ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Two Stage Successive Carotid Artery Occlusion Surgeries in Wistar Rat Reduce its Mortality and Depicts as a Better Model for Vascular Dementia.

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ABSTRACT

Chronic cerebral hypoperfusion induced vascular dementia (VD) constitutes a major cause for dementia in the geriatric population. Permanent-bilateral common carotid artery occlusion (P-BCCAO) in Wistar rat models are considered as accepted models for preclinical research on VD. It has been documented that the P-BCCAO rats undergo a brief period of acute severe ischemia immediately following P-BCCAO surgery. This offers a significant disadvantage in using this model to study chronic cerebral hypoperfusion induced VD in human beings. Moreover, the survival rate of this rat model is relatively low compared to other animal models being used for similar studies. Ten months-old male Wistar rats (n=12/group) were randomly divided into 4 groups as normal control, Sham-BCCAO, one stage P-BCCAO and two stage P- BCCAO. All rats were subjected to spatial and avoidance learning memory tasks and their brains were subjected to histopathalogical analysis. Both P-BCCAO groups of rats showed significant deficits ($p < 0.05$) in spatial and avoidance learning -memory tasks & neuronal injury in CA1 subregion of hippocampus compared to NC. This two stage P-BCCAO rat model has better survival rate, less acute ischemic stress and better represents the human chronic cerebral hypoperfusion brain injury compared to conventional one stage P-BCCAO rate model.

Key words: P-BCCAO, Chronic cerebral hypoperfusion, Vascular Dementia, Hippocampus

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ISSN: 0975-8585

INTRODUCTION

Stroke is the second leading cause for mortality in the world and an important cause for dementia in the geriatric age group. In 2010, worldwide prevalence of stroke was 33 million, with 16.1 million people having recurrent stroke episodes in their life. Stroke was the second-leading global cause of death behind heart disease, accounting for 11.13% of total deaths worldwide [1]. The prevalence of stroke in India is estimated to range between, 84-262 per 1,00,000 in rural population and 334-424 per 1,00,000 among urban population [2]. Studies have observed that stroke doubles the risk of dementia and post-stroke cognitive decline is more common than the recurrence stroke itself**.** The incidence of dementia increases gradually by 7% at first year and 48% after 25 years of ischemic brain injury in 30 % of the stroke survivors [3, 4]. It is estimated that 3.7 million Indians aged over 60 years suffer from dementia with approximately 4,00,000 new cases being reported every year [5].

Vascular dementia (VD) is a clinical cognitive disorder of cerebrovascular origin which is second most common cause of dementing illness after Alzheimer's disease (AD) worldwide [6, 7]. Though improved socio-economic conditions and increased life expectancy in India have created a large geriatric population, they are at a higher risk of ischemic stroke and VD**.** Since stroke is the second highest contributor to VD, the research on the management of VD should be a major thrust area for the future research. Preclinical studies are frequently conducted using rodent species due to its better survival rate, good recovery from surgery, reproducible behavioral testing and ethical acceptance. In spite of novel strategies and huge number of preclinical studies in VD, many studies failed to show comparable results in human clinical trials. Eminent neuroscientists and physician scientists upon evaluation of failed clinical trials encountered certain flaws that needed to be addressed. One of the important findings among them was failure of the animal models to reasonably mimic the actual human clinical conditions. This study aims to improve the existing animal models so that they can better represent VD in humans.

Models for Vascular dementia

Middle aged (10 months-old) Wistar rats are frequently used for preclinical research studies on VD. They are;

Ischemic model of VD in rat - Permanent bilateral common carotid artery occlusion (P-BCCAO) model

P-BCCAO model Wistar rats have been used in most of the preclinical research to study the effects of chronic cerebral hypoperfusion induced cognitive impairment. Studies using the P-BCCAO model have demonstrated features similar to those observed in progressive carotid artery occlusive disease including a pattern of cerebral hypoperfusion-related metabolic changes, learning and memory disturbances, failure of neuronal signaling, and neuropathological changes in the hippocampus [7]**.** P-BCCAO surgery in Wistar rats reduces the cerebral blood flow to 35- 40 % of the control level in cortical white matter and approximately by 60% in hippocampus [8]. Due to the absence of posterior communicating artery (PCA) and incomplete circle of Willis, abrupt ligation of both the carotid arteries in mice and gerbils results in acute ischemic stroke. Contrarily in Wistar rats PCA and other extra-cranial collaterals are well developed and P-BCCAO surgery will result in a brief period of acute ischemia then chronic cerebral hypoperfusion. Both acute and long term changes in cerebral blood flow (CBF), biochemical and histopathological changes following P-BCCAO are well studied and documented.

The changes following the P-BCCAO in adult rat can be classified into three phases [8];

a) **Acute phase:** Stage immediately following carotid artery occlusion to till two days post occlusion, where cerebral perfusion is drastically reduced due to abrupt carotid artery ligation and hemodynamic-vascular compensatory mechanisms then become active and try to restore CBF.

b) **Chronic hypoperfusion phase**: stage lasting form 1- 8 weeks, where the hemodynamic and flow induced acute compensatory mechanism are reset and blood flow declines leading to morphological changes that are similar to changes seen in cerebral hypoperfusion associated with ageing and dementia

c) **Restitution phase:** approximately 3 months later, the CBF gradually returns to baseline values and both cerebral hypoperfusion and suboptimal metabolism cease.

Hippocampus-related spatial learning is impaired in P-BCCAO rats when tested in the Morris water maze and eight-arm radial maze. Spatial memory and related cognitive impairment worsens as post P-BCCAO duration increases. Non-spatial memory in the object recognition test was also impaired in this P-BCCAO induced Wistar rat model. These results demonstrate that visual-spatial learning, fear conditioning and non-spatial memory are impaired by P-BCCAO. Since cognitive deficits worsen over time, the chronic cerebral hypoperfusion phase of P-BCCAO plays a major role in the gradual deterioration of learning performance [9]. But the mortality rate of conventional BCCAO surgery in Wistar rat model is unacceptably high (29-46%) [10] and these models also undergo a brief phase of acute ischemia, which make them unfit to be used for studies on chronic cerebral hypoperfusion induced VD.

Micro haemorrhage induced multiple infarct rat model of VD- Spontaneous Hypertension Model

Chronic hypertension induced cerebrovascular damage and post micro-hemorrhagic multifocal lacunar ischemic brain injury led VD are reported in geriatric patients. Spontaneously hypertensive rat (SHR) is the rat strain most extensively used for research on hypertensive brain damage and its treatment. These rats are normotensive at birth but develop a sustained hypertension at 6 months of age. They also exhibit an age dependent progressive increase in the arterial blood pressure, brain atrophy, loss of nerve cells and glial reaction similar to the hypertensive brain damage and cognitive impairment seen in hypertensive humans [11]. Hypertension induced cognitive impairment in these rat models can be made worse by supplementing them with a high fat and salt diet, (phenomena similar to that of accelerated aging and vascular impairment). Previous studies have also reported that SHR shows a progressive disruption of neurovascular unit and glial cells leading to loss of neuronal repair mechanisms and interfering with the beneficial neuroplastic changes. SHR shows impairment in both spontaneous and diurnal motor activity similar to that of exhibited by patients with VD. Additionally SHR exhibit significant reductions in the levels of acetyl choline (ACh) and choline in the cortex, hippocampus and cerebrospinal fluid (CSF), and decreased passive avoidance response latency [12, 13]. Similarly histopathological findings of brain in SHR reveals multiple infarcts similar to hypertension induced VD in humans. Recent research on VD have reported poor correlation between hypertension and VD in geriatric patients, indicating that the multiple ischemic infarcts that lead to VD in elderly could be more commonly attributed to chronic cerebral hypoperfusion rather than to cerebral micro-hemorrhages associated with hypertension [14]. So in the present study we explored the techniques to improve the existing rat model of P-BCCAO to more closely represent human clinical condition of chronic cerebral hypoperfusion associated with VD.

Objectives

To investigate and compare the behavioral and histopathological changes of progressive two stage P-BCCAO rat model with the conventional P-BCCAO Wistar rat model of brain injury.

MATERIALS AND METHODS

Animals

Eight - twelve 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 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 study was 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

Wistar rats (n=12 /group) were randomly allocated to the following groups;

1. Normal control rats [NC]
2. Sham BCCAO - The common carotid arteries were exposed and separated from the vagus but not occluded either temporarily or permanently.
3. Conventional one stage P-BCCAO - The common carotid arteries were exposed and the vagus nerve was separated. This was followed by an abrupt and permanent occlusion of both the carotids using surgical silk suture.
4. Two stage P-BCCAO - one of the common carotid arteries was exposed, vagus was separated and the artery was permanently occluded. Then the animals were allowed to recover for 60 days after which the second common carotid artery was occluded permanently. The rats undergoing sequential occlusion were serially numbered and the alternate side of the carotid artery was chosen for first occlusion in each subsequent rat. All odd number rats in the series underwent a right common carotid artery first occlusion and all even numbered rats underwent a left common carotid artery first occlusion.

Induction of Chronic cerebral hypoperfusion ischemic injury

Food was withdrawn 12 h prior to surgical procedure and water was allowed *ad-libitum*. Chronic cerebral hypoperfusion ischemic brain injury was induced in Wistar rats as described by Kim, et al (2008) [15]. Rats assigned to the surgical group were anesthetized by an intra-peritoneal injection using a cocktail of ketamine (50mg/kg b.w) and xylazine (5mg/kg b.w). Atropine sulphate and gentamycin were used as pre-anesthetic medication to minimize secretions and the risk of infection respectively. A midline incision was made and both common carotid arteries were exposed. Care was taken to avoid damage to the vagus nerves by gently separating them from the arteries using a glass rod. The carotid arteries were double ligated using silk sutures (one or two stage). During surgery, the rectal temperature of the rats were continuously monitored and the core body temperature was maintained at 37^0C - 37.9^0C using infra-red heating lamp. In sham-operated group of rats, with the exception of carotid occlusion, the surgical procedures were similar to the P-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 h with an infrared lamp. Appropriate post-operative care was provided by proper surgical wound dressing using betadine solution (Povidone ointment USP). During the 15 days of recovery, the rats were maintained on a special platform designed to allow easy accesses to food and water. Following this all the experimental animals were 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, a 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. It was specifically chosen over other spatial orientation tests because studies have shown that, the visually impaired rats with the intact working memory did not alter their performance significantly. Even in the absence of visual input, an intact vestibular system can still convey cues to the CNS and an intact cerebellum, thalamus and substantia innominate can still effectively process this afferent information thereby allowing a visually impaired rats with an intact working memory capable of performing this test successfully [16].

Spontaneous alternation test

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

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

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 enter one of the arms by blocking the other arm and was allowed to consume the pellet placed there. In the choice run, the forced arm was kept empty and the pellet was placed in the opposite arm. Both the arms were kept free for the rat to run into. If the rat entered the arm opposite to the forced arm, it was considered as a "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 for four successive days. "Percentage of correct responses" was calculated for each rat by using the following formula:

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

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

Passive avoidance test

Modified procedure of Buresova, et al (1989) [17] was adopted. On the first day of the test, each rat was allowed to briefly explore the two compartments for about 5 minutes. On the second day latency to enter the dark compartment for the first time was noted for each rat (control latency). The learning session immediately followed. 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 allowed to return to its home cage. Retention performance of each rat was assessed 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 the retention test (i.e. 48 h) after inescapable foot shock), was interpreted as good retention performance.

Morphological Assessment

Processing of brain tissue for cresyl violet staining

Subsequent to cognitive assessment, rats from all afore mentioned groups were deeply anesthetized using high doses of ketamine-xylazine injection IP. All rats were trans-cardially perfused with equivolumes of heparinized saline and 10% formalin. After perfusion, rats were decapitated and brains were removed, embedded in paraffin blocks according to standard protocols. 5µm thick coronal sections of the brain were obtained serially at the level of hippocampus using rotary microtome. The sections were then mounted on albumin coated slides and stored at 4 ^{0}C for future use.

Nissl staining of hippocampus

Nissl staining method was used for morphological evaluation of the extent of neural damage in hippocampus in all groups of rats. Every 15th best brain sections in the series were chosen for Nissl staining. Selected sections were immersed in 0.1% cresyl violet at 37^{0}C for 20 min. After rinsing with distilled water, sections were dehydrated, mounted with DPX and cover slipped and examined with a light microscope.

Analysis of neurons in CA1 sub-regions of the Hippocampus.

Photomicrography

The CV stained coronal sections of the rat hippocampus were observed and photographed using Motic camera microscope for studying the morphological features of pyramidal neurons in the CA1 region of the hippocampus.

Morphometric assessments

For morphometric analysis, CV stained sections were viewed under the Motic Red 200 microscope, mounted with Moticam 580-5.0 mp colour digital camera and attached to an image analysis system driven by Motic Images-Plus 2.0 software. The calibration was done with the provided Motic standard stage micrometre. High‐power (40 × objective) digital photomicrographs were captured and used for determining the nuclear area and density of pyramidal cells of CA1 sub region of hippocampus. Inverted image function of the Motic image plus-2 software was used to manually delineate the nuclear and cell membrane profiles. The Pyramidal neurons of CA1 sub-region 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. Briefly, all viable CA1 neurons from total of 36 good coronal sections from each group were counted and represented as percentage of surviving neurons. The total numbers of neuronal profiles falling in the each counting frame of micrographs were counted and the total numbers of surviving neurons were represented as percentage of surviving neurons. Neuronal architecture and the number of pyknotic neurons in the hippocampal sections were compared between NC, Sham BCCAO, one stage P-BCCAO and two stage successive P-BCCAO from the photomicrographs.

Statistical Analysis

Results are expressed as Mean ± S.E.M. Statistical significance of the difference in the means between various groups were determined using one way analysis of variance (one way ANOVA) followed by post hoc Bonferroni test using SPSS software v-16. The differences between groups was considered significant at $P<0.05$. The computations and diagrammatic representation of data was performed using Microsoft excel 2007.

RESULTS

Systemic changes following experimental cerebral hypoperfusion ischemic brain injury:

Body weight

On postoperative days 1 and 2 reduction in body weight was observed in both the sham operated and P-BCCAO operated groups. This could be attributed to damage to muscle groups in the ventral cervical region (e.g. the sternohyoid and the sternomastoid muscles) incurred during surgery and discomfort in moving the head, during mastication and swallowing the food post operatively. There was a gradual regaining of body weight starting from the 7^{th} – 10^{th} postoperative day of surgery in sham and unilateral carotid artery surgery group (two stage P-BCCAO group) but not significantly in single stage conventional P-BCCAO rat group. The preoperative body weight was however never achieved in one stage P-BCCAO rats and also by a few of the one stage BCCAO group of rats even after the 15 days of recovery period.

Acute epileptic episodes following P-BCCAO

One stage conventional P-BCCAO rat model, approximately 16.6% (2 out of 12) of rats had seizures even before completely recovering from anaesthesia and all of them died within 8- 12 h post P-BCCAO surgery. But in two Stage P-BCCAO rat model, only one out of twelve rats had seizures and died.

Death of BCCAO rats on 5-7 post-operative day- Delayed neuronal death

Two of one stage conventional BCCAO group of rats died on the 5^{th} and 7^{th} postoperative days. These rats had also exhibited a reduced motility, hunched back, rough- soiled hair coat, and porphyrin stained periorbital region before their death. One of the rat also had signs of facial ischemia with loss of facial hair, facial skin injury etc. Previous studies have reported such phenomena in the post-operative period and had attributed these changes to delayed neuronal cell death. Two stage BCCAO rats did not show any signs of facial ischemia.

ISSN: 0975-8585

Mortality rate between the groups

The mortality rate of two stage P-BCCAO groups of rats was less (16.6%) with only 2 out of 12 rats dying compared to one stage P-BCCAO (33%) rats where 4 out of 12 rats died post operatively. All the sham P-BCCAO rats survived and recovered completely by 2 weeks of surgery.

Hippocampal based spatial learning

Both the one stage P-BCCAO and two stage P-BCCAO groups of rats showed a significant ($p \leq 0.05$) reduction in mean number of alternations compared to age matched NC groups of rats. There was no difference in the mean number of alternations between the two P-BCCAO groups of rats (Fig.1). Moreover both P-BCCAO (one stage and two stage) group of rats demonstrated a significant ($P<0.05$) bias (always turning towards right) in T Maze spontaneous alternation test compared to age matched NC (Fig. 2). Both the NC and Sham P-BCCAO group of rats showed a significantly greater ($P< 0.005$) mean percentage of correct responses compared to the two (one stage and two stage) P-BCCAO groups of rats (Fig. 3).

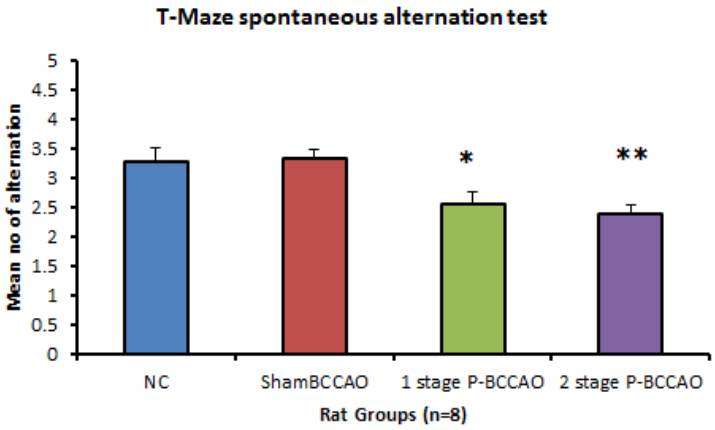

Figure 1: NC- Normal Control, Sham BCCAO-Sham surgical control, one stage P-BCCAO - chronic global cerebral hypo-perfusion injured and two stage P-BCCAO (carotid arteries were occluded consecutively with a gap of two months between the two occlusions). Values are expressed as Mean +SEM number of alternations in the T maze and further analyzed by one way ANOVA followed by Bonferroni's post hoc test. * P <0.05, **P<0.01 vs normal control.

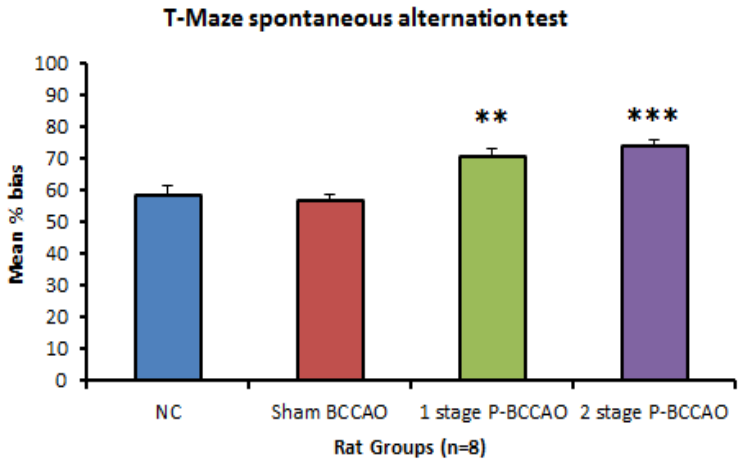

Figure 2: NC- Normal Control, Sham BCCAO-Sham surgical control, one stage P-BCCAO - chronic global cerebral hypo-perfusion injured and two stage P-BCCAO (carotid arteries were occluded consecutively with a gap of two months between the two occlusions). Values are expressed as Mean +SEM % bias on the T maze and further analyzed by one way ANOVA followed by Bonferroni's post hoc test. * P <0.05, **P<0.01 vs normal control.

ISSN: 0975-8585

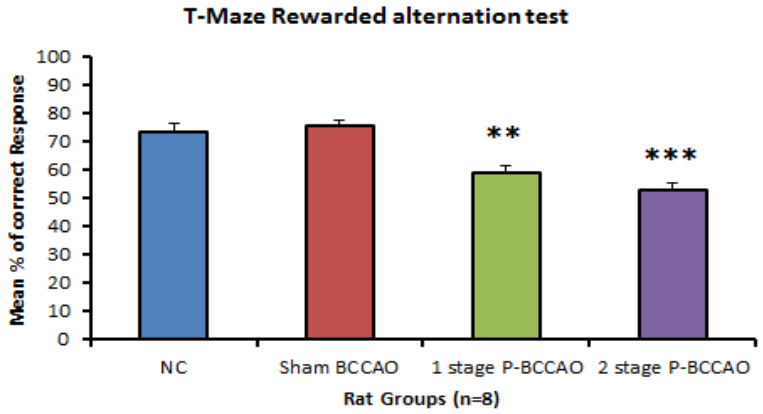

Figure 3: NC- Normal Control, Sham BCCAO-Sham surgical control, one stage P-BCCAO - chronic global cerebral hypo-perfusion injured and two stage P-BCCAO (carotid arteries were occluded consecutively with a gap of two months between the two occlusions). Values are expressed as Mean +SEM % correct response on the rewarded alternation test using the T Maze and further analyzed by one way ANOVA followed by Bonferroni's post hoc test. **P<0.01, ***P<0.001 vs normal control.

Amygdale based Inhibitory avoidance task

There was a significant deficit (P<0.001) in memory retention in both of the P-BCCAO group of rats compared to NC. Moreover, though there were no statistically significant, the two stage P-BCCAO rats exhibited a much shorter mean latency to enter the dark compartment compared to the one stage P-BCCAO rats indicating a relatively greater extent of injury in two stage P-BCCAO rats compared to the conventional P-BCCAO rats (Fig. 4).

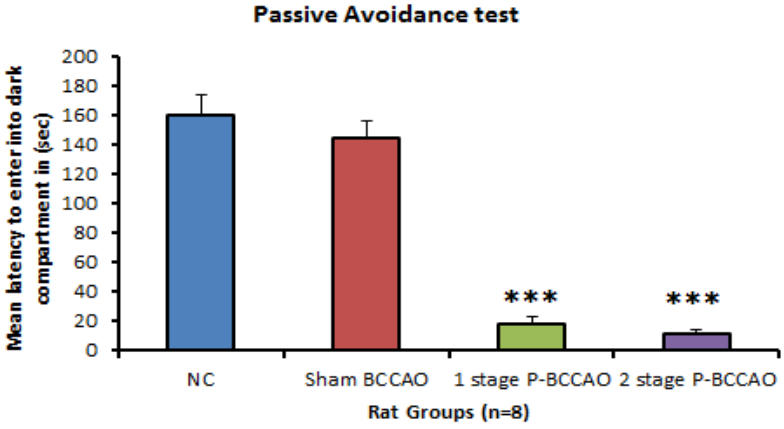

Figure 4: NC- Normal Control, Sham BCCAO-Sham surgical control, one stage P-BCCAO - chronic global cerebral hypo-perfusion injured and two stage P-BCCAO (carotid arteries were occluded consecutively with a gap of two months between the two occlusions). Values are expressed as Mean +SEM latency to enter the dark compartment [in seconds] on the passive avoidance test and further analyzed by one way ANOVA followed by Bonferroni's post hoc test. **P<0.01, ***P<0.001 vs normal control.

Qualitative analysis of CA1 region of the hippocampus

The clear and intact 2 – 3 layers of pyramidal shaped neuronal cell bodies in hippocampal sections from both NC and sham BCCAO groups of rats were observed in photomicrograph which is showed in Fig. 5; a, and b. Alternately Fig. 5; c & d shows a significant neuronal damage (p<0.05) with shrunken neuronal cell bodies, hyper dense neuronal soma cells and few pyknotic nuclei were observed in hippocampal sections from both the P-BCCAO groups of rats induced with chronic cerebral hypoperfusion ischemic brain injury compared to the same in age matched normal and sham BCCAO rats. The extent of neuronal damage in both one stage and two stage P-BCCAO group of rats are similar there was no significant difference between them.

ISSN: 0975-8585

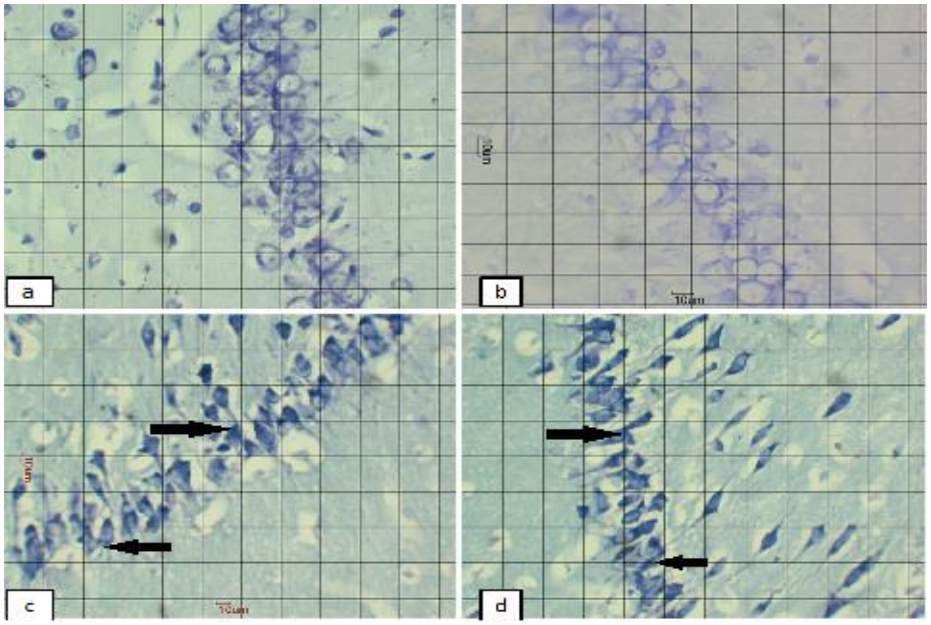

Figure 5: a,b,c & d: Representative photomicrographs of CA1 region of the Hippocampus in rat groups (n=8 / group) -a] Normal Control, b] Sham BCCAO-Sham control, c] one stage P-BCCAO- Bilateral common carotid artery occlusion and d] Two stage P-BCCAO (carotid arteries were occluded consecutively with a gap of two months between the two occlusions). Note: Arrow in Fig 5 c & d represents hyperdense dead CA1 neurons in both the BCCAO groups rats.

Quantitative analysis of the hippocampal neuronal cells

The percentage of surviving neurons are significantly ($p<0.001$) less both in conventional one stage P-BCCAO and two stage P-BCCAO rat model of chronic cerebral hypoperfusion induced ischemic brain injury compared to age matched NC and Sham BCCAO (Fig. 6). More than 48 % of neurons in one stage P-BCCAO rat model and 52.5% of neurons of two stage P-BCCAO rat model were found to be nonviable in CA1 sub-region of Hippocampus when compared to NC group of rats.

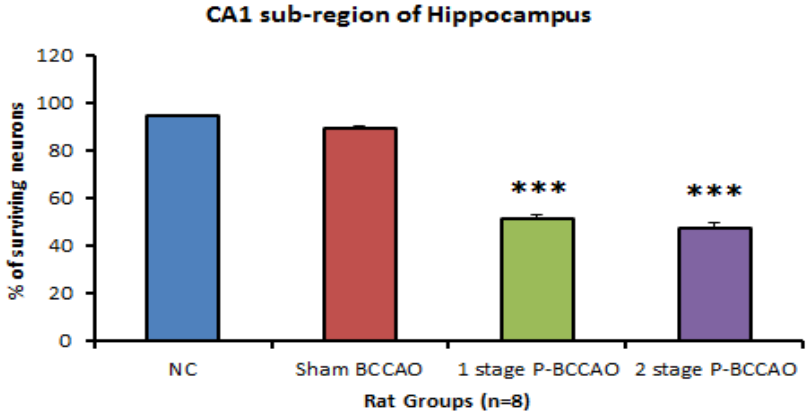

Figure 6: NC- Normal Control, Sham BCCAO-Sham surgical control, one stage P-BCCAO - chronic global cerebral hypo-perfusion injured and two stage P-BCCAO (carotid arteries were occluded consecutively with a gap of two months between the two occlusions). Values are expressed as Mean +SEM % of surviving neurons in the nissle stained sections of CA1 subregions of hippocampus and further analyzed by one way ANOVA followed by Bonferroni's post hoc test. ***P<0.001 vs normal control.

DISCUSSION

The present study indicates a significant reduction in the mortality in two stage P-BCCAO rats compared to one stage P-BCCAO. This could be due to lesser acute ischemic stress to the brain in two stage progressive P-BCCAO group compared to one stage P-BCCAO rats. Previous studies on conventional one stage

P-BCCAO rat models documented an initial stage of acute oligemic hypoperfusion followed by a gradual restoration of the cerebral blood flow (CBF) to normal levels in about six months [8]. Similarly two stage P-BCCAO group of rats had fewer episodes of seizures and other related co-morbid changes compared to one stage P-BCCAO group. This reduction in mortality and morbidity in two stage P-BCCAO group of rats could be attributed to the action of hemodynamic and vascular adaptive compensatory mechanisms which evaded the severe acute ischemia in progressive two stage carotid artery occlusion. Since the carotid arteries were occluded sequentially allowing a gap of two months between occlusions, this provided sufficient time for the neurovascular unit to adapt the state of hypoperfusion**.** This haemodynamic changes which restore CBF and improve ischemic threshold of the neurovascular unit include flow induced vasodilation and opening of unnamed collaterals. Chronic vascular remodeling changes include increased diameter of basilar, posterior cerebral, posterior communicating, internal carotid, middle cerebral and anterior cerebral arteries [18].

Extensive studies on P-BCCAO- Wistar rat model have clearly documented that occlusion of carotid arteries on both sides completely arrested blood flow through the anterior cerebral arteries and partially arrested through the middle cerebral arteries causing white matter lesions and gliosis in the frontal, parietal and temporal lobes and associated degenerative changes in the hippocampus [8]**.** Since CA1 region of hippocampus is more susceptible to hypoxic-ischemic injury, P-BCCAO rodents perform poorly in hippocampal based spatial learning and memory. Extensive research have clearly documented that discrete trial procedure of T-Maze is better in detecting hippocampal damage than radial arm or water maze. Rodents have an inherent tendency to explore novel environment and in the T-Maze, they tend to alternate their choice of goal arm. They rely on their 'working memory', to alternate their choices. A healthy rodent with intact hippocampus will retain memory of the arm it visited in a previous trial and in the subsequent trial will try to explore the opposite arm. Thus in a normal healthy rodent the response in every trial (choice in choosing the arm) T-Maze, varies based on the previous experience with more alternation and less percentage bias which is an indication of intact hippocampus.

Previous studies have shown that hippocampectomized animals notoriously adopt side preferences, e.g., always turning right on a T-Maze indicating the problem in either learning, retaining or retrieval of the information necessary for alternation [16]**.** In our study both one and two stage P-BCCAO group of rats demonstrated a high percentage bias to right side and reduced mean number of alternation in T-Maze spontaneous alternation test compared to age matched NC and Sham BCCAO. There was no significant difference between the two P-BCCAO groups in the performance of T-Maze spontaneous alternation test indicating comparable hippocampal damage among them.

Results of the present study on rewarded alternation test (Fig. 3) also indicate that ischemic brain injured (both P-BCCAO model rats) seem to choose a specific (right) side irrespective of forced or choice run during majority of the rewarded alternation trials thereby significantly reducing the percentage of correct response compared to sham BCCAO animals indicating a hippocampal lesion. Whereas the food deprived sham BCCAO and NC animals clearly retains the memory of forcefully visited arm and chooses the other arm for the food during the choice run indicating intact functional hippocampus. We have primarily chosen T-Maze for this P-BCCAO model because ligation of common carotid arteries deprive blood supply to the retina and causes degeneration of the optic tract and optic chiasma leading to partial visual impairment in BCCAO group of rats. Studies on cognitive neuroscience research indicate that T maze test are effective in identifying hippocampal lesions even in blind animals, since the rodent performance in this test relies on the presence of an intact cerebellum, basal ganglia and thalamus which gives cues in visual impaired animals.

Ye Xi Wang M et al [19] in their study have correlated increased oxidative stress, central cholinergic dysfunction and neurological damage leading to spatial learning and working memory dysfunction in the rat model of BCCAO. Similarly SK kim et al [20] have also reported that BCCAO induced rats perform poorly even in non-visual cues cognitive tests like T -Maze indicating cognitive dysfunction. Hania Shakil et al [21] in their study reported a significant deficit in spontaneous alternation test in BCCAO rats compared the treated groups. In the current study, both the BCCAO groups of rats were found to be significantly poor in retaining avoidance memories (Fig.4) for the passive avoidance task, compared to age-matched NC and Sham BCCAO rats. Ischemic brain injured 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 demonstrate poor retention memory as evidenced by short latency to enter the dark compartment when compared to stage-matched normal controls. Studies also 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 [22]. Several studies of BCCAO in rodents have also documented injuries to amygdala, hippocampus and white matter lesions with gliosis to prefrontal and parietal lobes [8]. Similarly, in the present study, P-BCCAO rats of both groups 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. Alireza Sarkaki [23] had documented that conventional P-BCCAO rats had impairment in both active and passive avoidance task and their cognitive performance with regard to both the avoidance memory had improved when supplemented with pomegranate extract for 15 days.

Present study also indicates severe neuronal damage in both models of P-BCCAO. Although not statically significant, the neuronal loss was greater in two stage P-BCCAO compared to one stage BCCAO. Previous studies have documented the susceptibility of CA1 sub field to ischemic injury. The most obvious signs of neurodegeneration are due to loss of neuronal cell bodies and synaptic contacts. At 2^{nd} week, 6–29% of the animals exhibited hippocampal injury in the CA1 subfield [24-26]. At 4^{th} week, this had increased to 55% [26], while at 8–13^{th} week, total hippocampal destruction was observed in 67% of the P-BCCAO rats [27, 28]. The chain of events that eventually lead to neuronal cell death in chronic cerebral hypoperfusion begins with neuronal energy failure due to the blood flow reduction and the consequent hypoxia and hypoglycemia. The energy failure in cerebral ischemia is reflected most evidently in the rapid depletion of ATP, also found in P-BCCAO rats [29, 30]. In ischemic brain injury or stroke, the loss of ATP is promptly followed by the dysfunction of energy dependent ion pumps, depolarization of the neurons, and generation of reactive oxygen species (ROS) lethal to neurons at high concentration. The ROS in turn initiate lipid peroxidation, generating lipid peroxides that are degraded to reactive aldehyde products such as malondialdehyde (MDA) [31]. Future studies can further investigate the nature of neuronal cell death among the two models of P-BCCAO group of rats.

CONCLUSION

Two stage P-BCCAO surgery improves survival rate of chronic cerebral hypoperfusion ischemic brain injury in Wistar rat model compared to conventional one stage P-BCCAO rat model. Similarly this modified model of two stage occlusion more appropriately represents human progressive carotid artery occlusive disease than the conventional one stage P-BCCAO where abrupt bilateral occlusion of carotid arteries results in the initial brief period of acute ischemia in Wistar rats which is unlike the pathogenesis of human carotid artery disease. The acute brain injury induced seizure episodes are far less in this modified two stage P-BCCAO model compared to the conventional one stage P-BCCAO model. A few rats which had undergone conventional common carotid artery occlusion procedures endured facial ischemic features whereas none of the rats from two stage P-BCCAO group showed such injuries. Thus two stage P-BCCAO model would be a more preferred model for preclinical research on chronic cerebral hypoperfusion induced VD.

ACKNOWLEDGEMENTS

We thank Vaishnavi Balaji and Akriti B Kanth of Manipal School of Life Sciences, Manipal University for their assistance in BCCAO surgery and also would like to convey our heartfelt thanks to Manipal University for providing us the infrastructure support and facility for doing this work.

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