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Research Article

Prophylactic combined supplementation of choline and docosahexaenoic acid attenuates vascular cognitive impairment and preserves hippocampal cell viability in rat model of chronic cerebral hypoperfusion ischemic brain injury

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ABSTRACT

Background: Stroke is the second cause of mortality in the world and third leading cause of disability in surviving victims. Cerebral ischemic cascade involves multiple pathways that can result in motor and cognitive deficits. The current treatment strategy focuses mainly on motor recovery, and the management of post-stroke cognitive impairment is largely neglected. Similarly, very few studies have explored the prophylactic combined synergetic treatment strategies that have the potential to target multiple pathways in the ischemic cascade to alleviate vascular cognitive impairment (VCI) in the event of an ischemic stroke. Choline and docosahexaenoic acid (Cho-DHA) are both essential neuronal membrane phospholipid precursors, known to be important in enhancing cognitive functions. The objective of present study was to explore the prophylactic efficacy of combined Cho-DHA supplementation (Cho-DHA suppl.) in attenuating VCI in a rodent model of ischemic brain injury.
Methods: An 10-months-old male Wistar rats were subdivided into four groups (n=8/group); normal control (NC), bilateral common carotid artery occlusion (BCCAO) induced ischemic brain injury group, sham BCCAO (S-BCCAO) group, and prophylactic combined Cho-DHA suppl. BCCAO group. Subsequently, all groups of rats were tested for cognition and neuro-morphological changes in the hippocampus.
Results: BCCAO rats showed significant learning and memory deficits ($p<0.05$) and neuronal injury compared to S-BCCAO and NC rats. These cognitive deficits and neuronal injury were significantly ($p<0.01$) attenuated in Cho-DHA suppl. BCCAO rats.
Conclusion: Prophylactic combined Cho-DHA suppl. may be envisaged as an effective preventive strategy to attenuate VCI and neuronal injury in high-risk individuals susceptible for a future event of an ischemic stroke.

Keywords: Chronic cerebral hypoperfusion brain injury, Combined choline and docosahexaenoic acid dietary supplementation, Stroke, Vascular cognitive impairment

INTRODUCTION

Stroke is the second leading cause of mortality in the world and third most common cause of disability in surviving victims.[1] Stroke incidence between 1970 and 2008 have increased by 100% in the low-income group of countries and reduced to 40% in high-income countries. Effective management of risk factors for ischemic stroke and its allied diseases by a high-income group of countries are found to be the primary cause for this change in trend.[2] Most of the post-stroke intervention programs predominantly focus on motor recovery and management of post-stroke vascular cognitive impairment (VCI) gains least attention even among the health care professionals. Moreover, cerebral ischemic cascade involves multiple complex biochemical pathways that are injurious to neurons leading to the overwhelming motor and cognitive deficits. Hence, therapeutic strategies targeting a single biochemical entity in an ischemic cascade may not effectively attenuate the neuronal injury. To overcome this, strategies exploring prophylactic synergetic neuroprotective treatments, which have the potential to target multiple biochemical pathways in the ischemic cascade may help to effectively enhance the ischemic threshold of neural cells and minimize VCI or other cerebral ischemic complications in the event of potential stroke.

Choline a non-toxic quaternary amine and is predominantly utilized for the synthesis of phosphatidylcholine (PC) in liver and brain that may be otherwise synthesized *de novo* by phosphatidylethanolamine methyltransferase (PEMT) enzyme activity.[3-5] Egg yolk, beef, chicken meat, and soyabean oil, are rich sources of choline in diet and it is also available as an oral supplement. Oral supplementation of choline increases its bioavailability as it is readily absorbed in the gut and crosses the blood–brain barrier. Cholinergic neurons in the brain, store a large pool of choline as choline-phospholipids and are readily available for acetylcholine (ACh) synthesis when required.[3] In addition, choline is also a precursor for the biosynthesis of PC, phosphatidylethanolamine, sphingomyelin, and other neural and sub neural membrane phospholipids. In addition, these neurons have a dynamic balance for utilization of choline in the two metabolic pathways that involves the synthesis of PC and ACh.[6,7] These two pathways compete for the available choline, with acetylation being favored when neurons are physiologically active[6] and if ACh is depleted due to glutamate excitotoxity as in cerebral ischemia, choline phospholipids, especially PC, is hydrolyzed to provide a source of choline. This phenomenon is called as auto-cannibalism.[8] As the central cholinergic system plays a crucial role in learning and memory,[6] any disruption to this system would lead to cognitive deficits. Similarly, neural cell membranes also contain lipids concentrated in amino phospholipids, including chiefly essential omega-3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).[9] Various studies on animals have shown that reduced DHA is associated with impairments in cognitive, behavioral, and motor performance.[10,11] A study on rodent model of traumatic brain injury has shown that pre-injury supplementation of DHA reduced the injury response and improved memory when compared to non-DHA supplemented (DHA suppl.) group.[12] Based on these studies, we hypothesize that combined Cho-DHA prior to chronic cerebral hypoperfusion injury will preserve cognitive functional integrity.

METHODS

Animals

An 8-12 months old male Wistar rats (250-300 g) were used for the study. Animals were housed in polypropylene cages and maintained under standard laboratory environmental conditions; temperature 25±2°C, 12 hrs light: 12 hrs 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 hrs). 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, New Delhi (India). The Institutional Animal Ethical Committee of KMC, Manipal approved the protocol of the study.

Experimental protocol for Cho-DHA supplementation

Male Wistar rats (n=8/group) were randomly allocated to the following groups.

1. Group1: normal control rats (NC)
2. Group 2: sham bilateral common carotid artery occlusion surgery rats (S-BCCAO): the common carotid arteries were exposed and separated from vagus but not occluded permanently and these rats were orally supplemented with of saline instead of Cho-DHA.
3. Group 3: BCCAO surgery-induced chronic cerebral hypoperfusion ischemic brain-injured rats, and these rats were also orally supplemented with of saline instead of Cho-DHA.
4. Group 4: prophylactic Cho-DHA suppl. BCCAO group rats, were orally supplemented with choline chloride (4.6 mmol/kg/bodyweight [BW]/day), (Loba Chemie, India), and DHA (300 mg/kg/day) (Nouveau Medicament Pvt., Ltd., India) via oral gavage for 15 days as pre-treatment and continued for 30 days after the induction of BCCAO surgery, till they are euthanized for analyzing neuronal morphology.

Induction of chronic cerebral hypoperfusion ischemic injury

The food was withdrawn 12 hrs prior to the surgical procedure, and water was allowed *ad-libitum*. The chronic cerebral hypoperfusion ischemic brain injury was induced in Wistar rats as described by Kim et al., 2008.[13] Briefly, animals assigned to surgical group were anesthetized with ketamine (50 mg/kg BW IP)-xylazine (5 mg/kg BW IP)

cocktail and with atropine sulfate (130 mg/kg BW) and gentamycin (4.4 mg/kg BW) as pre-anesthetic medication. A midline incision on the neck was made to expose common carotid arteries, and they were carefully separated from the vagus and other connective tissues. Then both carotid arteries were double ligated with silk suture permanently to deprive blood supply to brain completely via carotid circulation. In a 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. Appropriate post-operative care was provided, and all the rats were subsequently assessed for their cognitive efficacy.

Cognitive assessment

Spatial learning test (T-Maze)

To assess spatial learning ability and memory retention, rats were subjected to spontaneous alternation and rewarded alternation tests on the T-Maze as described in Rai et al. 2001.[14] All behavioral tests in the T-Maze were carried out in a sound attenuated room.

Spontaneous alternation test

Rats were starved for 2 days prior to the test in order to motivate them for a food reward. Each rat was placed for 30 mins daily, for 2 days, to orient them to the T-Maze environment. During these sessions, 15 pellets of food were kept in each goal area. On the following 4 days, six trials were given daily with the food pellets in each goal area. The total number of alternations made by the rat was noted, and percentage bias was observed as more frequently chosen side by the rat was calculated for each rat.

Rewarded alternation test

The test consisted of six trials per day for 4 consecutive days. Each trial had two runs *viz*. forced run and choice run. During 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. During the choice run, the forced arm was kept empty, and food pellet was placed in the opposite arm. Both the arms were free for the rat to run. Now the rat had to enter into the arm, opposite to the forced arm, where the pellet is placed 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. The experiment was repeated on four successive days. "Percentage correct responses" were calculated.

Passive avoidance test

Passive avoidance test was performed as explained in Rai et al. 2001.[14] Briefly, on 1st day of the test, each rat was allowed to explore the two compartments of the passive avoidance apparatus for 5 mins. On 2nd 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 hrs.

Morphological Assessment

Processing of brain tissue for cresyl violet staining

Subsequent to the cognitive assessment, rats from all aforementioned groups were deeply anesthetized with high doses of ketamine-xylazine injection IP. All rats were transcardially perfused with equ-volumes of heparinized saline and 10% formalin. After perfusion, rats were decapitated, and brains were removed, embedded in paraffin blocks according to standard protocols. 5 µm thick coronal sections of the brain were obtained serially at the level of the hippocampus using rotary microtome, which were then mounted on albumin coated slides and stored at 4°C for further use.

Nissl staining of hippocampus

Nissl staining method was used for morphological evaluation of the extent of neural damage in the hippocampus of rats of all the groups. Every 15th best brain sections in the series were chosen and processed for cresyl violet staining according to the standard protocol.[15,16]

Qualitative assessment of neurons in sub-regions of the hippocampus.

Neurons in CA1, CA2, CA3, and CA4 subregion of the hippocampus were viewed under the Motic Red 200 microscope, mounted with Moticam 580-5.0 mp color digital camera with an image analysis system driven by Motic Images-Plus 2.0 software. The calibration was done with the provided Motic standard stage micrometer. High-resolution high-power (×40 objective) digital photomicrographs were captured and used for determining the nuclear area and density of pyramidal cells of subregion of the hippocampus. The neurons of hippocampal sub-regions with well-defined nuclear and cell membrane and clearly visible nucleoli were considered as surviving and irregularly shaped hyper dense shrunken cells with pyknotic nuclei were considered as non-viable neuronal cells. The neuronal architecture was compared between NC, BCCAO, S-BCCAO, and prophylactic Cho-DHA suppl. BCCAO groups from the representative photomicrographs.

Statistical analysis

Results are expressed as a mean±standard error of the mean. The statistical difference in means between groups

was determined by one-way analysis of variance followed by *post-hoc* Bonferroni test using GPIS software. The computations and diagrammatic representation of data were performed using Microsoft Excel. The differences between groups were considered as significant at $p<0.05$.

RESULTS

Spatial learning and memory

In the current study, prophylactic dietary supplementation with both Cho-DHA to ischemic brain-injured rats significantly retained their cognitive functions and ability to learn on the spontaneous and rewarded alternation test of the T-Maze, as well as on the passive avoidance test. Cho-DHA suppl. rats showed a significant increase in mean number of alternations (prophylactic Cho-DHA=3.68±0.1 vs. BCCAO=2.59±0.2; $p<0.01$), (Figure 1) and reduced mean percentage bias (prophylactic Cho-DHA=58.81±1.2 vs. BCCAO=68.73±2.9; $p<0.01$), (Figure 2) compared to age-matched ischemic brain-injured rats (Figures 1 and 2). In addition, there was a deficit in the ability of BCCAO group (59.7±3.8) of rats to learn on the rewarded alternation test of the T-Maze, as evidenced by reduction in mean % of correct responses compared to age-matched NC (69.4±3.9), (Figure 3). Whereas ischemic brain-injured rats supplemented prophylactically with both Cho-DHA showed a significant increase (80.1±4.6; $p<0.005$) (Figure 3) in mean % of correct responses as compared to age-matched non-supplemented BCCAO rats. Supplementing with both Cho-DHA prior to ischemic brain injury significantly preserved hippocampal based spatial learning ability on the T-Maze task as compared to non-supplemented ischemic brain-injured rats.

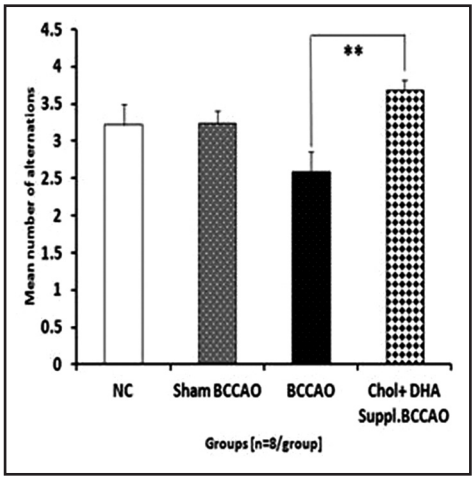

Figure 1: Rat groups-normal control, sham-bilateral common carotid artery occlusion (S-BCCAO) control, BCCAO - and choline and docosahexaenoic acid supplemented (Cho-DHA suppl.) BCCAO - prophylactic combined Cho-DHA suppl. to BCCAO rats (n=8/group). Values are expressed as a mean±standard error of the mean number of alternations on the spontaneous alternation test using the T-Maze and statistically analyzed using one-way analysis of variance followed by Bonferroni test. **p<0.01, (Cho-DHA suppl. BCCAO=3.68±0.1 vs. BCCAO=2.59±0.2).

Passive avoidance learning and memory retention

There was a significant deficit in memory retention of ischemic brain-injured rats on amygdala based inhibitory avoidance task compared to the same in sham control rats (24.6±6 vs. 122.3±24 sec; $p<0.01$) (Figure 4). Prophylactic supplementation of combined Cho-DHA to ischemic brain-injured rats significantly attenuated their learning deficits and also allowed for significant preservation of their ability to retain avoidance memory (153.7±12 sec; $p<0.001$), (Figure 4) compared to non-supplemented ischemic brain-injured rats on the passive avoidance task.

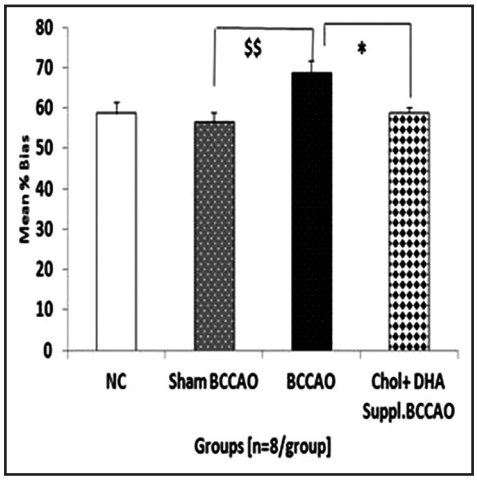

Figure 2: Rat groups-normal control, sham bilateral common carotid artery occlusion (S-BCCAO) control, BCCAO - and choline and docosahexaenoic acid supplemented (Cho-DHA suppl.). BCCAO - prophylactic combined Cho-DHA supplemented to BCCAO rats (n=8/group). Values are expressed as a mean±standard error of the mean. % Bias on the spontaneous alternation test using the T-Maze and statistically analyzed using one-way analysis of variance followed by Bonferroni *post-hoc* test. $^{\$\$}p<0.01$ (S-BCCAO=56.71±2.3 vs. BCCAO=68.73±2.9) and *p<0.05, (Cho-DHA suppl. BCCAO=58.81±1.2 vs. BCCAO=68.73±2.9).

Qualitative analysis of the hippocampal neuronal cells

CA1 region of the hippocampus

CA1 subregion of the hippocampus, as represented in Figure 5a-d photomicrograph, were observed to have a clear and intact 2-3 layers of pyramidal shaped neuronal cell bodies in hippocampal sections from both NC and S-BCCAO group of rats. Alternately, significant neuronal damage with

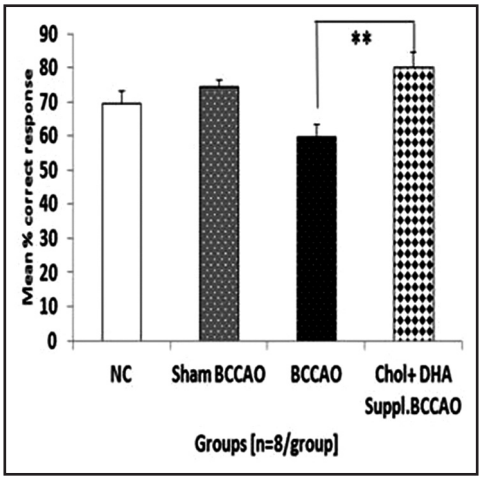

Figure 3: Rat groups-normal control, sham bilateral common carotid artery occlusion (S-BCCAO) control, BCCAO - and choline and docosahexaenoic acid supplemented (Cho-DHA Suppl.). BCCAO - prophylactic combined Cho-DHA suppl. to BCCAO rats (n=8/group). Values are expressed as a mean±standard error of the mean. % correct response on the rewarded alternation test using T-Maze. Values are further statistically analyzed by one-way analysis of variance and Bonferroni *post-hoc* test. **p<0.01, (Cho-DHA suppl. BCCAO 80.1±4.6 vs. BCCAO 59.7±3.8).

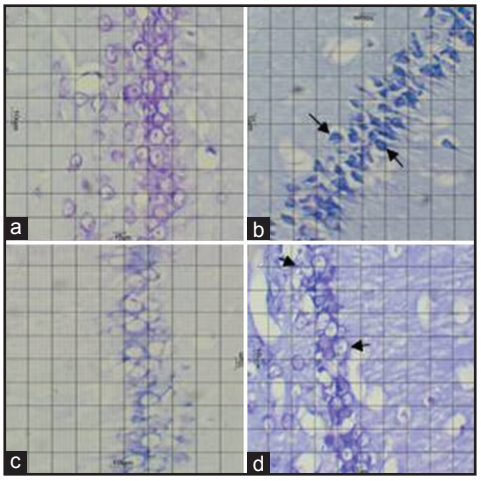

Figure 5: (a-d) Representative photomicrographs of CA1 region of the hippocampus in rat groups - (a) normal control, (b) bilateral common carotid artery occlusion (BCCAO), (c) Sham BCCAO (S-BCCAO), (d) choline and docosahexaenoic acid supplemented (Cho-DHA Suppl.). BCCAO - prophylactic combined Cho-DHA suppl. to BCCAO rats (n=8/group). Note: Arrow in Figure 5c represents hyperdense dead CA1 neurons in BCCAO group and in Figure 5d represents structurally intact surviving CA1 neurons in Cho-DHA suppl. BCCAO group.

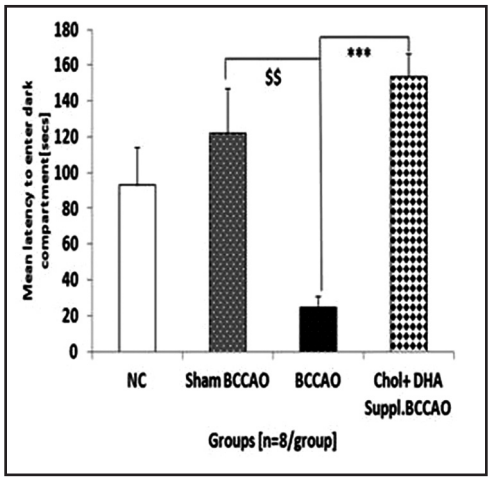

Figure 4: Rat groups-normal control, sham bilateral common carotid artery occlusion (S-BCCAO) control, BCCAO - and choline and docosahexaenoic acid supplemented (Cho-DHA suppl.). BCCAO - Prophylactic combined Cho-DHA supplemented to BCCAO rats (n=8/group). Values are expressed as a mean±standard error of the mean latency to enter dark compartment (seconds) on the passive avoidance test. Values are further statistically analyzed by one-way analysis of variance and Bonferroni *post-hoc* test. $^{$$}$p<0.01, (S-BCCAO=122.37±24.56 sec vs. BCCAO=24.65±6.35 sec), ***p<0.001 (Cho-DHA suppl. BCCAO 153.7±12 sec vs. BCCAO 24.6±6 sec).

shrunken neuronal cell bodies, hyperdense neuronal soma cells, and few pyknotic nuclei were observed in hippocampal sections from BCCAO rats as compared to the same in normal and S-BCCAO rats. Supplementation of combined Cho-DHA prior to BCCAO surgery in rats was observed to have better preserved neuronal cell viability compared to the same in age-matched BCCAO groups of rats.

CA2 region of the hippocampus

Microscopic examination of the representative photomicrographs of hippocampal CA2 subregion (Figure 6a-d) showed a reduction in the number of neuronal cell layers and cell size in hippocampal sections from BCCAO rats compared to the same in NC, S-BCCAO and prophylactic Cho-DHA suppl. groups.

CA3 region of the hippocampus

Compared to other regions of the rodent hippocampus, CA3 subregion is quite broad and has an approximately 4-5 layer of pyramidal cells and are more vulnerable to stress. Microscopic examination of the hippocampal sections from NC, S-BCCAO group of rats are observed to have clear spherical or pyramidal-shaped viable neuronal cells with distinct nucleus, cytoplasm and cell membrane, forming 4-5 layers (Figure 7a-d). Furthermore, cells are relatively densely packed with minimal inter-cell distance. Contrarily, CA3

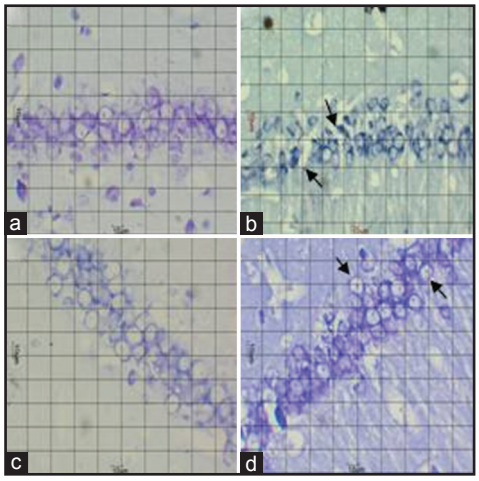

Figure 6: (a-d) Representative photomicrographs of CA2 region of the hippocampus in rat groups - (a) normal control, (b) bilateral common carotid artery occlusion (BCCAO), (c) Sham BCCAO (S-BCCAO), (d) choline and docosahexaenoic acid supplemented (Cho-DHA Suppl.). BCCAO - Prophylactic combined Cho-DHA suppl. to BCCAO rats (n=8/group). Note: Arrow in Figure 6c represents shrunken CA2 neurons with a pyknotic nucleus in BCCAO group and in Figure 6d represents surviving CA2 neurons in Cho-DHA suppl. BCCAO group.

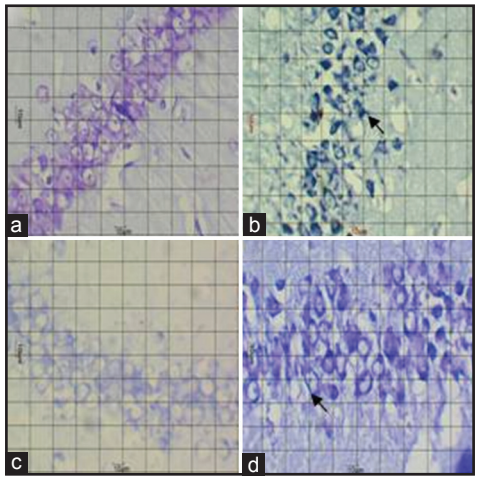

Figure 7: (a-d) Representative photomicrographs of CA3 region of the Hippocampus in rat groups – (a) normal control, (b) bilateral common carotid artery occlusion (BCCAO), (c) Sham BCCAO (S-BCCAO), (d) choline and docosahexaenoic acid supplemented (Cho-DHA Suppl.). BCCAO - Prophylactic combined Cho-DHA suppl. to BCCAO rats (n=8/group). Note: Arrow in Figure 7c indicates irregularly shaped hyperdense non-viable CA3 neuronal cell bodies in BCCAO group and in Figure 7d indicates clear spherical or pyramidal-shaped viable CA3 neuronal cells with distinct nucleus, cytoplasm, and cell membrane in Cho-DHA suppl. BCCAO group.

neuronal cells from the hippocampal sections of BCCAO group of rats were observed to have lesser cell density, with shrunken, irregularly shaped hyperdense cells with almost no distinct boundary between nucleus and cytoplasm. In addition, layers of CA3 neuronal cells in BCCAO group were observed to have relatively more spaces between them indicating the reduced cell size or cerebral edema. Alternately with fewer dead cells, better preservation of CA3 neuronal cell morphology was observed in prophylactic Cho-DHA suppl. group.

CA4 region of hippocampus

CA4 sub-region of the hippocampus is interposed between the two blades of dentate gyrus with 3-4 layers of scattered neuronal cells, less densely packed. In the present study, microscopic examination of neural cells in the CA 4 sub-region of the hippocampus was observed to have significant neuronal damage in BCCAO group of rats with least cell density as compared to the same in NC and S-BCCAO rats (Figure 8a-d). Prophylactic supplementation of combined Cho-DHA to rats preserved CA4 neuronal cells to a small extent although many CA4 neural cells appeared to be non-viable.

DISCUSSION

Extensive studies on Wistar rat model of BCCAO have clearly documented that occlusion of carotid arteries on both the sides deprives blood supply completely to anterior and partially to middle cerebral arteries causing white matter lesions and gliosis in frontal, parietal, and temporal lobes with degenerative changes in the hippocampus.[17] In the present study, Wistar rats with chronic cerebral hypoperfusion brain injury-induced by permanent BCCAO surgery have significant deficits ($p<0.01$) in both hippocampal based spatial learning and memory retention abilities and amygdala based avoidance memory retention abilities as compared to age-matched normal and S-BCCAO rats. Studies show that alternation tests performed in the T-Maze are better at detecting hippocampal dysfunction, probably even better than the Morris water maze. Previous studies have also documented that hippocampectomized animals notoriously adopt side preferences, e.g. always turning right on the alternation test performed in a T-Maze.[18,19] Results of our current study, on spontaneous alternation test (Figures 1 and 2) demonstrates that BCCAO group of animals have a high percentage bias, and less number of alternation compared to the same in age-matched NC. Similarly, ischemic brain-injured animals were observed to choose consistently a specific side irrespective of forced or choice run on the rewarded alternation trials. This significantly reduced the mean percentage correct response, indicating spatial memory deficits in BCCAO rats (Figure 3) compared to the same in age-matched NC and sham control animals.

In our previous study, supplementation of choline alone prior to BCCAO induced ischemic brain injury in rats did

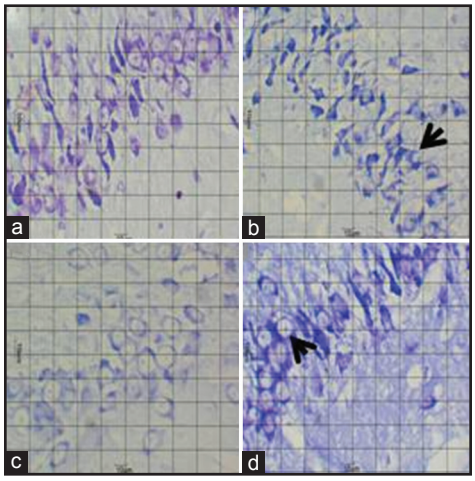

Figure 8: (a-d) Representative photomicrographs of CA4 region of the hippocampus in rat groups – (a) normal control, (b) bilateral common carotid artery occlusion (BCCAO), (c) Sham BCCAO (S-BCCAO), (d) choline and docosahexaenoic acid supplemented (Cho-DHA Suppl.). BCCAO - Prophylactic combined Cho-DHA supplemented to BCCAO rats (n=8/group). The arrow in Figure 8c indicates irregularly shaped non-viable CA4 neuronal cell bodies in BCCAO group, and Figure 8d indicates few of the surviving neuron in CA4 region of Cho-DHA suppl. BCCAO group.

not significantly preserve hippocampal based spatial learning ability on the spontaneous alternation test of the T-Maze although they did improve preservation of spatial learning on the rewarded alternation test of T-Maze as compared to the same in non-supplemented ischemic brain-injured rats.[20] This is consistent with studies by Guseva et al.,[21] where in dietary choline supplementation was observed to reduce traumatic brain injury-induced spatial learning deficit on the Morris water maze and reduced expression of cortical injury-induced α7nAChR and brain inflammation in these animals. Alternately, it is also observed that dietary choline deprived rats were less capable of learning active avoidance task compared to rats with normal diets, indicating that choline is essential for normal learning and memory retention and retrieval.[22] Moreover, Zhao et al.[23] have shown that intraperitoneal citicoline administration to focal ischemic brain-injured Wistar rats significantly reduced their mean escape latency and also significantly increased time spent in the former platform quadrant in water maze compared to untreated focal ischemic brain-injured rats.

Alternately in the current study, synergetic supplementation with both Cho-DHA prior to chronic cerebral hypoperfusion brain injury in rats significantly preserved their spatial learning and memory abilities. This is evident from their enhanced cognitive performance on the T-Maze, with an increase in mean number of alternations, decrease in mean % bias on spontaneous alternation test (Figures 1 and 2) and increase in percentage of correct response on rewarded alternation test (Figure 3) compared to the same in BCCAO rats. A recent study also shows that third generation DHA depleted mice on traumatic brain injury were observed to have poor motor cum cognitive recovery with exacerbated neuronal death and reduced NeuN positive cells compared to normal mice.[10] Alternately, dietary supplementation with DHA prior to traumatic brain injury reduces injury response, as measured by axonal injury counts, apoptosis, and memory assessment by water maze testing.[12]

In addition, in the current study, chronic cerebral hypoperfusion injured rats were found to be significantly poor in retaining avoidance memories (Figure 4) on the passive avoidance task, compared to the same in NC rats. BCCAO rats have either failed to learn or failed to retrieve memory of the previous unpleasant experience of foot shock delivered in the dark compartment and demonstrates poor retention memory as evidenced by short latency to enter the dark compartment compared to the same in NC. Studies show that consolidation and retrieval of memory in a step-down inhibitory avoidance task requires integrated and sequential activity of the hippocampus, amygdala, entorhinal, parietal, and prefrontal cortical structures.[24] Several studies of BCCAO in rodents have also documented injuries to the amygdala, hippocampus, and white matter lesions with gliosis to prefrontal and parietal lobes.[17] Similarly, in the present study, BCCAO rats possibly have extensive injuries in most of these regions, leading to alterations in integration and sequence for the observed failure of learning or memory retrieval in the passive avoidance task. Alternately, supplementation of both Cho-DHA to rats prior to ischemic brain injury significantly enhanced their abilities to learn or retain avoidance memories of the unpleasant experience of foot shock delivered in the dark compartment. This is evident from the significantly increased mean latency (in seconds) to enter dark compartment by the supplemented ischemic injured rats compared to the same in non-supplemented chronic cerebral hypoperfusion brain-injured rats. Earlier studies have documented that stroke-prone spontaneously hypertensive rats with depleted choline concentrations in hippocampus perform poorly in passive avoidance task and dietary supplementation of DHA significantly improves both hippocampal choline-Ach levels and enhances memory retention in passive avoidance task[25] whereas synergetic supplementation of combined choline chloride and glucose improved passive avoidance behavior and Ach concentration in hippocampus of mice.[26]

In our present study, histopathological screening of cresyl stained brain sections from BCCAO rats confirms extensive neurodegenerative changes in CA1, CA2, CA3, CA4 sub-regions as documented in earlier studies.[17] Further studies on vulnerability of different subregions of hippocampus to ischemia indicates that even a brief period of transient ischemia causes damage to CA1 region and prolonged global cerebral hypoperfusion in gerbils kills almost 96% of CA1 neurons by the 4th day. Alternately, CA3 and few other interneurons of the hippocampus are relatively resistant to acute transient ischemia but susceptible to long term

hypoperfusion.[27] The degeneration of CA1 interneurons and CA3 pyramidal cells may also occur secondary to ischemia-related delayed neuronal cell death of CA1 pyramidal neurons.[28]

Representative photomicrographs of our current study have shown that prophylactic combined Cho-DHA suppl. to BCCAO rats have prevented neurodegeneration, improved neuronal survival and cell density in CA1, CA2, CA3, and CA4 regions of hippocampus compared to non-supplemented BCCAO rats. This is consistent with earlier studies on forebrain ischemic brain-injured gerbils supplemented with cytidine 5′-diphosphocholine (CDP)-choline, where it was found that choline moiety of CDP-choline and not cytidine was involved in neuroprotection of hippocampal CA1 region compared to the same in untreated transient forebrain ischemic gerbils.[29] Previous studies on rat model of traumatic brain injury treated with CDP-choline also reported reduced hippocampal neuronal damage in CA2 and CA3 sub-regions, cortical contusion volume, and neurological dysfunction in rats.[30] Similarly, studies have shown that rats with cerebral ischemia supplemented with fish n-3 fatty acids (DHA and EPA) along with their regular food had significantly reduced tunnel positive apoptotic neurons in CA1, CA2, CA3, and dentate gyrus hippocampal sub-regions compared to the same in untreated rats.[31] Further, studies also report that oral pretreatment with ethyl-DHA significantly attenuates ischemia- reperfusion-induced delayed neuronal death in CA1 sub-region of gerbil hippocampus.[32] Moreover, studies have also shown that a higher choline intake increases PEMT activity resulting in greater PC-DHA enrichment of the PC molecule whereas higher DHA intake in the absence of PEMT activity as in PEMT-/- mice influences PC-DHA, PS-DHA, and PI DHA that restores fetal hippocampal neurogenesis.[33] Thus with increased prophylactic availability of both choline-DHA and greater PC-DHA enrichment of the PC molecule, neural membranes may be largely better stabilized to withstand injurious effects of chronic cerebral hypoperfusion and promote neuronal repair by enhancing neural plasticity, neurogenesis that might attenuate cognitive deficits.

Thus, the role of prophylactic combined Cho-DHA suppl. in providing neuroprotection to attenuate VCI in ischemic brain-injured rats are also supported by some of the following previous researchers. Prior supplementation of both Cho-DHA increases the source of substrates for neural cell membrane biosynthesis in injury-induced neurogenesis in the dentate gyrus,[34] CA1 and other subregions. This helps to restore complex circuits of the hippocampus, as well as normal neural signaling, thereby improving the functionality of hippocampal based spatial learning and memory.[35] Further, metabolites of Cho-DHA augment brain antioxidant mechanisms and thereby prevent neuronal damage during the ischemic cascade.[8,36] Studies also show that ischemic biochemical cascade leads to glutamate excitotoxicity that results in depletion of ACh, which further causes choline to be derived from hydrolyzing neuronal membrane phospholipid called as "auto-cannibalism." Thus, exogenous choline supplementation helps to replenish brain Ach levels during ischemic cascade induced excitotoxicity and prevents the need for auto cannibalism.[37]

CONCLUSION

Post-stroke VCI is more common than the recurrence of stroke itself. Results of our present study confirm the need for supplementing two important neural membrane phospholipid precursors - Cho-DHA that prevents the risk of post-stroke VCI by providing synergetic neuroprotection to the hippocampal neural cells. Thus, dietary supplementation of both Cho-DHA is an easy and cost effective method to provide prophylactic neuroprotection in a high-risk population for cerebro- vascular events leading to cognitive deficits. Such a combined prophylactic dietary supplementation may also benefit patients undergoing coronary artery bypass grafting surgery, carotid endarterectomy, endovascular therapy, and cardiac valvular replacement surgeries, who are at risk of developing ischemic brain injury as a complication during the procedure. Further, translational research with large scale clinical trials and population-based studies are needed to ascertain the true advantage of this supplementation to at-risk geriatric and middle-aged groups for meaningful public health benefits. Subsequent to confirmatory studies, both DHA and choline may be fortified with regular health drinks to supplement the high-risk.

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