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Evaluation of neuro-protective activity of Brihatvata Chinthamani Rasa

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ABSTRACT

The objective of the study was to screen the neuroprotective role of Brihatvata Chinthamani Rasa, a compound mineral formulation extensively used in stroke condition in the Indian system of medicine. The selected animals were grouped into four different categories and administered with group specific drugs for seven consecutive days. On seventh day an hour after drug administration all the rats were anesthetised. A midline incision was made and soft tissues were pulled apart and both bilateral common carotid arteries were exposed. The cerebral ischemia were induced by simultaneous occlusion of both common carotid artery for 30min, followed by acute ischemic reperfusion injury was produced by untying the temporary ligature and releasing the thread. At the end of the experiment, under anaesthesia animals were sacrificed and brain has been removed. Anti oxidant and histopathological investigation were carried out of brain tissue. Control group rats have shown increased lipid peroxidation and decreased levels of catalase and glutathione peroxidase activity, while the test drug administration significantly elevated catalase and considerable decrease in the lipid peroxidation in comparison to control group. Histopathological examination revealed there is a decreased cellularity and predominance of immature neurons in the granular layer was observed in hypothalamus in control rats while the test drug has shown normal cytoarchitecture. In conclusion the test drug possesses storing anti oxidant and retained normal cellular integrity of different brain regions.

Keywords: Brihatvata Chinthamani Rasa, Anti-oxidants, Hippocampus, Lipid peroxidation, Catalase, Glutathione peroxidase.

INTRODUCTION

Ischemic brain injury is one of the leading causes of mortality and adult disability worldwide [1]. Cerebral ischemia is complex pathological process involving oxidative damage, neuro-inflammation, calcium overload and cellular apoptosis which ultimately lead to irreversible brain injury. During ischemia followed by reperfusion causes production of huge quantity of reactive oxygen species (ROS), which leads to the oxidation of lipids, proteins, DNA, apoptosis and subsequent cellular damage [2]. This can be minimised by using efficacious antioxidants. Inflammation occurs within few hours of ischemic reperfusion injury and it contributes for secondary damage caused by the activation of microglia, macrophages and peripheral inflammatory cells. The neutrophils plays a central role in the development of ischemic brain damage and depletion of circulatory neutrophils or inhibition of neutrophils infiltration causes ameliorative effect in ischemic brain damage [3].

Brihatvata Chinthamani Rasa (BVCR) a compound mineral preparation, widely used in the management of neuro-psychiatric illnesses in Indian system of medicine. It contains Bhasma (ash) of gold, iron, mica, silver, calcium, pearl and mercuric sulphide. The ingredients of compound formulary were indicated as a stimulant, nervine, nootropic and rejuvenate which improves the acuity of mind as well as directly indicated in the management of stroke in Ayurveda [4]. The test drug contains the ingredients having potent anti inflammatory [5], anti oxidant and are used in rheumatoid arthritis [6]. The resent study is aimed to evaluate the neuroprotective activity of test drug in bilateral common carotid artery occlusion (BCCAO) induced neuronal damage in wistar albino rats.

MATERIALS AND METHODS

Test Drug

The market available Tablet of Brihatvata Chinthamani Rasa prepared by Sri Dhootapaapeshwara Ltd, (Batch no.130269, Manufacture date Sept 2013) was used throughout the experimental study.

Dose fixation

The dose of BVCR for the purpose of neuroprotective activity in human is 250mg. The rat dose was

derived from human dose based on body surface area ratio by referring standard conversion table of Pagets and Burnes 1964 [7]. The rat dose was calculated using the following formula. i.e. Rat dose = human dose X 0.018X 5/ Kg body weight and it was found to be 22.5/kg body weight. The test drug made suspension with 0.5% gum acacia and administered through orally with the help of oral catheter.

Experimental animal

Wistar albino rats of either sex weighing between 200±50g were used for the experimentation. The experimental animals were procured from animal house facility attached to SDM Centre for Research in Ayurveda and Allied Sciences. The rats maintained at standard laboratory condition with natural day and night cycles with ideal laboratory condition of 25±20 ^{0} c temperature and 55% humidity. They were fed with normal rat pellet supplied by Sri Durga feeds from Bangalore and tap water given *ad libitum.* The study protocol was approved from Institutional ethical committee (SDMCRA/IAEC-2012-13, MNS 02) and principles of laboratory animal care guidelines were followed throughout the experimentation.

Experimental design

The selected rats were grouped into four groups with six animals in each group**.** Group I administered with 0.5% CMC at a dose of 5ml/kg body weight served as control group. Group II administered with combination of Memantidine and Clobedrolin a dose of 20mg/kg and 3mg/kg body weight respectively, served as reference standard. Group III administered with BVCR 22.5mg/kg in 0.5% CMC. Group-IV administered with BVCR 45mg/kg in 0.5% CMC considered as Test I & Test II respectively. Rats were anaesthetised by administering combination of Ketamine (100mg/kg) and xylazine (3mg/kg). Using 70% ethanol, sterilized the surgical area at the ventral regions of neck. A midline incision was made and soft tissues were pulled apart and both bilateral common carotid arteries were exposed. The cerebral ischemia were induced by simultaneous occlusion of both common carotid artery for 30min, followed by acute ischemic reperfusion injury was produced by untying the temporary ligature and releasing the thread. 48h after cerebral ischemic reperfusion all animals were sacrificed under anaesthesia. The brain tissue was excised, a part of brain was used for anti oxidant activity and a part of brain was preserved in 10% formalin for histopathological investigation [8, 9].

Anti oxidant activity in brain homogenate

Preparation of brain homogenate

Excised brain was cleaned with ice cold saline and stored at-20^{0}C. Before subjecting the brain tissue to antioxidant activity, the brain tissue samples were thawed and homogenized with 10 times (w/v) ice-cold 0.1M Phosphate Buffer (pH 7.4). Aliquots of homogenates from rat brain were used to determine catalase and glutathione peroxidise activity along with lipid peroxidation employing standard procedures. Catalase activity in tissue was measured according to the procedures of Sinha *et al.,* 1972 [10]. 1ml of homogenate solution was taken in 5 ml of phosphate buffer. To this 4 ml of 0.2 MH_2O_2 in phosphate buffer was added and time was noted. Exactly after 180 seconds of adding H_2O_2, a set of 1ml of reaction mixture from the above was taken in 2 ml dichromate acetic acid. Then it was kept in boiling water bath for 10 minutes. Cool all the tubes under running tap water and finally reading was taken at 570 nm against reagent blank. Catalase activity in the tissue was expressed as µmoles H_2O_2 consumed /mg protein /min. Lipid peroxidation activity was determined by measuring the content of the Thiobarbituric acid reactive substances (TBARs) following the procedure of Ohkawa *et al.,* 1979 [11]**.** A standard stock solution of malondialdehyde was prepared in distilled water using 1,1,3,3 tetraethoxypropane. The solution was stored at 4ºC and diluted just before use as the working standard contained 50 nmoles/ml. Pipette 0.1ml of homogenate, 0.2 ml of 8.1% sodium dodecylsulfate (SDS), 1.5 ml of 20% acetic acid solution and 1.5 ml of 0.8% aqueous solution of TBA into suitably labelled test tubes. Reaction mixture was made up to 4.0 ml with distilled water and heated at 95°C for 60 min in water bath. After cooling the test tubes under tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of n-butanol and pyridine (15: 1, v/v) was added and the mixture is shaken vigorously. Centrifuge the test tubes at 4000 rpm for 10 minutes and the absorbance of the upper layer is measured at 532 nm. Standard malondialdehyde is processed in similar fashion. Level of lipid peroxide was expressed as µmoles of MDA formed /g wet tissue. Glutathione peroxidase was estimated by taking 0.2 ml of EDTA, sodium azide, reduced glutathione and H_2O_2 in a test tube. Added 0.4 ml of buffer and 0.2 ml of homogenate mix and incubate at 37 ^{0}C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of TCA and tubes were centrifuged. Taking 0.5 ml of supernatant and add 4 ml of disodium hydrogen phosphate and 0.5 ml of DTNB. The colour developed was read at 420 nm immediately. Standard was also treated in the similar way. The glutathione peroxidase activity is expressed as µM of glutathione utilized per mg protein per minute at 37^{o}C

Histopathological study was carried out by transferring brain into 10% formalin immediately after excision from rats. Sections of less than 5µm thickness of fore brain were prepared and stained with haematoxyline and eosin for microscopical observations [12].

RESULTS AND DISCUSSION

Anti oxidant activity

In the present study test group has showed significant increase in the catalase activity of brain tissues at therapeutic dose and non-significant decrease in the Glutathione Peroxidase and Lipid Peroxidation in comparison to that of BCCAO control group (Table 1).

Table 1: Effect of test drug on antioxidant activity in the brain homogenate

Group	Catalase activity µmoles H_2O_2 consumed /mg protein /min	Glutathione Peroxidase activity (µM of glutathione utilized per mg protein per minute at 37^{o}C)	Lipid Peroxidation µmoles of MDA formed /g wet tissue
BCCAO Control	24.52±0.86	22912.94±5409.1	1959.43±571.07
Reference standard	25.49±0.39	3458.93±327.33*	1567.37±35.73
Test I	29.18±0.96*	20645.74±6147.7	1537.35±107.23
Test II	26.32±1.72	16428.12±2538.5	1226.79±75.48

Data expressed in Mean ± SEM, * p<0.05 compared with BCCAO control, one way ANOVA followed by Dunnet's multiple comparison test.

Histopathological Study

Hippocampus-Microscopic examination of sections containing hippocampus especially the dentate gyrus from BCCAO control rats revealed almost normal cellular organization with distinct granular layer, hilar region, and molecular layers. The cells of the granular layer containing compactly arranged mature granular cells. Cellularity was found to be decreased in section from one rat. Predominance of immature neurons in the granular layer was observed in sections from two rats. Standard drug administered group exhibited almost normal cytoarchitecture. The molecular layer, the granular layer and the hilus region exhibited normal profile. However, oedema was observed in section from one rat in the hippocampus region. The sections of hippocampus from test-1 and test -2 treated groups exhibited almost normal cytoarchitecture (Figure -1).

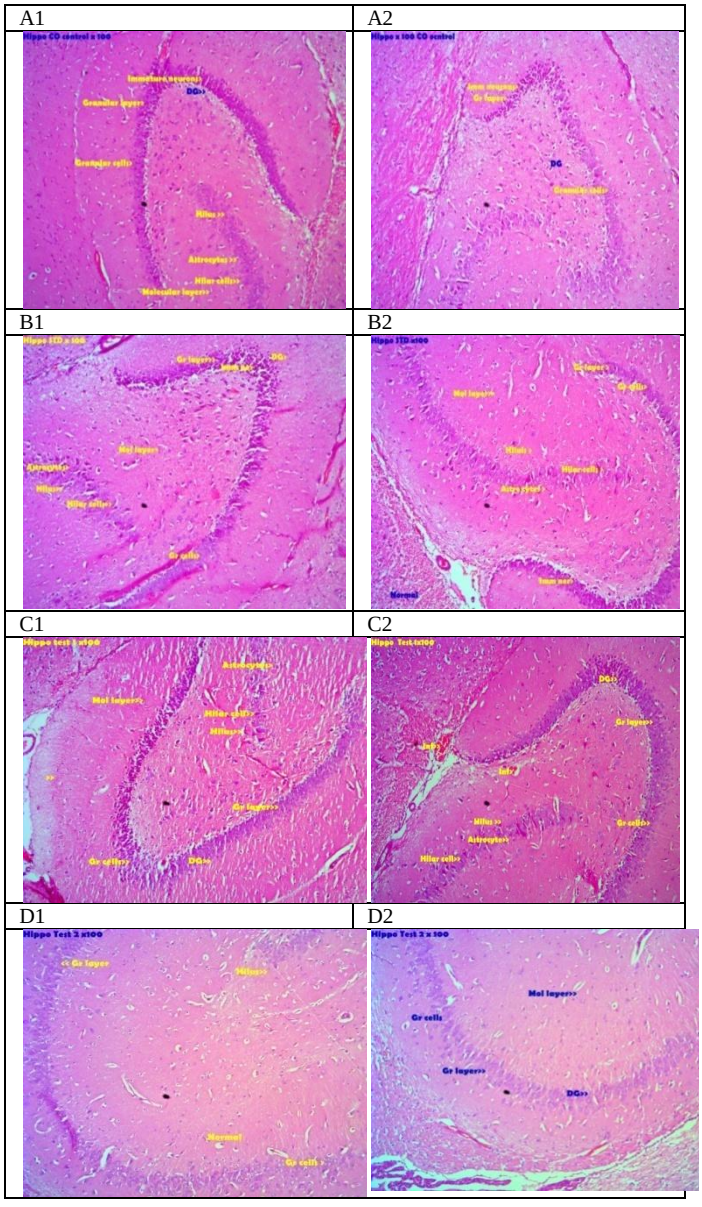

Figure- 1: Photomicrograph of representative brain sections focused on hippocampus region of BCCAO positive control group (A 1& 2) , Reference standard treated with Memantidine and Clobedrolin the dose 20mg/kg and 3mg/kg body weight respectively (B1& 2), Test group BVCR administered at TED and TED X2 (C1& 2, D1& 2).

BCCAO control rats cellularity was found to be decreased & predominance of immature neurons in the granular layer was observed where as standard and test drug administered group exhibited almost normal cytoarchitecture of molecular layer, granular layer and the hilus region.

Midbrain-The midbrain sections from the BCCAO control group, three rats showed periventricular and perivascular oedema as indicated by less sparsely cellular and light staining areas. In few sections focal round cell infiltration was observed especially adjoining the areas to meninges. The standard drug given groups exhibited much less disturbance in comparison to the BCCAO control rats. Oedematous changes were much less. Cell infiltration though observed was much less in severity in comparison to the BCCAO control rats. Examination of the sections of midbrain from test-1 groups given groups showed mild to moderate oedematous changes in sections from two rats. Cell infiltration was found to be less in comparison to the BCCAO rats. The test-2 groups showed mild to moderate oedematous changes in sections from two rats. Cell infiltration was found to be less in comparison to the BCCAO rats Figure 2).

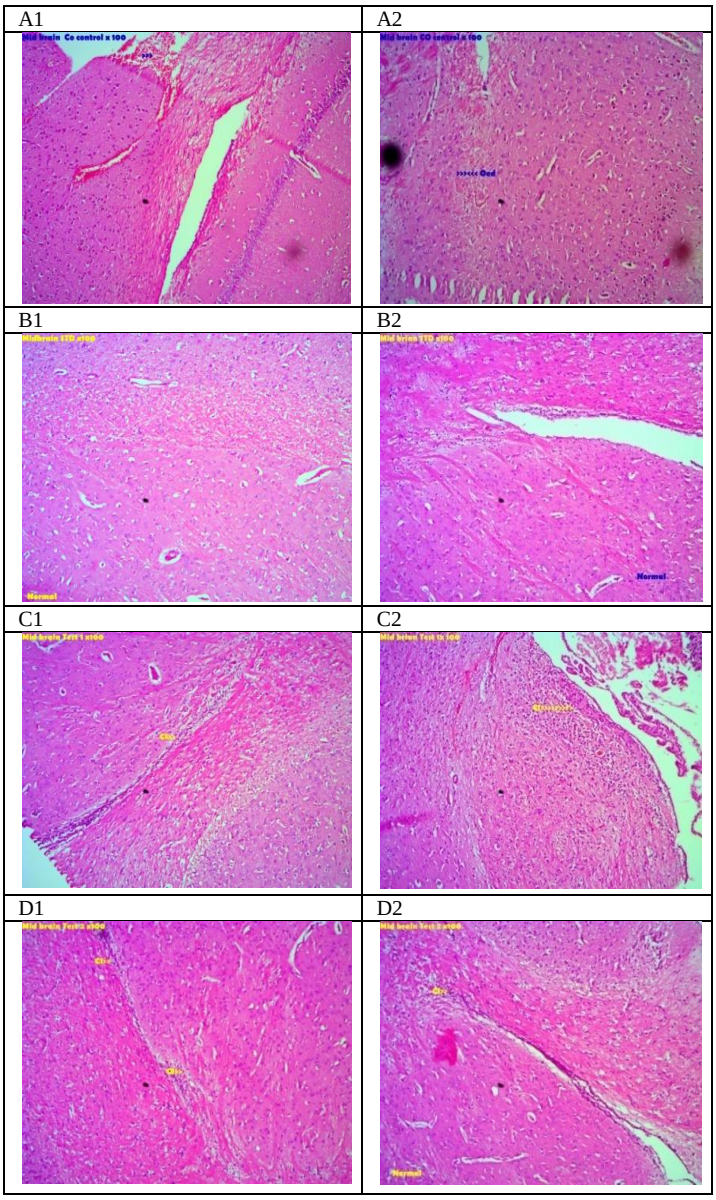

Figure- 2: Photomicrograph of representative brain sections focused on mid brain region of BCCAO -positive control group (A 1& 2) , Reference standard treated with Memantidine and Clobedrolin the dose 20mg/kg and 3mg/kg body weight respectively (B1& 2), Test group BVCR administered at TED and TED X2 (C1& 2, D1& 2).

BCCAO control group rats have showed periventricular and perivascular oedema as indicated by less sparsely cellular and light staining areas. In few sections focal round cell infiltration was observed especially adjoining the areas to meninges. The standard drug given groups exhibited much less disturbance in comparison to the BCCAO control rats. Oedematous changes were much less. Cell infiltration though observed was much less in severity in comparison to the BCCAO control rats. Test-1 group showed mild to

moderate oedematous changes, cell infiltration was found to be less in comparison to the BCCAO rats. The test-2 groups showed mild to moderate oedematous changes in sections from two rats. Cell infiltration was found to be less in comparison to the BCCAO rats (Figure 2). CI- Cell infiltration, Oed-Oedema

Forebrain-The fore brain sections from BCCAO group exhibited almost normal cytoarchitecture. The sections from reference standard, test-1 and test-2 groups also exhibited normal cytoarchitecture (Figure 3).

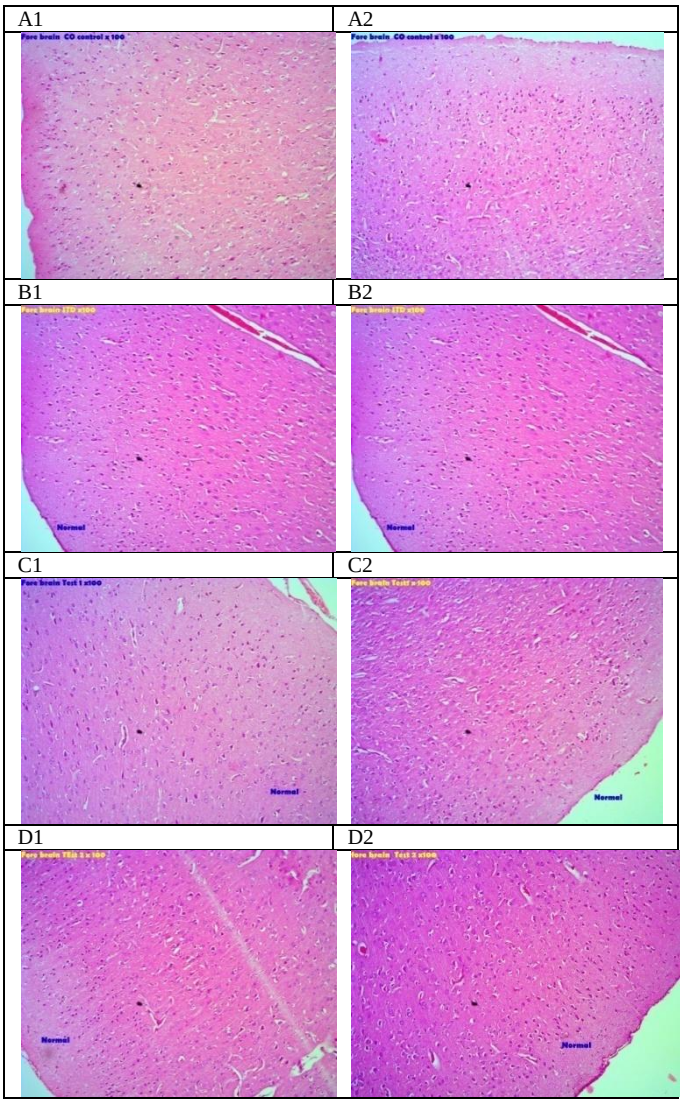

Figure- 3: Photomicrograph of representative brain sections focused on fore brain region of BCCAO -positive control group (A 1& 2) , Reference standard treated with Memantidine and Clobedrolin the dose 20mg/kg and 3mg/kg body weight respectively (B1& 2), Test group BVCR administered at TED and TED X2 (C1& 2, D1& 2).

The fore brain sections from BCCAO group exhibited almost normal cytoarchitecture. The sections from reference standard, test-1 and test-2 groups also exhibited normal cytoarchitecture.

Anti-Oxidant Study

In the present study the test group showed significant increase in the Catalase activity of brain tissues in therapeutic group and non-significant decrease in the Glutathione Peroxidase and Lipid Peroxidation in comparison to that of BCCAO control group. In the focal cerebral ischemia, cerebral blood flow is significantly reduced and hence there is impairment in the oxygen supply to different regions of brain. Reperfusion injury produced during BCCAO procedure causes re-oxygenation mean while generation of reactive oxygen species by enzymatic oxidation reactions. In addition it also increases level of oxygen which cannot be utilised by mitochondria under normal physiological condition. It has been reported that about 2-5% of electron flow in isolated brain mivtochondria produces superoxide radicles and hydrogen peroxide [13]. The free radicles generated during natural oxidation reaction were detoxified by antioxidant system such as ascorbic acid and vitamin E.

Thus during reperfusion there is a great depletion of antioxidants and failure to adequate replenish antioxidants is ischemic brain tissue is generally assessed by estimation of lipid peroxidation, DNA damage and protein oxidation [14, 15]. Catalase as mentioned above is part of the body's endogenous anti-oxidant system – increased level of this enzyme is indicative of enhanced anti-oxidant system. Lipid peroxidation is the primary process which is responsible for the generation major part of free radicals. Deceased lipid peroxidation is indicative decreased generation of free radicals. Glutathione peroxidase is involved in the metabolism of glutathione. Increased activity of this enzyme may lead to decreased anti-oxidant system through decreased availability of glutathione. In the present study-catalase activity was found to be significantly enhanced in test-1 group and moderate increase in test-2 group in comparison to the BCCAO control group. This is indicative of enhanced anti-oxidant activity but was not dose dependent. Glutathione peroxidase activity was found to be moderately increased in standard group and moderately decreased in test drug treated group- the decreased activity of this enzyme may increase glutathione activity and might contribute to the anti-oxidant activity. Lipid peroxidation was found to be moderately decreased in both reference standard and test drug administered groups. Thus the presence of anti-oxidant promoting and lipid peroxidation decreasing effect might contribute significantly to the observed neuroprotective effect. Glutathione peroxidase activity decrease may also have a role.

CONCLUSION

To conclude it can be suggested that the test drug, especially at higher dose level, produced significant neuroprotection against BCCAO induces lesions especially in the mid brain region as revealed in reversal of BCCAO induced changes in activity cage performance and changes in microscopic profile. The presence of anti-oxidant effect and lipid peroxidation attenuating effect may contribute to the observed neuroprotection.

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Conflict of Interest

There is no conflict of interest from any of our co authors.

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