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Original Article

Prophylactic Choline Supplementation Attenuates Vascular Cognitive Impairment in Rodent Model of Ischemic Stroke

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Abstract

Introduction: Stroke is the second leading cause of mortality in the world and estimated to be about 0.22% in India. Current management of post stroke vascular cognitive impairment (VCI) is not satisfactory. Choline is an important component of neuronal membrane phospholipids known to enhance cognition.

Objective: To explore the prophylactic efficacy of choline in attenuating VCI in a rat model of ischemic brain injury.

Materials and Methods: 10 month old male Wistar rats (n=8/group) were assigned as normal control, Bilateral common carotid artery occlusion (BCCAO), Sham BCCAO and Prophylactic BCCAO choline supplemented groups. Subsequently, all the groups of rats were subjected to cognitive function tests.

Results: BCCAO rats showed significant deficits ($p< 0.05$) in cognitive functions compared to age matched sham BCCAO and NC rats. Prophylactic supplementation of choline in chronic cerebral hypoperfusion ischemic brain injured rats significantly restored learning and memory abilities ($P<0.001$) compared to age matched BCCAO rats.

Conclusion: Prophylactic dietary choline supplementation attenuates vascular cognitive impairment in rodent model of ischemic stroke. Thus, after appropriate human clinical trials, dietary choline supplementation may be considered as a preventive strategy to attenuate post stroke cognitive impairment in high risk individuals.

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Introduction

Stroke is the second leading cause of mortality in the world and major cause of permanent disability in surviving victims (1). Prevalence of stroke in India is estimated to be about 90-222 per 100,000 and 1.44-1.64 million cases of new acute strokes are reported every year (2). According to Framingham study, ischemic brain injury doubles the risk of dementia and post-stroke cognitive decline is more common than stroke recurrence (3). Post-stroke VCI affects 30% of survivors, and the incidence of new-onset dementia increases from 7% after one year of stroke to 48% after 25 years (4). It is estimated that 3.7 million Indians aged over 60 years have dementia and approximately 400,000 new cases are reported per year (5). As life expectancy is projected to increase, India will likely face a significant socioeconomic burden to meet the costs of managing stroke and stroke induced cognitive impairment (6). Stroke being one of the important contributors for cognitive impairment, the management of post stroke cognitive decline should be a major thrust area for research.

Current methods of treatment in ischemic stroke includes tissue plasminogen activator (t-PA) to dissolve clot, surgical removal of tumor compressing blood vessel, re-canalizing the stenosed artery by stents and stem cell transplantation techniques. Recent neuro-protective therapeutic strategy studies with NMDA receptor antagonists, calcium antagonists, sodium and potassium channel modulators have shown reduction of ischemic damage to some extent in animal models of stroke and are presently in human clinical trials (7).

Therapeutic strategies for ischemic brain injury are focused mainly on treatment of motor recovery and improving the activities of daily living following stroke. Management of post stroke cognitive impairment gains least attention among both health professionals and supporting family members. Though, clinical trials with calcium channel blockers, angiotensin antagonists, ACE inhibitors and statins are found to have positive effects on post stroke cognitive skills, they have limitations due to other systemic side effects.

Prophylactic therapy for ischemic brain injury is a relatively new concept, focusing on enhancing the ability of neural cells to withstand against potential ischemic insults. Although most of the current treatment strategies are primarily aimed at treatment and rehabilitation subsequent to the occurrence of ischemic brain injury or focused on prophylactic therapy for the risk factors, very few research studies are aimed at addressing the issue related to prophylactic neuro-protection.

Various essential nutritional components like Vit B12, folic acid, choline, omega 3 fatty acids play an important role in neural development and sustaining neural cell functions. Choline is a quaternary amine (trimethyl-ß-hydroxy-ethylammonium) predominantly utilized for the synthesis of phosphotidylcholine (PtdCho), an essential component of neuronal cell membrane phospholipids (8). Choline is richly present in dietary components like egg yolk, beef, chicken meat, soyabean oil, cod fish oil and minimally in many other sources of our daily food (9). Choline is also available in the form of an oral supplement. Bioavailability of choline following oral supplementation is better as it is readily absorbed in the gut and cross the blood– brain barrier (10). Choline is critical during fetal development and is also a precursor for acetylcholine synthesis an important neurotransmitter involved in learning and memory. Choline deficiency increases rate of neuronal cell death (11). A previous study in rodent model of hypoxic ischemic brain injury shows that CDP-Choline (Citicoline) an intermediate compound in the bio-synthesis of phosphatidylcholine reduces caspase-3 activation and Hsp70 expression (12). Further a double blind randomized control clinical trial on severe and moderate head injury patients shows that citicoline improves motor and cognitive recovery (13). So based on previous studies we hypothesize that prophylactic choline supplementation to individuals with high risk for stroke will enhance the threshold of neural cells against ischemic insult and will minimize post-stroke cognitive impairment. The objective of the present study was to test the efficacy of prophylactic choline supplementation in attenuating learning and memory deficits in post-stroke vascular cognitive impaired Wistar rats.

Materials and Methods

Animals:

The animals were housed in polypropylene cages and maintained under standard laboratory environmental conditions; temperature 25°±2°C, 12 h light: 12 h dark cycle, and 50±5% relative humidity with free access to food and water *ad libitum*. All the experiments were carried out during the light period (08:00-18:00 h). The studies were carried out in accordance with the guidelines given by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi (India). The Institutional Animal Ethical Committee of KMC, Manipal approved the protocol of the study (IAEC/KMC/66/2010-2011).

Experimental protocol and choline supplementation:

8-12 month old Male Wistar rats (n=8 /group) weighing ~250-300 g were randomly allocated to the following groups

1. Group 1 – Normal control rats

2. Group 2 – Sham Bilateral Common Carotid Artery Occlusion surgery rats. In sham operated animals, identical volumes of physiological saline solution were administered orally for 30 days.

3. Group 3 – Bilateral Common Carotid Artery Occlusion (BCCAO) surgery induced chronic cerebral hypo-perfusion ischemic brain injured rats

4. Group 4 – Prophylactic Choline BCCAO Group-Rats were orally supplemented with Choline chloride (LOBA Chemie Pvt. Ltd, Mumbai, India) that was dissolved in normal saline (Dosage-4.6 mmol/kg/bodyweight/day) for 15 days as pretreatment and continued after the induction of BCCAO surgery for 30 days.

Experimental procedure:

Induction of Chronic cerebral hypoperfusion ischemic injury:

The food was withdrawn 12 h prior to surgical procedure and water was allowed *ad-libitum*.

The chronic cerebral hypoperfusion ischemic brain injury was induced in Wistar rats as described by Kim, Seul-Ki *et al* (14). Briefly, animals assigned to surgical group were anesthetized with intra-peritoneal injection with a cocktail of ketamine (50 mg/kg b.w) and xylazine (5 mg/kg b.w) with atropine sulphate (40 mg/kg b.w) and gentamycin (4.4 mg/kg b.w) used as pre-anesthetic medication for reducing secretions and infections respectively.

A midline incision was made and both common carotid arteries were exposed. Care was taken to avoid damage to the vagus nerves by separating them out using a glass rod. The carotid arteries were double ligated using silk sutures. During surgery, rectal temperature of the rat was maintained at 37°C-37.9°C with infra-red heating lamp.

In sham-operated group of rats, with the exception of occlusion of the carotid arteries, surgical procedures were the same as those in the BCCAO-operated rats.

Post-surgery, all animals were injected with 2 ml (i.p) of dextrose normal saline to prevent surgery induced hypoglycemia and dehydration. Body temperature was maintained at 37±1°C after the surgical procedure for 8 hour with an infrared lamp.

Appropriate post-operative care was provided by proper surgical wound dressing using betadine solution (Povidone ointment USP). During 15 days of recovery period, rats were maintained on a special platform designed to allow easy accesses to food and water. All the experimental animals were subsequently assessed for cognitive efficacy.

Cognitive assessment:

Behavioral Analysis by T-Maze test:

To assess the spatial learning ability, rats were subjected to spontaneous alternation and rewarded alternation tests on the T-Maze. The T-Maze consists of a start box, a stem, choice area and two arms.

At the end of two arms were the goal areas containing food pellets. The T-Maze was placed in a sound attenuated room.

Spontaneous alternation test:

Rats were starved for two days prior to the test in order to motivate them for food reward. Rats were placed in the T-maze for 30 minutes daily, for 2 days, to orient them to the T-maze environment. During these sessions 15-20 pellets of food were kept in each goal area. On the following 4 days, six trials were given daily. Percentage bias was calculated for each rat using the following formula:

$$\text{Percentage bias} = \frac{\text{Total number of choices of more frequently chosen side} \times 100}{\text{Total number of trials}}$$

More number of alternations and less % bias was considered as an index of improved learning ability.

Rewarded alternation test:

This test was done after completion of spontaneous alternation test. Test consisted of six trials per day for 4 consecutive days. Each trial had two runs viz. forced run and choice run. In the forced run, the rat was forced to one of the arms by blocking the other arm and allowing it to consume the pellet there. In the choice run, the forced arm was kept empty and pellet was placed in the opposite arm. Both the arms were kept free for the rat to run. Now the rat had to enter into the arm, opposite, to the forced arm, if it had to be considered as “correct response”. The forced arm was predetermined and it was same for all rats on any given day. It was changed on subsequent days. Experiment was repeated on four successive days. “Percentage of correct responses” was calculated for each rat by using the following formula :

$$\text{Percentage correct response} = \frac{\text{Total number of choices of correct response} \times 100}{\text{Total number of trials}}$$

Increase in mean% correct response was considered as improved learning and memory.

Passive avoidance test:

Modified procedure of Buresova and Bures (1983) was adopted. Briefly, on the first day of the test, each rat was allowed to explore the two compartments for 5 minutes. On the second day latency to enter the dark compartment for the first time was noted for each rat. The learning session was followed immediately. The plexiglass door between the two compartments was closed and the rat was confined to the dark compartment. Three inescapable electric foot shocks (50 Hz, 1.5 mA, 1 sec) were delivered to the rat. The rat was then returned to its home cage. Retention performance of each rat was tested by noting the latency to enter the dark compartment after a period of 48 hours. Increase in the latency to enter the dark compartment during retention test (i.e. 48 hrs) after inescapable foot shock, was interpreted as good retention performance.

Statistical analysis:

The results are expressed as mean±S.E.M. Statistical difference between means of groups were determined by one way analysis of variance (ANOVA) followed by Bonferroni’s test. The computations and diagrammatic representation of the data was performed by using SPSS software package version6 and Microsoft Excel. The differences were considered significant at $p<0.05$.

Results

Spatial learning and memory:

Prophylactic choline supplementation to ischemic brain injured rats improved their cognitive functions and their ability to learn on the spontaneous alternation test of the T maze by showing an increased mean number of alternations ($p=0.88$, $F=5.739$) and reduced mean percentage bias ($p=0.124$, $F=7.270$) compared to ischemic brain injured rats although the results were not statistically

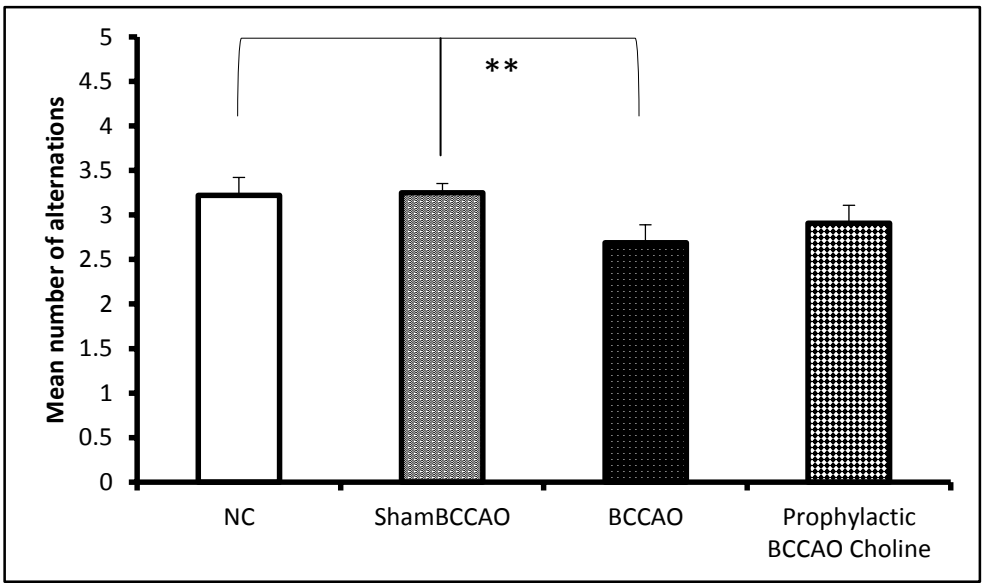

Fig. 1 : Mean (+SEM) number of alternations in the T maze of Normal Control (NC), Sham BCCAO, BCCAO- and Prophylactic BCCAO Choline group of rats (n=8 / group). There was no statistically significant difference in mean number of alternations between BCCAO and Prophylactic BCCAO Choline groups on the spontaneous alternation test.

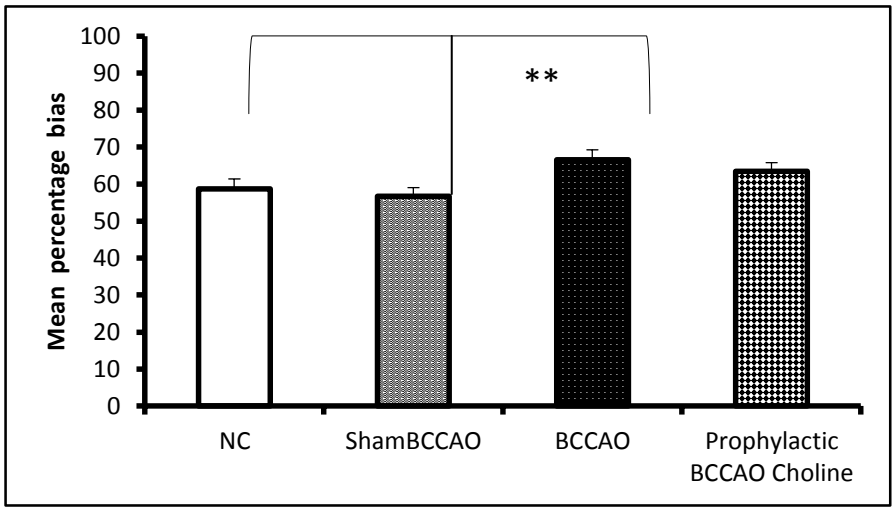

Fig. 2 : Mean (+SEM) % bias on the T maze of Normal Control (NC), Sham BCCAO, BCCAO and Prophylactic BCCAO Choline group of rats (n=8 / group). There was no significant difference in mean % bias between BCCAO and Prophylactic BCCAO Choline on the spontaneous alternation test.

significant (Fig. 1, 2 and Table I). Alternately, there was a significant deficit ($p< 0.01$) in the ability of BCCAO group of rats to learn the rewarded alternation test on the T maze as evidenced by the significant reduction in mean % of correct responses as compared to age matched sham BCCAO control rats (Fig. 3 and Table I). Supplementing choline prior to ischemic brain injury significantly preserved ($p=0.000$, $F=18.88$) their hippocampal based spatial learning ability in the T maze rewarded alternation test as compared to the same in non-supplemented ischemic brain injured rat groups.

Avoidance learning and memory retention

There was a significant deficit ($p<0.001$) in memory

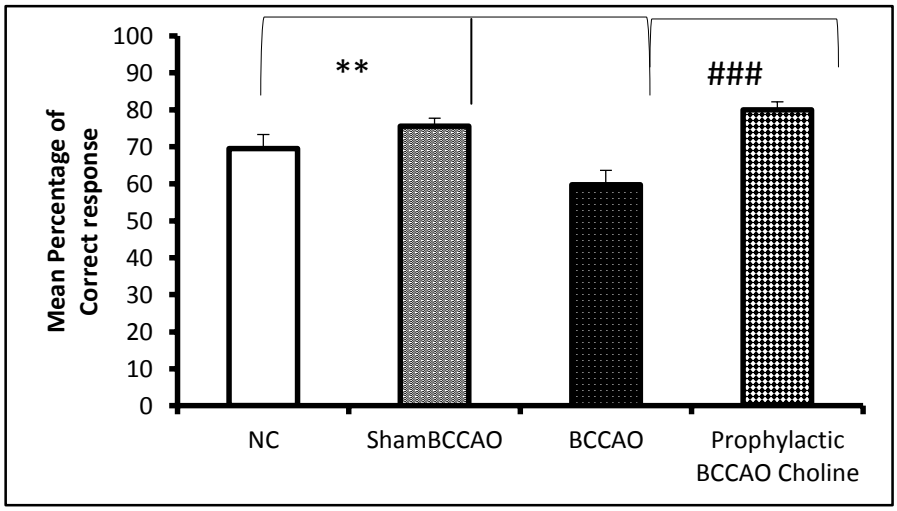

Fig. 3 : Mean (+SEM) % correct response on the rewarded alternation test using the T Maze of Normal Control (NC), Sham BCCAO, BCCAO and Prophylactic BCCAO Choline group of rats (n=8 / group). **p<0.01 significantly lower % correct responses by BCCAO group compared to Sham BCCAO group and ###p<0.001 significantly greater % correct responses by Prophylactic BCCAO Choline group compared to BCCAO group.

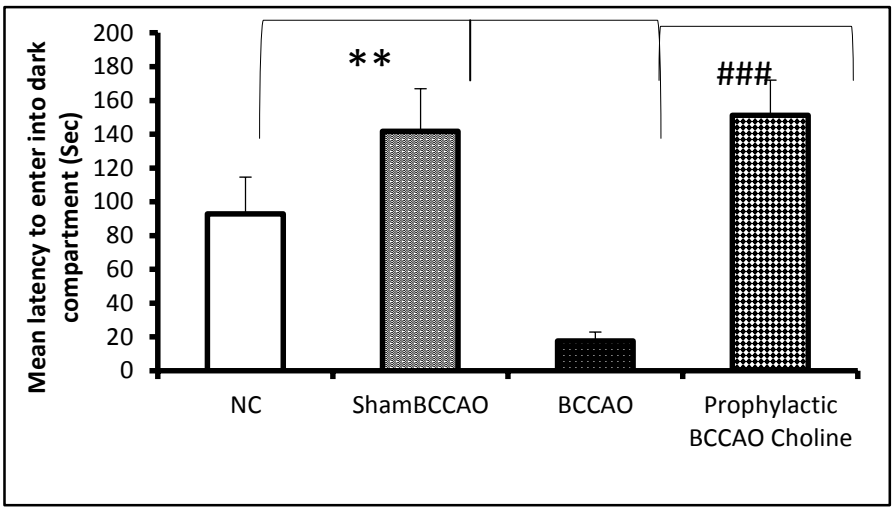

Fig. 4 : Mean (+SEM) latency to enter the dark compartment (seconds) on the passive avoidance test of Normal Control (NC), Sham BCCAO, BCCAO and Prophylactic BCCAO Choline group of rats (n=8 / group). Significantly lower latency to enter dark compartment by BCCAO group compared to Sham BCCAO group and significantly greater latency to enter dark compartment by Prophylactic BCCAO Choline supplemented group compared to BCCAO group was observed. **p<0.01, ###p<0001.

retention of untreated ischemic brain injured rats on amygdala based inhibitory avoidance task compared to normal control rats. Prophylactic choline supplementation to ischemic brain injured rats significantly preserved ($p<0.001$, $F=9.547$) memory retention compared to non-supplemented ischemic brain injured rats on the passive avoidance task (Fig. 4 and Table I).

Discussion

In BCCAO-Wistar rat model, it has been clearly documented that occlusion of carotid arteries on both sides deprives blood supply completely to anterior and partially to middle cerebral arteries causing white matter lesions and gliosis in frontal, parietal and

TABLE I: Effect of prophylactic BCCAO choline supplementation on spatial memory (T maze) and memory retention (passive avoidance test) in wistar rats.

Rat Groups (n=8)	*T-Maze*			*Passive avoidance*
	Spontaneous alternation test Mean±SEM		*Rewarded alternation test Mean±SEM*	
	Mean number of alternations	*Mean percentage bias*	*Mean percentage of correct response*	*Mean latency to enter into dark compartment (sec)*
NC	3.2±0.26	58.7±2.7	69.4±3.9	92.8±21.6
Sham BCCAO	3.2±0.16	56.7±2.3	75.5±2.2	141.5±25.23
BCCAO	2.1±0.17**	71.3±2.1**	51.5±2.7**	17.6±5.2**
Prophylactic BCCAO Choline	2.9±0.2	63.5±2.3	80±2.23###	151±20.7###
P-value	**NC/Sham BCCAO Vs BCCAO p=0.007/0.006 Prophylactic BCCAO Choline VS BCCAO p=0.88 F=5.739	**NC/Sham BCCAO Vs BCCAO p=0.005/0.001 Prophylactic BCCAO Choline VS BCCAO p=0.124 F=7.27	**NC/Sham BCCAO Vs BCCAO p=0.001/0.000### Prophylactic BCCAO Choline VS BCCAO p=0.000 F=18.88	**NC/Sham BCCAO Vs BCCAO p=0.05/0.001### Prophylactic BCCAO Choline VS BCCAO p=0.000 F=9.54

temporal lobes with degenerative changes in hippocampus (15).

In the present study, behaviorally a non-significant improvement on the spontaneous alternation test indicates either

1) a relatively lesser loss of neurons in septal-hippocampal system crucially needed for spontaneous alternation in hippocampal based spatial learning and memory (16, 17) or

2) BCCAO rats with an intact vestibular – basilar artery spares blood flow to all other structures like the cerebellum, brain stem nucleus including vestibular nucleus (15). It is possible that the vestibular and cerebellar systems can still acquire postural and motor cues for alternation to perform equally well in T-Maze spontaneous alternation task compared to normal controls,

3) the short inter-trial interval of one minute duration used in the current study was not sensitive enough to identify hippocampal damage significantly (16). Usually, in T-Maze spontaneous alternation, when two trials are given in quick succession (inter-trial interval is zero), on the second trial the rodent tends to choose the arm not visited earlier, reflecting the retention and retrieval of memory, indicating intact hippocampal based spatial learning and memory. Alternately, by increasing the inter-trial interval to more than 1 minute, the cognitive demands (memory load) can be increased, potentially increasing the chance of detecting even the mild cognitive impairment due to hippocampal lesions.

Alternation tests performed on the T-Maze are better at detecting hippocampal dysfunction, probably even better than the Morris water maze (16), (18), (19), (20). Previous studies have documented that hippocampectomized animals notoriously adopt side preferences, e.g., always turning right on the rewarded alternation test performed in a T-maze both during the choice and forced run.

Results of the present study on rewarded alternation test (Fig. 3 and Table I) indicate that ischemic brain injured animals seems to choose a specific side irrespective of forced or choice run during majority of the rewarded alternation trials thereby significantly reducing the mean percentage of correct response compared to sham control animals indicating a hippocampal lesion.

Further, supplementation of choline fifteen days prior to induction of cerebral ischemia significantly reduced ischemic brain injury-induced spatial learning and

memory deficits as assessed during the retention phase of cognitive evaluation, on the rewarded alternation test of the T maze. Guseva *et al* (21) has also shown a reduction in traumatic brain injury-induced spatial learning deficit on the Morris water maze, decrease in α7nAChR expression and brain inflammation following dietary choline supplementation.

Additionally in the passive avoidance task (Fig. 4 and Table I) the retention memory in chronic cerebral hypoperfusion rats were significantly poor compared to age matched normal controls. Ischemic brain injured rats have either failed to learn or failed to retrieve the memory of the previous unpleasant experience of the foot shock delivered in the dark compartment and demonstrated a poor retention memory as evidenced by short latency to enter into the dark compartment compared to age-matched normal controls.

It is well documented that hippocampus and amygdala have bilateral connections along with dynamic interactions to other brain structures like prefrontal cortex, entorhinal cortex, thalamus that are essentially involved in encoding and consolidation to strengthen emotionally charged memories (22), (23), (24), (25), (26), (27). Additionally ,various studies have also established that in the rat model of BCCAO, there is severe cerebral hypoperfusion to all regions of brain structures supported by carotid arteries which includes frontal cortex, amygdala, hippocampus and parts of basal ganglia (15). With these evidences, it is clear that in the present study, the BCCAO group of rats have severe deficit in learning and emotionally charged memory of the foot shock thus failing to avoid the dark compartment in the passive avoidance task.

Alternately, prior supplementation of choline to ischemic brain injured rats led them to delay/ completely avoid their entry into the dark compartment and persistently remain in brightly illuminated compartment despite its inherent tendency to hide in the dark, demonstrating significant retention of memory in passive avoidance task. Similarly, other studies have shown that choline succinate administration in experimental ischemic brain injured rats improved cognitive performance in passive avoidance task compared to untreated brain injured rats (28). A recent study by Pacelli *et al* (29) have also shown that dietary choline deprived rats were less capable of learning the active avoidance task compared to rats with normal diets, indicating that choline is essential for normal learning and memory retention and retrieval. Zhao *et al* (30) have shown that intra-peritoneal citicoline administered to focal ischemic brain-injured Wistar rats exhibited significant reduction in mean escape latency and also spent significantly more time in the former platform quadrant in water maze compared to untreated focal ischemic brain injured rats.

In the present study prophylactic choline supplementation to rats is observed to have attenuated post-stroke vascular cognitive impairments. This could be possibly due to the augmentation of endogenous antioxidant mechanisms via Ado-Met pathway. Adibhatla et al (31) have shown that choline can be metabolized to glutathione (GSH) through the Ado-Met pathway which provides significant neuro-protection in ischemia induced oxidative stress. Further choline also attenuates lipid peroxidation induced neuronal membrane breakdown. Previous studies by Menku et al (32) have shown that propofol and citicoline combination therapy significantly reduced the malonyldialdehyde (MDA) levels of brain in rat model of traumatic brain injury. Moreover choline also inhibited the activation of phospholipase A2 (PLA2) and hydroxyl radical generation in ischemic cascade and thereby prevents phospholipid membrane breakdown. Adibhatla et al (33) have shown that citicoline, inhibits mitochondrial PLA2 activity following cryogenic brain injury in rabbits. Additionally increased choline availability may also prevent auto-cannibalism of neuronal membrane phospholipids (31). *In-vitro* studies by Adibhatla et al (33) has shown that choline deficiency in neurons causes loss of neuronal membrane phosphatidylcholine and sphingomyelin, resulting in apoptosis. Wurtman et al (34) have shown that ischemic biochemical cascade due to glutamate excitotoxicity can lead to depletion of ACh. Re-synthesis of ACh requires choline that is derived from breakdown of neuronal membrane phospholipid (PtdCho) which is referred to as auto-cannibalism.

Conclusion:

Prophylactic dietary choline supplementation minimizes post stroke cognitive impairment in the event of ischemic brain injury. This could be due to the role of choline in augmenting the efficacy of endogenous brain defence and neuronal repair mechanisms.

Based on clinical evidences of safety on oral choline supplementation and its risk benefit ratio, dietary choline supplementation could be suggested for both short and long term neuroprotection to high risk individuals (patients with transient ischemic attack, patients posted for valvular replacement surgeries etc).

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