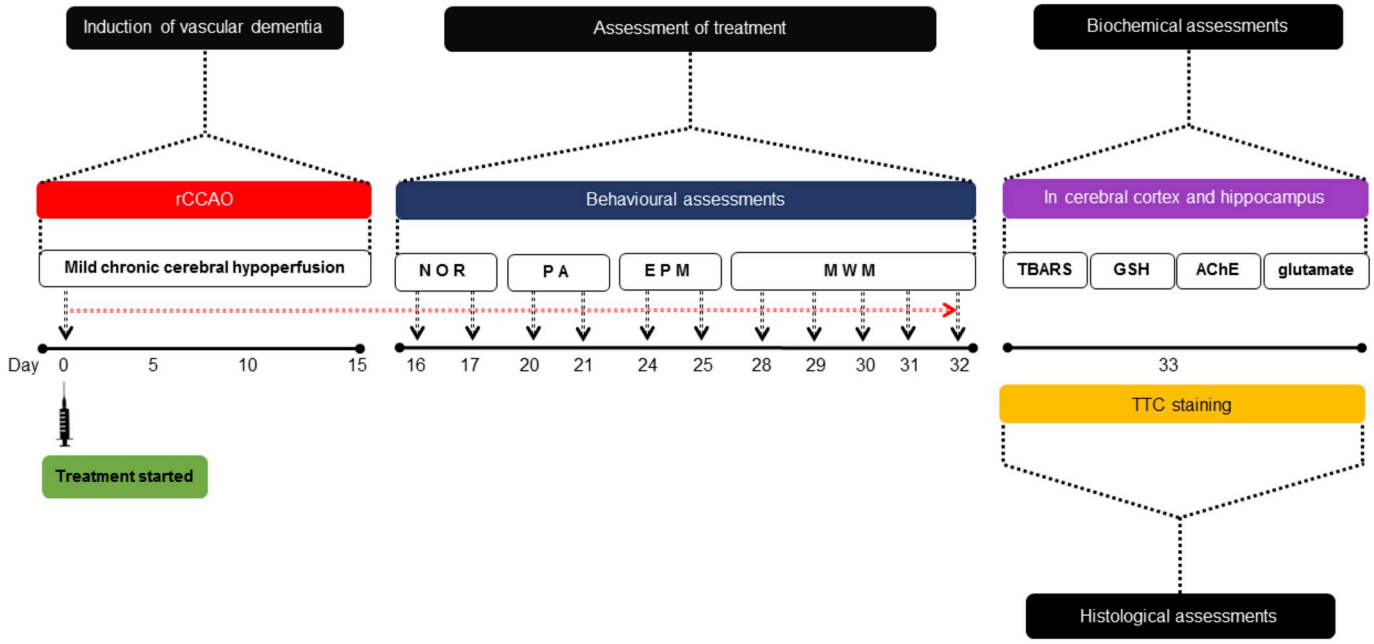

Fig. 1. Schematic illustration of the study design. rCCAO: right Common Carotid Artery Occlusion; NOR: Novel Object Recognition task; PA: step-through latencies in the Passive Avoidance task; MWM: Morris Water Maze test; TBARS: Thiobarbituric Acid Reactive Substances; GSH: Glutathione; AChE: acetylcholinesterase; TTC: 2,3,5-triphenyl tetrazolium chloride.

28 and 32 and repeated two times whereby latency to fall averaged. Mouse fall was detected by a pressure sensitive lever, which automatically stops and records the time of the fall.

2.5. Behavioral assessments

Behavioral test battery consists of an object recognition test, step-through passive avoidance test, elevated plus maze test and the Morris water maze test. All behavioral testing was carried out between 9:00 a.m. and 6:00 p.m. in a dedicated soundproof behavior testing room with controlled illumination. Mice were transferred to this room 30 min before beginning these tests. These tests were monitored and recorded by high definition CC- television.

2.5.1. Object recognition test

Day 16, mice were subjected to the object recognition test as described [23] with slight modifications. Briefly, this test was carried out in two sessions, i.e., familiarization on day 16 and test session on day 17. During the familiarization session, the individual mouse is presented with two similar objects whereas one of the objects replaced by a novel, unfamiliar object during the test session. The amount of time taken to explore the new object considered as the index of recognition memory. The analyzed parameters are as follows: object exploration time (time mouse spent with its nose at least 0.5 cm from object), rearing (time mouse spent in a standing position without leaning on the box's walls), leaning (time mouse spent upright leaning against the wall) and grooming. Climbing onto the object (unless the mouse sniffs the object it has climbed on) or chewing the object does not qualify as exploration. The discrimination index (DI) calculated as follows:

$$DI = \frac{time\ exploring\ the\ novel\ object - time\ exploring\ the\ familiar}{time\ exploring\ novel + familiar} * 100$$

2.5.2. Step through passive avoidance test

Day 20, mice were subjected to a step-through passive avoidance test as described [24] with slight modifications. Briefly, this test was carried out in two sessions, i.e., acquisition/conditioning session on day 20 and test session on day 21. During the acquisition/conditioning phase individual mouse is placed in the white compartment and given free access about both compartments for 30 s with guillotine door open. When mouse innately crosses to the black compartment, the guillotine door closed and it receives a mild foot shock (0.6 mA) for 15 s. The test phase was performed 24 h after the acquisition phase whereby individual mouse again placed in the white compartment, and the passive avoidance response was evaluated by measuring latency to enter dark compartment for up to maximum 300 s.

2.5.3. Elevated plus-maze

Day 24, mice were subjected to an elevated plus maze test as described [25] with slight modifications. In this test, measurement of transfer latency as a parameter (time taken to move from open to closed arm) was used to assess learning and memory. On the first day, i.e., day 24, a mouse is placed at the end of an open arm with its head directed away from the central platform and given access to all the arms and allowed to move freely about the maze for 90 s. If the mouse did not enter one of the closed arms within 90 s, it gently pushed into one of the two closed arms. Hereafter, the mouse can explore the maze freely for another 60 s and then returned to its home cage. Retention of this learned-task (memory) examined on day 25, i.e., 24 h after the learning.

2.5.4. Morris water maze test (MWM)

Day 28, mice were subjected to Morris water maze test as described (Bromley-Brits et al., 1992). During training sessions (day 28–31) individual mouse underwent 4 training sessions per day at each of 4 cardinal drop points (north, south, east, west) in random order with the time gap of 5 min for 4 consecutive days whereby escape latency (EL), duration of time required to locate a submerged escape platform recorded as the index of acquisition or learning. During the test session, i.e., day 32, the escape platform was removed, and mouse was allowed to swim freely in the water pool for 120 s to locate escape platform and time spent in all quadrant recorded. A total duration of time spent in the target quadrant (TSTQ) taken as an index of retrieval or memory.

2.6. Biochemical estimations

2.6.1. Sample preparations

Mice (n = 6) were sacrificed by cervical dislocation on day 33, i.e., after behavioral assessments. The brains were isolated and ipsilateral hemisphere is dissected into the cerebral cortex and hippocampus [26]. Dissected parts were weighed and divided into two equal parts. First part was homogenized in ice-cold 10% w/v (0.05 M, pH 7.4) phosphate buffer and centrifuged at 6000 g for 20 min at 4 °C (REMI C-24BL, cooling centrifuge, REMI, India) and the clear supernatant utilized for estimation of TBARS, GSH, and AChE. The second part homogenized in freshly prepared ice-cold 10% w/v perchloric acid followed by centrifugation at 14,000 g for 30 min at 4 °C (REMI C-24BL, Cooling Centrifuge, REMI, India). The clear supernatant utilized for estimations of glutamate.

2.6.2. Estimation of brain thiobarbituric acid reactive species (TBARS)

TBARS level in the cerebral cortex and hippocampus was measured by the method of [27] to analyze the oxidative stress. A standard calibration curve was prepared using 5–50 nM of 1,1,3,3-tetra methoxy propane and absorbance was measured spectrophotometrically at 532 nm.

2.6.3. Estimation of reduced glutathione (GSH)

GSH level in the cerebral cortex and hippocampus was measured as described [28] to analyze the oxidative stress. A standard calibration curve was prepared using 0.1–10 μM of the reduced form of glutathione and absorbance was measured spectrophotometrically at 412 nm.

2.6.4. Estimation of acetylcholinesterase (AChE) activity

AChE activity in the cerebral cortex and hippocampus assessed as described [29]. Change in absorption per min of the sample was read spectrophotometrically at 412 nm.

2.7. Histological assessments

2.7.1. In-vivo 2,3,5-triphenyl tetrazolium chloride staining (TTC)

TTC staining is commonly applied for visualization of hypoxic brain tissue and for defining the size of cerebral infarction; however, it is often difficult to apply immersion staining to severely injured or soft tissue like a brain of rodent. For this reason, we used in-vivo perfusion labeling method that is based on the osmotic opening of blood-brain-barrier with mannitol pre-treatment as described [30]. Briefly, the blood-brain barrier was disturbed by intraperitoneal administration of mannitol to one randomly selected male mouse from each group on day 33 (1 M, 0.1 ml/g of body weight). After 30 min, mice were anesthetized (50 mg/kg thiopental sodium, i.p.) and perfused transcardially with 20 ml of 2% TTC. At 10 min after transcardial perfusion, whole brains were dissected and placed into 4% paraformaldehyde for fixation.

2.7.2. Quantification of brain infarction

The fixed brains were sectioned six into ~2 mm thick coronal slices, and infarct volume was calculated by volume method as described [31] using the National Institutes of Health Image J software (NIH ImageJ version 1.52). The infarct volume expressed as mm3.

2.8. Drug target identification

The databases on the links between genes and diseases were used to search for genes associated with vascular dementia: PROTEOMETM, DisGeNET, Disease-Connect, DISEASES. A list of 147 genes associated with VaD created. The DIAMOnD algorithm [32] was used to predict 600 new associations between human proteins and vascular dementia from the network of functionally related genes. The possibility of interactions between 1976 known phytocomponents from 256 plants used in traditional Indian medicine and selected targets were estimated by PASS (Prediction of Activity Spectra for Substances) software [33] 175 genes associated with 187 mechanisms of actions (MoA) predicted by PASS selected from 600 ones. The analysis of this MoA revealed ten most promising targets of potentially related with vascular dementia whereby, glycogen synthase kinase 3β (GSK3β) seems to be one of the most promising targets for treatment of vascular dementia.

2.9. Statistical analysis

Non-parametric one-way ANOVA followed by Student Newman Keuls multiple comparisons test was performed on behavioral and neurochemical data, using Graph Pad Prism version 8.00 (GraphPad Software, La Jolla California USA). The results expressed as mean standard error mean. Differences were considered significant at $P < 0.05$.

2.10. Drugs and chemicals

Analytical grade drugs and chemicals used. Thiopental sodium purchased from Neon Laboratories Limited, Mumbai (India). Acetylthiocholine, glutamate, lithium carbonate from Sigma-Aldrich, Co., St. Louis, MO, USA. Ethylenediaminetetraacetic acid, glutathione and perchloric acid from SD fine chem. Limited, Mumbai (India). 2,3,5-triphenyl tetrazolium chloride, ascorbic acid, heptane sulfonic acid, mannitol, orthophosphoric acid, paraformaldehyde, sodium dihydrogen phosphate, thiobarbituric acid from Loba Chemie Pvt, Ltd, Mumbai (India). Donepezil from Wockhardt Ltd, Baddi (India). Non-absorbable surgical suture 6-0 from Johnson & Johnson LTD, Aurangabad (India).

3. Results

3.1. Discrimination ability in the novel object recognition task

In object recognition task, the naïve mice readily discriminate between a familiar and new object (Fig. 2A and B). This discrimination ability was significantly impaired ($F(7,40) = 10.46$, $P < 0.05$) in artery occluded mice as evident from the lower value of discrimination index in artery occluded group and higher value in the naïve group (Fig. 2A and B). In contrast, discrimination ability was significantly preserved ($P < 0.05$) in sham-operated mice along with donepezil and lithium-treated mice at a medium and high dose, as evident from higher values of discrimination index in these groups (Fig. 2A and B). In vehicle-treated, discrimination ability remains significantly impaired ($P < 0.05$).

3.2. Step-through latencies in the passive avoidance task

In passive avoidance task, the naïve mice readily learn that moving to the dark compartment has negative consequences. This fear motivated passive avoidance response was significantly impaired ($F(7,40) = 13.40$, $P < 0.05$) in artery occluded mice as indicated by the lower value of step-through latency in artery occluded mice and higher value in the naïve group (Fig. 3). In contrast, passive avoidance response was significantly ($P < 0.05$) preserved sham-operated mice along with donepezil and lithium-treated mice at medium and high dose (Fig. 3). In vehicle-treated, passive avoidance response remains significantly impaired ($P < 0.05$).

3.3. Transfer latency in an elevated plus maze task

In elevated plus maze task, naïve mice readily learn to move from open arm to closed arm (Fig. 4A and B). This learning and memory formation was significantly impairment in artery occluded mice as evident from higher value of transfer latency in artery occluded mice

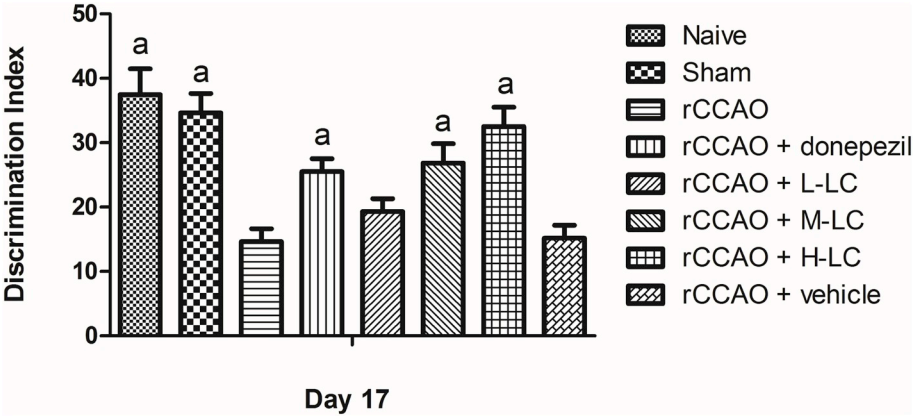

Fig. 2A. Discrimination index in the novel object recognition task. Discrimination index (DI) calculated as (time exploring the novel object – time exploring the familiar)/(time exploring novel + familiar) * 100. Each value expressed as mean ± standard error mean. Differences were considered significant with P < 0.05 (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

and lower value in naïve mice in both acquisition (F (7,40) = 7.28, P < 0.05) and retrieval test (F (7,40) = 10.55, P < 0.05). In contrast, learning and memory formation were recorded in sham-operated mice along with donepezil and lithium-treated mice at a medium and high dose as evident from lower values of transfer latency in these groups (Fig. 4A and B).

3.4. Escape latency and time spent in the target quadrant in the water maze task

During first four days of cued learning phase i.e. from day 28th to 31st, the average escape latency of artery occluded mice (55 ± 5) was significantly (P < 0.05) higher than naïve (29 ± 3), sham (31 ± 4), donepezil-treated (36 ± 4), medium dose of lithium (37 ± 4) and high dose of lithium (36 ± 3) treated mice (Table 1). It indicates normal learning abilities in naïve, donepezil and lithium-treated mice and learning impairment in artery occluded mice. In retention trials (i.e., day 32nd) time spent by naïve, sham, donepezil, lithium (medium and high dose) in target quadrant was significantly (F(7,40) = 7.31, P < 0.05) higher than artery occluded mice which indicates normal memory formations in former and significant memory impairment in later (Fig. 5A and B).

3.5. Neuromotor co-ordination was normal

Neurological deficit score remains zero throughout the study. Moreover, difference of fall of latency between the groups was also insignificant (F (4,7) = 0.591, P = 0.669) on day 16, 20, 24, 28 and 32 (F (4,7) = 0.173, P = 0.9903) which indicates normal neuromotor co-ordination in all groups (Table 2).

3.6. Thiobarbituric acid reactive substances (TBARS) levels in cerebral cortex and hippocampus

The naïve group represents the baseline level of TBARS in cerebral cortex and hippocampus (Fig. 6A and B). This level was significantly increased in cerebral cortex (F (7,40) = 8.48, P < 0.05) and hippocampus (F (7,40) = 11.38, P < 0.05) of artery occluded mice. In

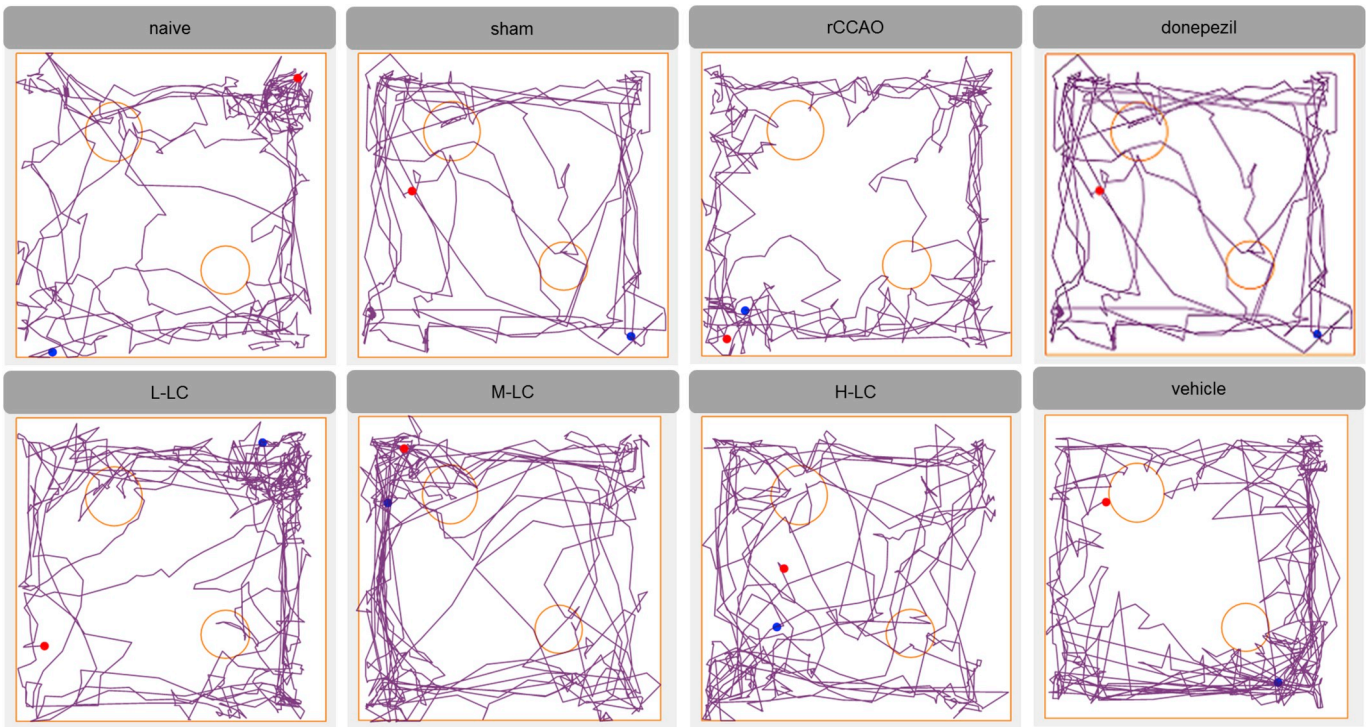

Fig. 2B. Exploration path in the novel object recognition task. Upper left circle represents a novel object whereas lower right circle represents a familiar object. The video track was carried out by using ANY-maze, trial version, Behavioral tracking software.

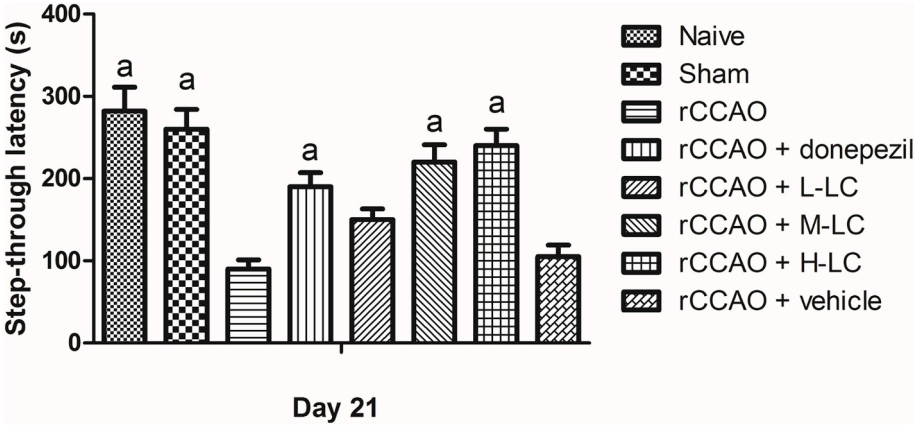

Fig. 3. Step-through latencies in the passive avoidance task. Differences were considered significant with $P < 0.05$ (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

contrast, this level significantly ($P < 0.05$) reduced to normal in donepezil and the lithium-treated group at a medium and high dose (Fig. 6A and B).

3.7. Glutathione (GSH) level in the cerebral cortex and hippocampus

The naïve group represents baseline GSH in cerebral cortex and hippocampus (Fig. 7A and B). This level was significantly reduced in cerebral cortex ($F(7,40) = 15.58$, $P < 0.05$) and hippocampus ($F(7,40) = 18.21$, $P < 0.05$) of artery occluded mice. In contrast, this level was significantly ($P < 0.05$) increased to normal in donepezil and the lithium-treated group at a medium and high dose (Fig. 7A and B).

3.8. Acetylcholinesterase (AChE) activity in cerebral cortex and hippocampus

The naïve group represents baseline acetylcholinesterase activity in cerebral cortex and hippocampus (Fig. 8A and B). This activity was significantly increased in cerebral cortex ($F(7,40) = 14.62$, $P < 0.05$) and hippocampus ($F(7,40) = 7.35$, $P < 0.05$) of artery occluded mice. In contrast, activity was significantly ($P < 0.05$) reduced to normal in donepezil and the lithium-treated group at a medium and high dose (Fig. 8A and B).

3.9. Glutamate levels in the cerebral cortex and hippocampus

The naïve group represents baseline glutamate in cerebral cortex and hippocampus (Fig. 9A and B). This level was significantly high in cerebral cortex cortical ($F(7,40) = 5.740$, $P < 0.05$) and hippocampus ($F(7,40) = 8.250$, $P < 0.05$) of artery occluded mice along with donepezil-treated mice and lithium-treated mice as compared to baseline levels in naive and sham-operated mice (Fig. 9A and B).

Table 1
Effect of sham operation, right carotid artery occlusion, donepezil and lithium carbonate treatment (low, medium & high dose of 20, 40 & 80 mg/kg, respectively) on escape latency in water maze test.

Groups	Day 55 ELT (s)	Day 58 ELT (s)
Naive	55 ± 5	29 ± 3[ab]
Sham	57 ± 7	31 ± 4[ab]
rCCAO	64 ± 8	55 ± 5
rCCAO + donepezil	61 ± 7	36 ± 4[ab]
rCCAO + L-LC	62 ± 6	47 ± 5
rCCAO + M-LC	59 ± 6	37 ± 4[ab]
rCCAO + H-LC	58 ± 5	36 ± 3[ab]
rCCAO + vehicle	63 ± 6	53 ± 6

Differences were considered significant with $P < 0.05$ (Two way ANOVA with Bonferroni *post hoc*). [a] shows the comparison with artery occluded mice on day 58; [b] shows the comparison from day 55–58 in artery occluded mice.

3.10. Cerebral infarct volume

In case of artery occluded mice, donepezil treated mice and vehicle treatment mice the volume of cerebral infarction was relatively larger than lithium-treated mice at a medium and high dose (Fig. 10A and B).

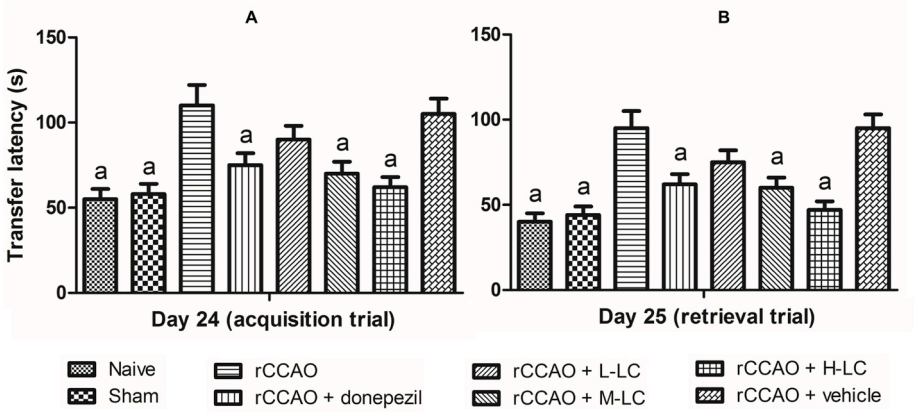

Fig. 4. Transfer latency during (A) acquisition trial (B) retrieval trial in elevated plus maze. Differences were considered significant with $P < 0.05$ (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

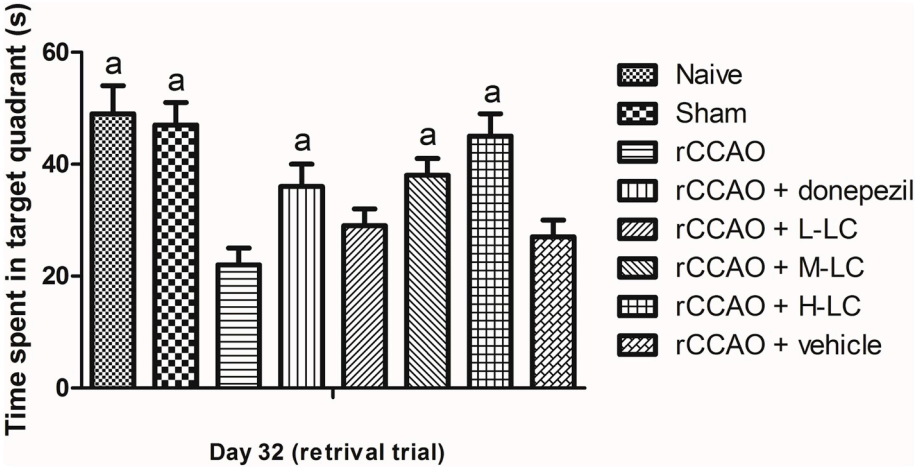

Fig. 5A. Time spent in target quadrant in Morris water maze test. Differences were considered significant with $P < 0.05$ (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

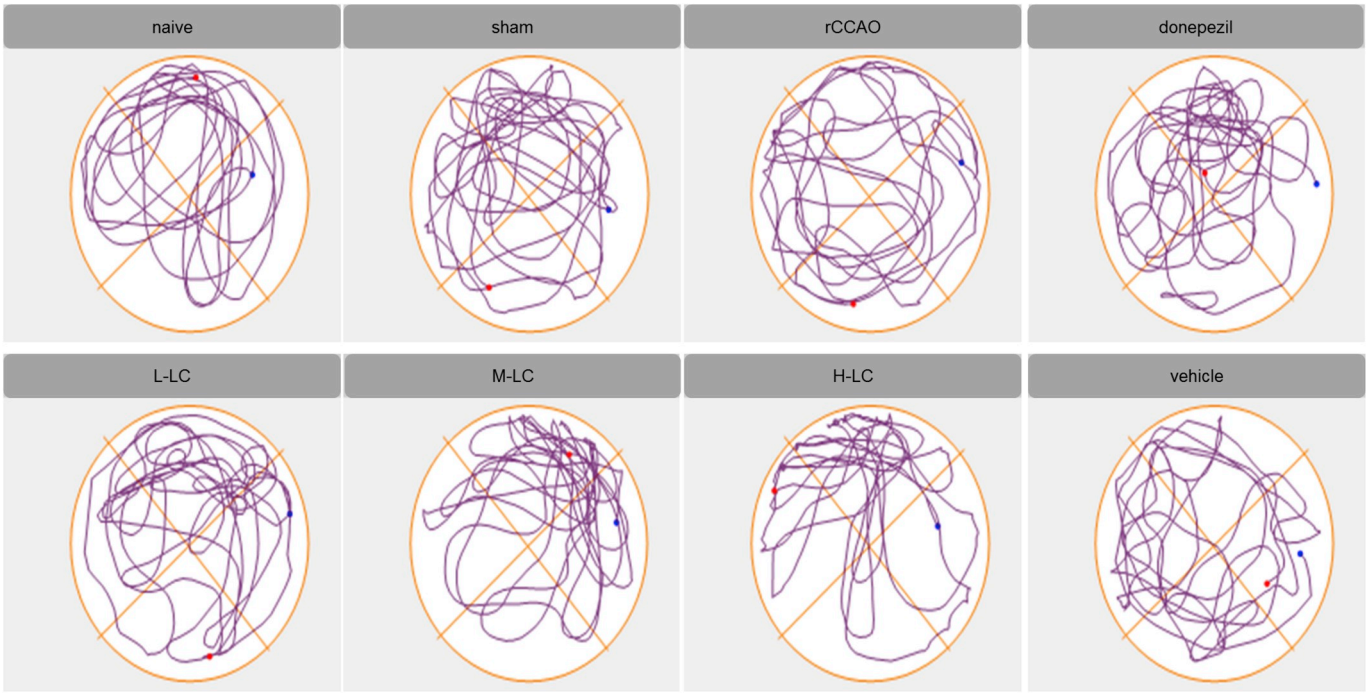

Fig. 5B. The path followed during retrieval trial in a water maze test. The blue dot and upper left represent the starting point to the starting point represent target quadrant. The video track was carried out by using ANY-maze, trial version, Behavioral tracking software.

Table 2
Effect of sham operation, unilateral carotid artery occlusion, donepezil and lithium carbonate treatment (low, medium & high dose of 20, 40 & 80 mg/kg, respectively) on fall off latency in rotarod test.

Fall of latency on	Day 16 (s)	Day 20 (s)	Day 24 (s)	Day 28 (s)	Day 32 (s)
Naive	203 ± 17	199 ± 18	193 ± 15	191 ± 17	190 ± 19
Sham	197 ± 16	195 ± 17	196 ± 16	192 ± 18	187 ± 13
rCCAO	195 ± 17	192 ± 19	193 ± 14	187 ± 13	182 ± 16
rCCAO + donepezil	191 ± 16	189 ± 17	185 ± 17	184 ± 16	175 ± 16
rCCAO + L-LC	193 ± 16	187 ± 18	187 ± 12	186 ± 17	183 ± 13
rCCAO + M-LC	195 ± 18	189 ± 17	183 ± 15	193 ± 18	188 ± 16
rCCAO + H-LC	198 ± 16	185 ± 16	185 ± 18	196 ± 20	185 ± 16
rCCAO + vehicle	196 ± 17	193 ± 17	189 ± 17	185 ± 19	180 ± 22

The difference in the fall of latency between groups was insignificant on day 16, 20, 24, 28 and 30. It indicates normal neuromotor coordination in all groups.

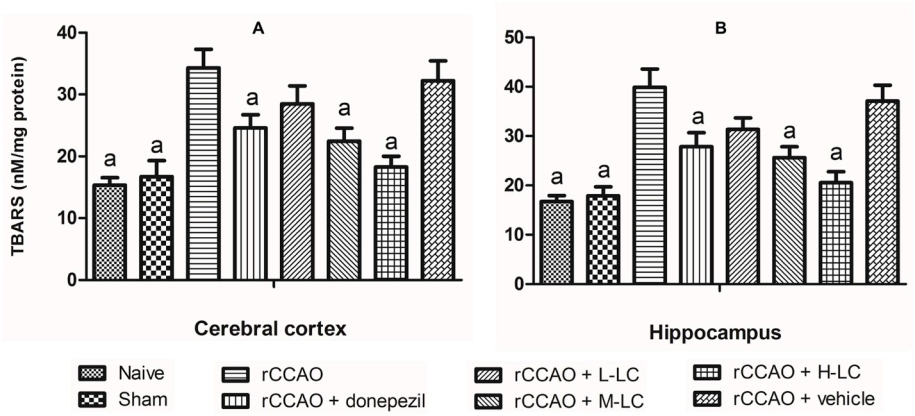

Fig. 6. Thiobarbituric acid reactive substances (TBARS) level in (A) cerebral cortex (B) Hippocampus. Each value expressed as mean ± standard error mean. Differences were considered significant with P < 0.05 (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

4. Discussion

In the present study, we demonstrate that lithium carbonate can attenuate cognitive impairment resulting from mild chronic cerebral hypoperfusion in mice. Mild chronic hypoperfusion is a well-recognized cause of cerebral ischemia and vascular dementia [18]. The mild chronic hypoperfusion can also be induced in mice by occlusion of either one or both the common carotid arteries [34]. The right common carotid artery occlusion leads to gradual hypoperfusion of cerebral hemisphere. This model mimic core feature of vascular dementia viz. neuroinflammation, white matter lesion and impairment in the cognitive functions with zero mortality rate in a 30 days' protocol without any motor impairment which is assessed by neurological deficit score and accelerated rotarod 120 min before every behavioral task [19]. All post operated mice were included for the behavioral task as there was no motor deficit and no mortality and results are consistent with literature reports [19].

Female subjects are underrepresented in animal research across disciplines, however and lack of pre-clinical research on female subjects has likely resulted in poorer treatment outcomes for women [35]. Therefore, both sex of mice (male and female) were included in this study. These mice were provided with various behavioral tasks at specified intervals, with or without test treatments (Fig. 1), whereby four different aspects of learning and memory were studded because impairment in the executive functions, addition to learning and memory has been reported in vascular dementia [16,36]. For the assessment, The recognition and discrimination ability evaluated by a novel object recognition task [37]. Emotional learning and memory by step-through passive avoidance task [24], an elevated plus maze test employed for evaluation of fear-motivated learning and memory [25] whereas spatial reference learning and memory evaluated by water maze test [38].

Behavioral tests are described in the chronological order (Fig. 1). In these tasks, naïve mice readily learn to identify and discriminate between novel and familiar object (Fig. 2A and B). Similar results were observed in passive avoidance task (Fig. 3), elevated plus maze task (Fig. 4A and B) and water maze task (Fig. 5A and B). These results and previous reports are consistent [23,38,39]; [40]. In contrast, the artery occluded mice exhibited learning and memory impairments. These impairments are evident from failure to discriminate between novel and familiar object in the object recognition task. A failure to learns and memorize that moving to the dark compartment has negative consequences in the passive avoidance task (Fig. 3). A failure to move more rapidly to close arm in elevated plus maze test (Fig. 4A and B) and failure to locate escape platform (Table 1), and spend less time in target quadrant in water maze test (Fig. 5A and B). These results and previous reports are also consistent [19,41] which indicates that occlusion of right common carotid artery allows development of reliable and reproducible dementia. In contrast, no such effect observed in sham-operated mice.

Due to deficiencies in the safe and affecting treatment of vascular dementia, it is reasonable to use acetylcholinesterase inhibitors (donepezil, galantamine, and rivastigmine) for at least symptomatic management because cholinergic dysfunction has been implicated in VaD [42]. Thus, we selected donepezil as positive control whereby significant cognitive improvement observed with this drug in artery occluded mice. This observation is consistent with previous studies showing cognitive improvement with donepezil treatment in patients [43], rodents and non-human primates [44].

GSK-3 is an evolutionary conserved serine-threonine kinase that

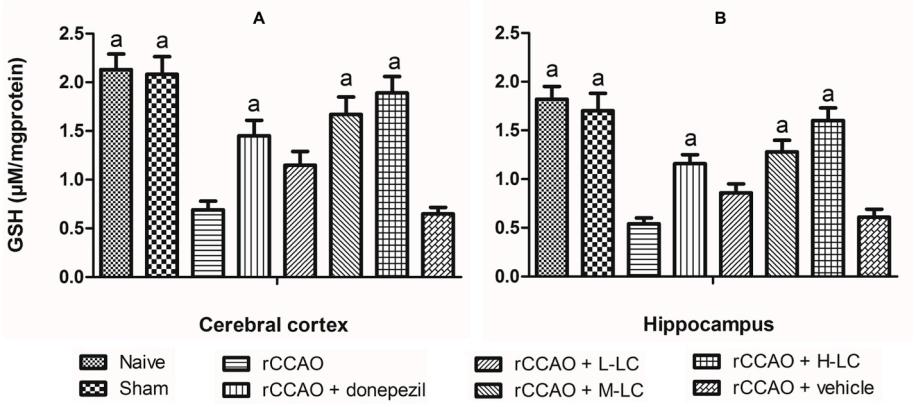

Fig. 7. Reduced form of glutathione (GSH) level in (A) cerebral cortex (B) Hippocampus. Each value expressed as mean ± standard error mean. Differences were considered significant with P < 0.05 (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

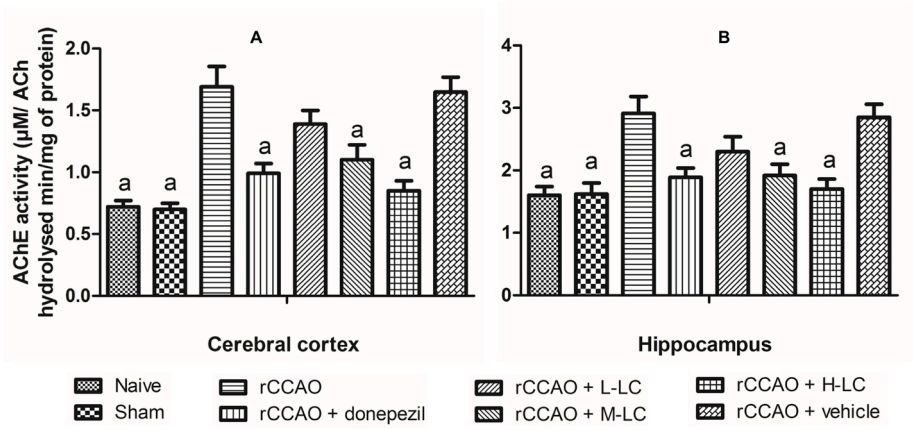

Fig. 8. Acetylcholinesterase (AChE) activity in (A) cerebral cortex (B) Hippocampus. Each value expressed as mean ± standard error mean. Differences were considered significant with P < 0.05 (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

consists of alpha and beta isoforms. The activity of these enzymes are positively regulated by phosphorylation on tyrosine residues 279 for GSK-3α and 216 for GSK-3β [45] and negatively regulated by inhibitory phosphorylation of the N-terminal serine 21 for GSK-3α and 9 for GSK-3β [46]. Lithium directly inhibits GSK3α and β by the competitive binding for magnesium ($Mg2+$), disrupting the catalytic functioning of GSK3. Lithium also indirectly inhibits GSK3 by increasing serine phosphorylation, through P13K-mediated phosphorylation/activation of Akt [47].

Lithium carbonate facilitates recovery of spatial learning and memory after transient global cerebral ischemia [17] and a drug of choice for mania and effective in patients with bipolar disorder [15]. However, we used this drug to evaluate changes in the cognitive parameters under mild chronic unilateral cerebral hypoperfusion at three doses (20, 40 and 80 mg/kg). Lithium carbonate improved learning and memory performance. These mice interacted more with the novel object in novel object recognition task (Fig. 2A and B); had increased latency time in step through-passive avoidance task (Fig. 3); reduced transfer latency in elevated plus maze task (Fig. 4A and B); had reduced escape latency (Table 1) and spent more time in target quadrant in Morris water maze task (Fig. 5A and B), in reference to corresponding control artery occluded mice. These results suggest that lithium carbonate may attenuate cognitive deficits under mild chronic cerebral hypoperfusion resulting from occlusion of the right common carotid artery.

After understanding of vascular dementia, we believe that pharmacological deactivation GSK-3 by lithium carbonate can treat this syndrome through pathways enlisted in Fig. 11 which predicted through the lens of bioinformatics. In order to reveal these pathways, we predicted some mechanism using SIGNOR 2.0 database [48]. Two types of interactions used in the study: activating and inhibiting. Shortest paths from GSK-3 protein to proteins which have known relationships to vascular dementia calculated. The paths where GSK-3 was interacting with vascular dementia-related proteins immediately or through one intermediate protein selected. All activating and inhibiting interactions between proteins from selected shortest paths of SIGNOR 2.0 database were retrieved. Through extensive analysis of literature, we found relationships between proteins with known associations to vascular dementia and similar pathological processes leading to the disease. Analysis of literature revealed that GSK-3β inhibition may also modulate other processes relevant to VaD and shown in Fig. 11, such as thrombosis [2], atherosclerosis [3], diabetes mellitus [4], disruption of blood-brain barrier [5], oxidative stress [6], neuroinflammation (Chang et al., 2016), neuronal death [8], synaptic plasticity [9], mood [10], learning [11] and memory [12]. We speculate that the lithium carbonate induced cognitive improvement mediated through these biological pathways (Fig. 11). Further, these pathways validated through neurochemical and histological assessments.

Improved performance of lithium carbonate treated mice in the object recognition task, step through-passive avoidance task, elevated plus maze task and water maze test is an indication of altered cortical and hippocampal function [38,49]. In similar to this, unilateral occlusion of common carotid artery reduces the blood flow to half of the original value in the cortical and hippocampal region [19]. Therefore, a neurochemical assessment was primarily carried out in these discrete regions of the brain.

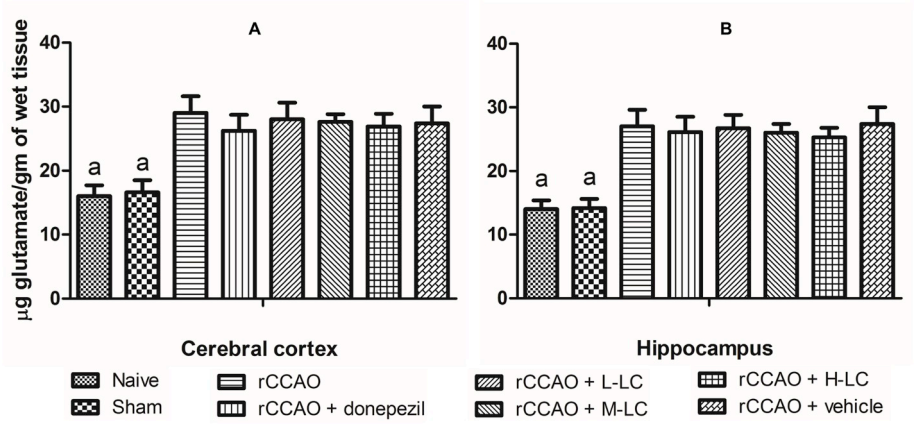

Fig. 9. Glutamate levels in (A) cerebral cortex (B) Hippocampus. Each value expressed as mean ± standard error mean. Differences were considered significant with P < 0.05 (one-way ANOVA with Newman-Keuls multiple comparison post hoc). a shows the comparison with artery occluded mice.

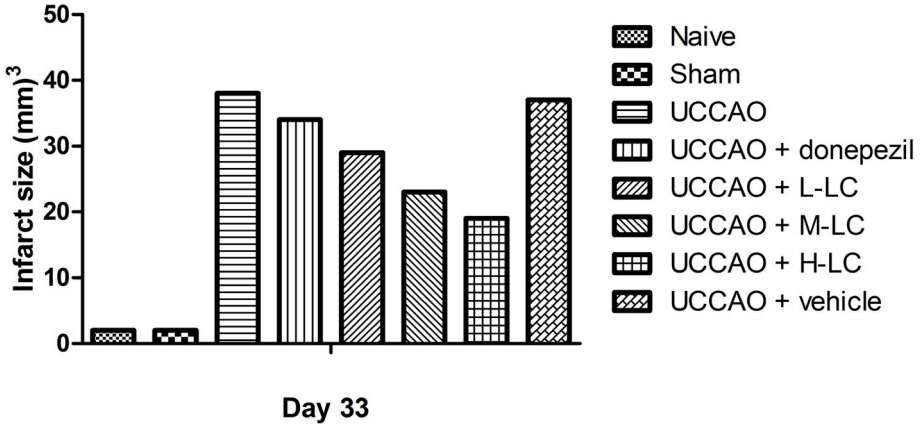

Fig. 10A. Cerebral infarct volume measured by Image J software (NIH ImageJ version 1.52).

Current evidence suggests that cholinergic agents may delay progression of VaD by enhancing cerebral blood flow [50], in addition to symptomatic treatment of cognitive impairments [43]. Significantly high acetylcholinesterase(AChE) activity shows cholinergic impairment in the hippocampus, and cerebral cortex of artery occluded mice (Fig. 8A and B). This observation is consistent with earlier reports suggesting the involvement of cholinergic dysfunction in vascular dementia [42]. GSK-3 activity is under negative regulation by phosphorylation of the serine 21 for GSK-3α, and 9 for GSK-3β [46] and this phosphorylation is promoted by lithium [47] and AChE inhibitor in the cerebral cortex and hippocampus [51]. Based on our experimental results and literature report, we believe that GSK-3 inhibition by lithium carbonate may enhance learning and memory abilities by enhancing cholinergic tone by increasing AChE activity as lithium carbonate treated mice had intact cholinergic functions at a medium and high dose, indicated by low AChE activity (Fig. 8A and B).

Vascular dementia arises from diseased vessels supplying inadequate blood to the brain resulting in either neuronal damage or death due to oxidative stress [52], an environment where pro-oxidant species overwhelm antioxidant species. We believe that pharmacological deactivation GSK-3 by lithium carbonate can relieve oxidative stress. Therefore, we assessed cortical and hippocampal oxidative stress as memory formation and learning is concerned with the hippocampus [53] and, executive functions are predominantly concerned with the pre-frontal cortex [54]. The artery occluded mice showed increased TBARS and decreased GSH levels in both hippocampus and cerebral cortex with a detrimental effect on learning and memory in our test battery (Figs. 6 and 7A, B). Consistent with previous reports [55], mice treated with lithium carbonate decreased TBARS and increased GSH levels in the cortex and hippocampus at a medium and high dose. Thus, we believe that intact learning and memory abilities in lithium carbonate treated mice were attributable to antioxidant actions of GSK-3.

We have performed histological studies for assessment of hypoxic brain tissue and cerebral infarct volume, along with the estimation of glutamate level in cerebral cortex and hippocampus because the neuronal injury caused by ischemia after occlusion of cerebral arteries is believed to be mediated by accumulation of glutamate and excessive activation of NMDA receptors [56]. In similar to these literature reports significantly high glutamate and a large infarct volume was observed in the right common carotid artery occluded mice. We believe that pharmacological deactivation GSK-3 by lithium carbonate can treat vascular dementia through neuroprotection because lithium carbonate may decrease glutamate excitotoxicity through downregulation of NMDA receptors [57] and ipsilateral hemisphere of lithium carbonate treated mice were protected from glutamate-induced excitotoxicity as evident from a small volume of cerebral infarction (Fig. 10A and B). Thus, these results suggest that lithium carbonate may act as a neuroprotective agent against glutamate-mediated excitotoxicity that results from mild

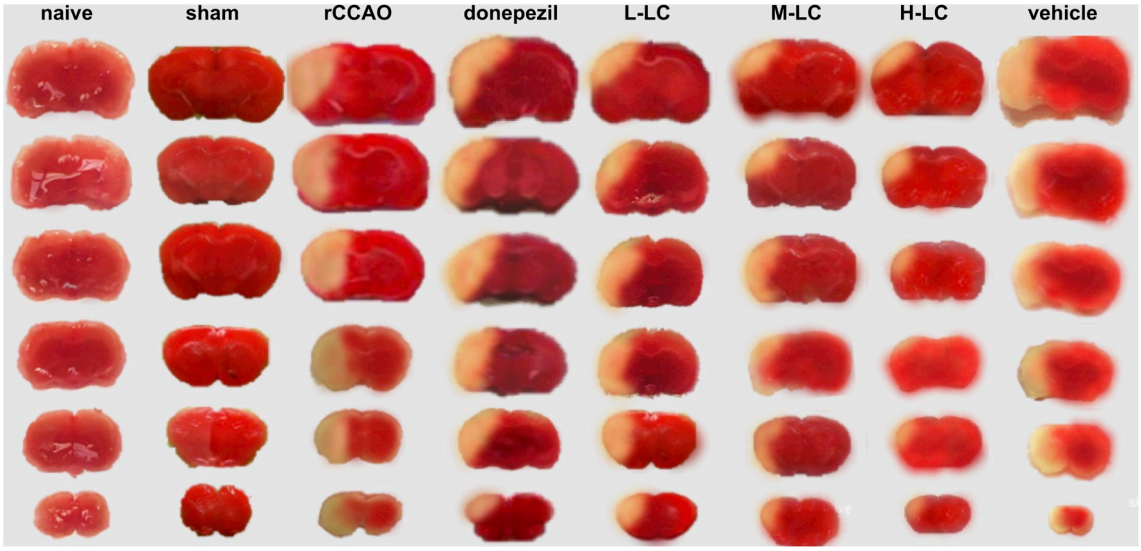

Fig. 10B. Cerebral infarction in naïve, sham-operated, artery-occluded, donepezil and lithium-treated (low, medium & high dose of 20, 40 and 80 mg/kg, respectively) mice. The infarction area appears white while the viable area in reddish in these coronal sections after in-vivo TTC staining.

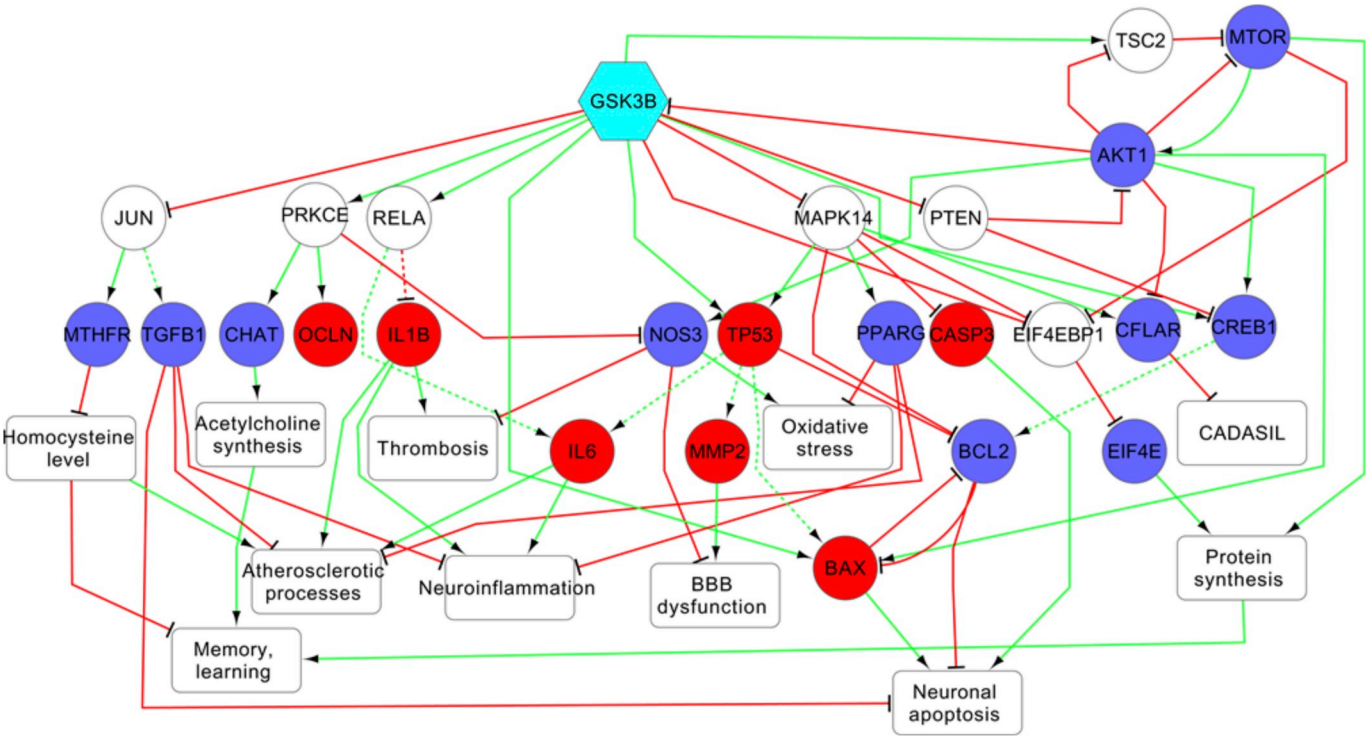

Fig. 11. Mechanisms related to the possible therapeutic effect of GSK-3 inhibition. The green edges are activating interactions, red edges are inhibiting interactions, solid lines are direct interactions, dotted lines are indirect interactions through transcription regulation, red nodes are proteins whose expression/function increased in VaD, and blue nodes are proteins whose expression/function decreased in VaD. CADASIL - cerebral autosomal dominant arteriopathy with subcortical infarcts.

chronic cerebral hypoperfusion [58] induced by occlusion of right common carotid artery of mice.

5. Conclusion

We have systematically collected and analyzed the biomedical literature and then validated the most promising drug target, i.e., GSK-3. Pharmacological inhibition of glycogen synthase kinase-3 enzyme successfully attenuated the cognitive disabilities under mild chronic cerebral hypoperfusion which mimic core features of vascular dementia. These behavioral findings agree well with neurochemical and histological findings. Collectively, based on our experimental results and in-silico predictions, we propose that glycogen synthase kinase-3 is a potential pharmacological target for vascular dementia.

Conflicts of interest

The authors declare no conflicts of interest.

Funding source

This work is supported by the Department of Science & Technology (India) grant number INT/RUS/RFBR/P-244 and Russian Foundation for Basic Research (Russia) grant number 16-54-45016.

Acknowledgments

The authors are grateful to the Department of Science & Technology and Department of Pharmaceutical Sciences & Drug Research, Punjabi University, Patiala (India) for providing necessary technical facilities for this work.

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