ORIGINAL ARTICLE

Role of *Centella asiatica* on cerebral post-ischemic reperfusion and long-term hypoperfusion in rats

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Centella asiatica (CA), a well known adaptogenic agent in Indian system of Medicine (Ayurveda), is believed to have beneficial effects in improving memory, treating anxiety and eczema. It also possesses antioxidant, cognitive enhancing and antiepileptic properties. Acute ischemia followed by reperfusion is known to bring about biochemical and histopathological alterations. In the present study the effect of *Centella asiatica* on acute cerebral reperfusion and long-term cerebral hypoperfusion in rats was investigated. Transient cerebral ischemia was induced under Ketamine anaesthesia by blocking bilateral common carotid arteries (BCCAO) for 30 min and then reperfusion was allowed for 45 min by releasing the block. Lipid peroxidation, superoxide dismutase (SOD) and brain protein were estimated, behavioral and histopathological studies were done for both acute ischemia-reperfusion and chronic hypoperfusion studies. One way ANOVA followed by post hoc Tukey test was used. In the present study, acute ischemia-reperfusion induced increases in lipid peroxidation and superoxide SOD activity. CA pre-treatment (100 mg/kg p.o. for 5 days) attenuated the reperfusion induced biochemical alterations. Long-term cerebral hypoperfusion in rats caused a propensity towards anxiety and listlessness (open field paradigm and elevated plus maze test) accompanied by deficits in learning and memory (Morris' water maze testing) and tendency towards depression (Porsolts swim test). Additionally, histopathological observations in forebrain revealed changes like gliosis, astrocytosis, cellular edema and inflammatory changes. CA treatment (100 mg/kg p.o. for 28 days) alleviated these behavioral, cognitive and histopathological changes. The results suggest that CA may be useful in cerebrovascular insufficiency conditions.

Key words: *Centella asiatica*, cerebral hypoperfusion, ischemia-reperfusion injury

INTRODUCTION

Recent research on natural products has proved potentiality of many plants for diverse clinical conditions. In Indian system of medicine, a number of herbal preparations those are known to promote health and longevity by increasing defence against disease, arresting the aging process and revitalizing the body in debilitated conditions have been designated as "rasayanas".[1,2] A subgroup of rasayanas called Medharasayanas, are used to promote memory and intellect (medha).[1-4]

Extensive works on *Ocimum sanctum* and *Withania somnifera*, for example, have been shown to possess significant anti-inflammatory anti-oxidant, immunomodulatory, antistress, radioprotective, neuroprotective effects.[5-13] They were also found to possess antidepressant, anxiolytic effect and to enhance cognition in animal models.[14-18]

In reperfusion injury, arrival of oxygen to the ischemic area following restoration of blood flow instead of alleviating ischemic state worsens the damage. The main causative factor for this reperfusion injury is believed to be reactive oxygen species.[19-21] Permanent occlusion of bilateral common carotid arteries (BCCAO) causes chronic cerebral hypo-perfusion and induces a state of chronic low-grade ischemia in rat forebrains over an extended period.[22] Impaired learning/memory and structural alterations in rats have been documented following permanent BCCAO.[23,24]

Centella asiatica (*CA*) belongs to family of umbelliferae and is a perennial, herbaceous creeper growing up to 30 cm in height with fan shaped leaves. C.A has been used in traditional medicine in Asia for hundreds of years.[25] Major constituents of C.A are triterpene saponins, mainly asiaticoside, sapogenin asiatic acid, madecassoside and madecassic acid.[26] It is believed to have beneficial effects in improving memory, treating anxiety and eczema.[27] Ayurvedic medicine has used C.A in treatment of inflammation, bronchitis, fever etc.[28] The aqueous extract of C.A possesses antioxidant, cognitive enhancing and antiepileptic properties.[29]

In clinical settings, C.A has been documented to improve microcirculation, venous insufficiency and venous hypertension of lower limbs.[30-32] Alcoholic extract of Hydrocotyle asiatica Linn has been shown to possess cardioprotective activity as it limited the ischemia-reperfusion induced myocardial injury.[33]

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A survey of literature shows that though C.A has been reported to have a number of beneficial effects on diverse experimental parameters, its possible role in experimental cerebral ischemia has not yet been explored. The present study was undertaken to evaluate the effect of CA, if any, on cerebral ischemia-reperfusion injury and chronic cerebral hypoperfusion in rats.

MATERIALS AND METHODS

Animals

After Institutional Animal Ethics Committee's approval, the present study was conducted on standard inbred Charles-Foster (CF) albino rats of either sex weighing 200-250 g. The animals were kept in colony cages at an ambient temperature of 25 ± 2°C at relative humidity of 45-55% with 12 h : 12 h dark and light cycle and they were allowed free access to standard rodent pellet diet and drinking water. The food was withdrawn 12 h prior to surgical procedure, however, water was allowed ad libitum. Through out the experiment, Principles of Laboratory Animal care guidelines were followed.[34]

Chemicals and Reagents

Thiobarbituric acid (TBA), NADH, Nitroblue tetrazolium (NBT) and Phenazine methosulfate (PMS) were obtained from Sigma, USA. 1,1,3,3 -Tetraetoxypropane (TEP) was obtained from Merck, Germany. All other chemicals and reagents were purchased locally and were of highest analytical grade.

Plant Preparation

Pharmacognostically identified plants of C.A collected from Ayurveda Faculty, Institute of Medical Science (Ayurveda), Banaras Hindu University and were authenticated. They were dried under shade, powdered and extracted with methanol and then concentrated over steam bath. The yield was 3.2%. The dried extract was stored in refrigerator until use. A voucher specimen was kept in the department for future reference. The extract was used in the dose of 100 mg/kg/day, p.o. This does was selected on the basis of earlier studies.[33,35,36]

Experimental Procedure

Ischemia-reperfusion injury

Transient cerebral ischemia was induced as per the method of Iwasaki *et al.* (1989).[37] In short, under Ketamine anaesthesia (100 mg/kg, i.p.), common carotid arteries were approached by midline incision of neck and were carefully separated from accompanying vago-sympathetic trunk. Rat brain ischemia was induced by blocking bilateral common carotid arteries (BCCAO) for 30 min and then reperfusion was allowed for 45 min by releasing the block. Body temperature was maintained at 37°C ± 1°C during the surgical procedure with the help of a heating lamp. Sufficient degree of ischemia was confirmed by ascertaining brain lactate levels (37.13 ± 2.95 nM/mg protein in sham operated animals versus 67.52 ± 3.22 nM/mg protein after 30 min BCCA block and 45 min reperfusion, p £0.001, n = 6 in each group)

Hypoperfusion injury

For chronic hypoperfusion studies, after separating them from nerves, the carotid arteries were doubly ligated with 3-0 silk and cut in between.[38] Animals were returned to home cage after suturing the skin

Steady state experiment

For acute ischemia-reperfusion study the animals were divided into 5 groups. First one, normal rats with out any interventions. Second, sham operated animals, which received double distilled water. Per se group received methanolic extract of C.A 100 mg/kg, po for 5 days. Fourth group underwent 30 min BCCA block followed by 45 min reperfusion and served as control. The fifth, i.e., treatment group received C.A 100 mg/kg, p.o. for 5 days before subjecting them to ischemia-reperfusion injury. At the end, animals were humanely sacrificed by decapitation, brains were taken out and lipid peroxidation and SOD activity were estimated.

Similarly for chronic hypoperfusion studies, the rats were divided in to 5 groups. First, normal animals with out any interventions, second sham-operated animals, which received double distilled water, third per se, which received methanolic extract of C.A 100 mg/kg, p.o. for 28 days. Fourth group underwent permanent BCCA occlusion (hypoperfusion control). In the fifth, i.e., treatment group, C.A 100 mg/kg, p.o. was administered 1 h before subjecting them to permanent BCCA block, and then the same dose was continued for 28 days. Animals were subjected for behavioural assessment from 28th day onwards 1 h after the last dose and at the end, animals were sacrificed under pentobarbitone (PB) over dose, and histopathology of the brain samples was studied.

Biochemical Estimation

Sampling technique

At the end of experiments animals were sacrificed by decapitation, forebrains were collected; and rinsed with ice cold 0.9% NaCl solution. Both halves of forebrain were transferred to appropriate homogenizing medium.

Lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) a by-product of lipid peroxidation. One g of wet rat forebrains were transferred to 9 ml of ice cold 1.15% KCl (1:9 w/v) and homogenized with the

help of Teflon homogenizer under ice cold condition. 200 µL of this homogenate was taken for further assay. Results were expressed as n mols of MDA/g of wet tissue. 1, 1′, 3, 3′-tetraethoxypropane was used as external standard to prepare standard curve.[39]

SOD activity

Rat forebrains were homogenized with Teflon homogenizer in ice-cold 0.052 M sodium pyrophosphate (pH 8.3) and 200 µL of this homogenate was used for further assay. The inhibition of reduction of NBT to blue coloured tetrazolium, in the presence of PMS and NADH, by SOD was measured at 560 nm. One unit of enzyme activity is defined as enzyme concentration required to inhibit the optical density of chromogen production at 560 nm, by 50% in one min under experimental condition, and was expressed as specific activity in milliunits/mg of protein.[40]

Brain protein

It was estimated by following the method of Lowry *et al*. 1951.[41]

Behavioural Study

Open field test

Locomotor activity and exploratory behaviour was evaluated using open field test. Open field apparatus consists of cubicle box made up of plywood, and is of 96 × 96 cm dimension. All the walls are painted black except for the floor which is divided into 16 squares by 6 mm thick white lines. Animals were placed in a corner individually and for the next 5 min ambulation (number of squares crossed), immobility period (in sec), grooming (number), rearing (number) and faecal pellets (number) were observed.[42]

Elevated plus maze test

Anxiety parameter was tested by elevated plus maze test. This maze consists of two open arms (50 × 10 cm) crossed with two enclosed arms (with walls 40 cm high) of same dimension at right angles to each other. The arms are connected with a central square (10 × 10 cm) to give the apparatus a plus sign appearance. The elevated plus maze was kept elevated 50 cm above the floor in a dimly lit room. The rats were placed individually on the central square of the plus maze facing one enclosed arm. The time spent and the number of entries made by the rat during the next 5 min on the open and closed arms was recorded. An arm entry was defined when all four limbs of the rat were on the arm.[43]

Behavioural despair/Porsolt's swim test

The rats were placed individually in a cylinder (45 × 20 cm) containing 38 cm water (25 ± 2°C), so that the rats could not touch the bottom of the cylinder with its hind limbs or tail or climb over the edge of the chamber. Two swim sessions were conducted, an initial 15 min pre-test followed by a 5 min test, 24 h later. Drug was administered after pre-test. The period of immobility (duration of floating in water without struggling and making only those movements necessary to keep its head above the water level), during 5 min test period was noted.[44]

Morris' water maze test

On 28th day of surgery, spatial learning and memory were tested in a water maze.[45] The maze consisted of a black circular pool (diameter 214 cm, height 80 cm) filled to a depth of 44 cm with water (25 ± 2°C) made opaque by adding Indian ink. On 27th day the rats received habituation (exposure in water maze for 1 min) during which no platform existed. Then, on 28th day a circular platform (9 cm in diameter) was kept hidden 2 cm below the water level in centre of a quadrant. The platform remained in same position during training days (reference memory procedure). At the beginning of each session, a random sequence of four starting poles along the perimeter of pool was generated. All animals followed this sequence for that session. Each rat was placed in the water facing the wall at the start location and was allowed 90 sec to find the hidden platform. The animal was allowed a 20 sec rest on the platform. The latency to reach the platform was recorded. If the rat was unable to locate the hidden platform, it was lifted out and placed on the platform for 20 sec. The procedure was repeated for all the four start locations.

Four sessions of four trials each were conducted on the first day of testing separated by 4 h and one session of four trials was conducted on the next day. After that, the platform was removed and a probe trial (without platform) was conducted 4 h later. Each rat was placed in the pool at the same randomly selected starting pole and swimming path was observed and time spent in the quadrant of the pool that initially contained platform was measured.

On completion of probe trial, a black platform that extended 1 cm above water surface was placed in a quadrant other than that chosen for submerged platform. Each rat was then given four trials of 90 sec to locate it. The latency to reach the platform was recorded (working memory procedure).

Histopathological Study

At the end of behavioural testing rats were sacrificed by decapitation under PB anaesthesia brains were taken out and transferred to 10% formalin. Five mm frontal sections of forebrain were prepared and stained with hematoxylin and eosin.

Statistical Analysis

One way ANOVA followed by post hoc Tukey test was used for statistical analysis of data. A P value < 0.05 was considered statistically significant.

RESULTS

Lipid Peroxidation

Effect of C.A pre-treatment (100 mg/kg/day for 5 days) on rat brain oxidant and anti-oxidant status in ischemia-reperfusion injury is shown in [Table 1]. IR injury resulted in 1.9 fold increase in MDA concentration. C.A pre-treatment attenuated this increase by 35.79%. A comparison between groups indicated that ischemia-reperfusion injury induced a significant increase in lipid peroxidation by-product MDA ($P < 0.001$) and this rise was significantly attenuated with C.A pre-treatment ($P < 0.001$) [F (4, 25) =18.410]. When comparison was made between normal, sham-operated and C.A *per se* animal groups, there was no significant difference ($P > 0.05$).

SOD Activity

SOD activity in ischemia-reperfusion control group was significantly higher than sham- operated animals ($P < 0.001$), and no such significance was noticed between normal, sham-operated and C.A per se animals. Ischemia-reperfusion injury produced 2.1 fold increase in SOD activity, while C.A pre-treatment decreased this up regulation by 25.24% ($P < 0.001$) [F (4, 25) = 90.563].

Open Field Test

The results are summarized in Table 2. In hypoperfused animals ambulations were decreased by 65.43% as compared with sham-operated animals. This alteration was ameliorated by C.A treatment ($P < 0.001$) [F (4, 25) =50.276]. Hypoperfused animals had increased immobility period as compared to that of sham-operated animals ($P < 0.001$). C.A treatment attenuated this change significantly ($P < 0.001$) [F (4, 25) = 19.019] Number of rearings, and faecal pellets did not show significant changes among any of the study groups. {Faecal pellets [F (4, 25) = 0.413], Rearing [F (4, 25) = 1.637]. There was significant decrease in number of groomings in hypoperfused animals ($P < 0.01$) but with CA treatment though there was a reversal trend the difference was not statistically significant [F (4, 25) = 4.047].

Elevated Plus Maze Test

Table 3 shows that hypoperfused animals spent more time in closed arms as compared to sham-operated animals [F (4; 25) = 6.512, $P < 0.01$]. C.A treatment in the hypoperfused animals significantly less time in closed arm compared to hypoperfused group ($P < 0.01$). The ratio of time spent in open arm to closed arm shows, sham operated vs. hypoperfused ($P < 0.001$) and hypoperfused vs. C.A treatment ($P < 0.001$) [F (4, 25) = 23.564}.and ratio of entries in open arm to closed arm shows between sham operated vs. hypoperfused ($P < 0.001$) and hypoperfused vs. C.A treatment ($P < 0.01$) [F (4, 25) = 3.760] (Time spent in open arm F = 23.05, Number of entries in to closed arm F = 5.599, Number of entries in to open arm F = 3.111).

No statistically significant difference was found between normal, sham-operated and C.A per se animals with respect to different parameters, viz, open arm entry, closed arm entry, time spent in open arm and time spent in closed arm, when compared with sham-operated animals ($P > 0.05$).

Behavioural Despair/Porsolt's Swim Test

[Table 4] shows that compared to sham-operated animals, hypoperfused animals stayed for significantly more time ($P < 0.01$) without despair. No statistical difference was found among sham-operated, normal and C.A per se animal groups ($P > 0.05$). C.A treatment significantly reduced this time period ($P < 0.05$), when compared with hypoperfused animals. [F (4, 25) =10.533].

Morris' Water Maze Test

In Morris' water maze test [Table 5], no significant difference

Table 1: Effect of *Centella asiatica* (100 mg/kg, p.o. x 5 days) on biochemical parameters of oxidative stress in rat forebrain following cerebral ischemia –reperfusion injury (I-R) (30 min BCCAO followed by 45 min reperfusion injury)

Groups	MDA (n mols/g tissue)	SOD (m units/mg protein)
Normal	883.33 ± 22.048	342.67 ± 13.8
Sham-operated	870.83 ± 25.35	339.8 ± 16.6
C.A. *per se*	908.33 ± 36.9	348.0 ± 18.94
I-R control	1654.16 ± 149.8*	717.8 ± 16.41*
C.A. treatment	1062.5 ± 71.22*	533.5 ± 21.44*

All data are expressed as Mean ± S.E.M. Number of animals in each group = 6. Sham operated group is compared with I-R group and C.A treatment group is compared with I-R group. *indicate *P* value < 0.001. Statistical analysis was done by One – way ANOVA followed by post hoc Tukey test

Table 2: Effect of *Centella asiatica* (100 mg/kg p.o. x 28 days) on open field parameter in long-term hypoperfused rats

Group	Ambulations (number)	Immobility (sec)	Rearing (number)	Grooming (number)	Faecal pellets (number)
Normal	50.33 ± 3.64	81.5 ± 7.94	23.67 ± 2.70	8.0 ± 0.78	1.83 ± 0.48
Sham-operated	49.67 ± 1.9	60.33 ± 2.31	19.67 ± 1.31	10.83 ± 0.88	1.33 ± 0.42
C.A. per se	50.0 ± 1.77	53.17 ± 1.78	21.83 ± 1.74	9.67 ± 0.96	1.33 ± 0.21
Hypoperfusion	32.5 ± 1.98*	101.17 ± 4.75*	18.0 ± 0.93	6.5 ± 0.96a	1.33 ± 0.33
C.A. treatment	79.33 ± 2.11*	66.33 ± 1.45*	19.67 ± 1.38	7.33 ± 0.80	1.67 ± 0.33

All data are expressed as Mean ± S.E.M. Number of animals in each group = 6. Sham-operated group is compared with hypoperfusion group and hypoperfusion group is compared with C.A treatment group. *Indicate *P* value < 0.001, and [a]indicate *P* value < 0.01 Statistical analysis was done by one – way ANOVA followed by post hoc Tukey test

Table 3: Elevated plus maze test- Effect of *Centella asiatica* (100 mg/kg p.o.x 28 days)

	Time spent in seconds		Number of entries		Ratio of time spent	Ratio of number of entries
	CA	OA	CA	OA	OA/CA	OA/CA
Normal	235.33 ± 3.04	37.0 ± 2.42	6.16 ± 1.08	5.0 ± 0.86	0.158 ± 0.011	0.913 ± 0.206
Sham operated	231.33 ± 11.06	36.66 ± 3.95	4.0 ± 0.58	5.0 ± 0.73	0165 ± 0.024	1.475 ± 0.367
Per se	242.83 ± 2.96	35.83 ± 4.42	3.83 ± 0.60	2.83 ± 0.40	0145 ± 0.020	0.883 ± 0.260
Hypoperfusion	250.66 ± 2.08[a]	15.16 ± 2.06[a]*	7.5 ± 0.76	2.33 ± 0.42	0.061 ± 0.008*	0.303 ± 0.036*[a]
Treatment	207.66 ± 6.38[a]	62.16 ± 3.09*	8.33 ± 0.84	4.5 ± 0.76	0.301 ± 0.018*	0.558 ± 0.110*

All data are expressed as Mean ± S.E.M. Number of animals in each group = 6. Sham-operated group is compared with hypoperfusion group and C.A treatment group is compared with hypoperfusion group. *Indicate *P* value less than 0.001, and [a]indicate *P* value less than 0.01. Statistical analysis was done by one – way ANOVA followed by post hoc Tukey test

Table 4: Effect of CA (100 mg/kg p.o. x 28 days) on Porsolts swim test in long term hypoperfused rats

Group	Immobility time in sec
Normal	22.5 ± 1.57
Sham operated	23.5 ± 1.23[a]
Per se	24.5 ± 1.46
Hypoperfusion	37.0 ± 2.59[ab]
Centella asiatica treatment	28.33 ± 1.96[b]

All data are expressed as Mean ± S.E.M. Number of animals in each group = 6. Sham- operated group is compared with hypoperfusion group and C.A treatment group is compared with hypoperfusion group. [a] indicate *P* value < 0.01, and [b] indicate *P* value < 0.05. Statistical analysis was done by one – way ANOVA followed by post hoc Tukey test. F=8.622 and 0.0930

was observed among normal, sham-operated and C.A per se group animals ($P > 0.05$), where as hypoperfusion control group animals showed a trend in which time taken to reach the submerged platform was more and was significant from the first trial itself ($P < 0.001$), as compared to sham-operated animals. The C.A treated animals found the submerged platform much quicker than hypoperfused animals ($P < 0.001$) in all the five escape latency trials (escape latency1 [F (4, 25) = 37.662], escape latency 2 [F (4, 25) = 26.386], escape latency3 [F (4, 25) = 15.280], escape latency 4 [F (4, 25) = 14.816], escape latency 5 [F (4, 25) = 20.542]). In probe trial, when compared with sham-operated animals, these hypoperfused animals, swam for lesser time (60.54%) in the quadrant in which the platform was kept earlier during escape latency sessions. These changes were attenuated by C.A treatment (32.76%) [F (4, 25) = 3.475]. In new platform test, sham-operated and C.A treated hypoperfused animals, found new visible platform kept at a different quadrant, much quicker than hypoperfused animals; however, the difference was not statistically significant [F (4, 25) = 3.326].

Histopathological Study

Forebrain sections of chronically hypoperfused rats showed an increase in number of inflammatory cell infiltration in the perivascular areas, cellular oedema, microgliosis and reactive astrocytosis [Figure 1a and 1b]. These changes are not evident in sham operated [Figure 2] and C.A *per se* animals [Figure 3]. C.A treatment in hypoperfused animals for 28 days reduced these changes [Figure 4].

DISCUSSION

Bilateral common carotid occlusion for 30 min followed by reperfusion for 45 min was associated with increased generation of reactive oxygen species (ROS) and free radicals.[12,13] These ROS will cause cell membrane lipid peroxidation. Increased levels of MDA, a by-product of lipid peroxidation, reflect membrane damage induced by these free radicals.[46] Increased SOD activity, a marker for oxidative stress, suggests the same. Generation of ROS by activated polymorphonuclear leukocytes (PMNL) represents an important mechanism of injury induced by transient cerebral ischemia and reperfusion. These are believed to be the source of increased lipid peroxidation and SOD activity.[20]

Table 5: Effect of *Centella asiatica* (100 mg/kg, p.o. x 28 days) on learning and memory in long-term hypoperfused rats in Morris' water maze

	Escape latencies					Probe trial	New platform
	1	2	3	4	5		
Normal	16.42 ± 1.98	11.21 ± 2.16	10.33 ± 1.01	10.29 ± 1.5	9.29 ± 1.7	27.13 ± 3.4	13.75 ± 1.92
Sham operated	13 ± 1.33*	10.38 ± 2.45*	10.54 ± 1.71*	6.21 ± 1.33*	6.92 ± 0.8*	29.25 ± 3.3	12.79 ± 1.5
Per se	20.87 ± 3.3	10.83 ± 3.67	9.13 ± 1.64	6.91 ± 1.1	6.45 ± 1.03	27.88 ± 2.12	7.5 ± 0.87
Hypoperfusion	67.04 ± 5.67*	48.83 ± 3.1*	52.46 ± 9.82*	30.18 ± 8.96*	24.13 ± 2.6*	17.71 ± 0.9	23.46 ± 7.1
Treatment	25.5 ± 3.95*	14.87 ± 4.3*	11.5 ± 3.6*	8.96 ± 1.4*	10.42 ± 1.4*	27.29 ± 1.8	9.04 ± 1.4

All data are expressed as Mean ± S.E.M. Number of animals in each group = 6. Sham-operated group is compared with hypoperfusion group and C.A group is compared with hypoperfusion group. *Indicate *P* value < 0.001 and [+]indicate *P* value < 0.05. Statistical analysis was done by one – way ANOVA followed by post hoc Tukey test

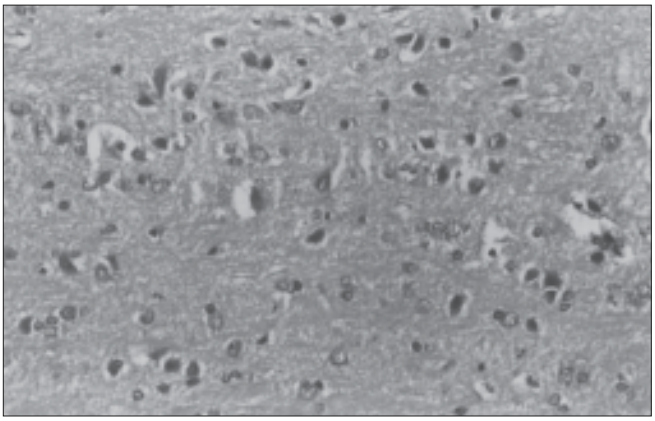

Figure 1: Histological appearance of forebrain of Sham operated animals. Note the normal brain picture (H and E, ×100)

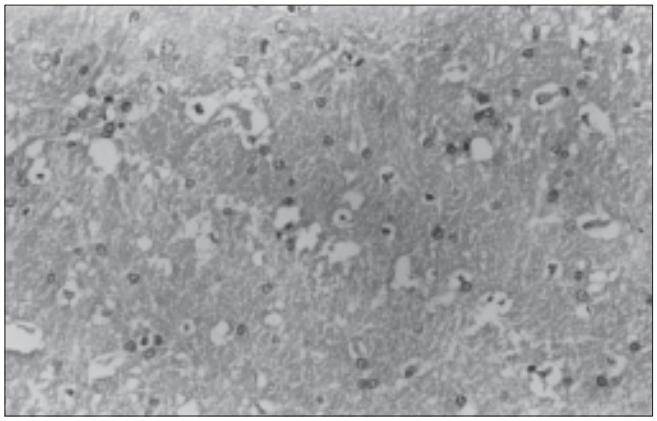

Figure 2: Histological appearance of forebrain in *Centella asiatica* per se animals Note normal brain picture (H and E, ×100)

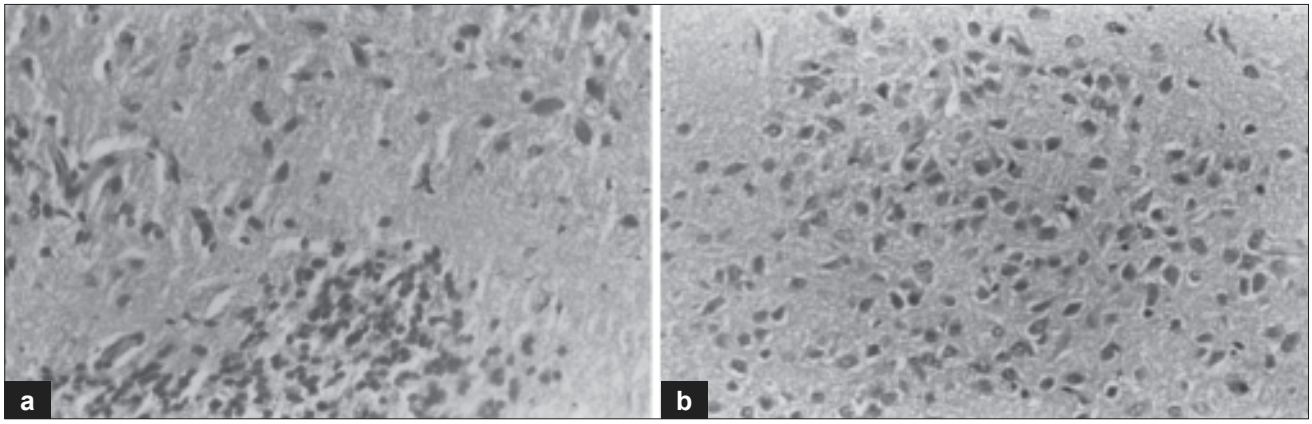

Figure 3 (a,b): Histological appearance of forebrain subjected to hypoperfusion study. Note the increase in cellularity (lymphocytic infiltration), gliosis, perivascular edema (H and E, ×100)

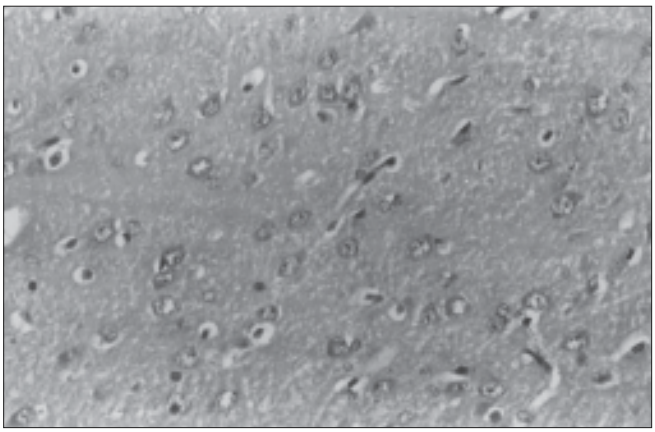

Figure 4: Histological appearance of forebrain of animals subjected to hypoperfusion and treated with *Centella asiatica* Note less prominent gliosis, lymphocytic infiltrations, cellular edema (H and E, ×100)

There will be an up regulation of SOD activity following ischemia reperfusion injury, and this up regulation indicates that the antioxidant machinery of brain has been activated in response to excessive generation of free radicals.[47] Ischemia- reperfusion injury is viewed as inflammatory response, as PMNL accumulation in the ischemic area is documented.[48] These PMNLs are the source of free radicals especially superoxide anions.[20] SOD converts these superoxide anions into H_2O_2 and molecular oxygen. This H_2O_2 is more toxic to tissues than superoxide. In the presence of Fe^{2++}, H_2O_2 is converted into hydroxyl radicals.[19] So in this setting elevation of SOD activity is rather detrimental than protective.[19] C.A pre-treatment prevented rise in MDA levels and down regulated SOD activity indicating the capability of CA to attenuate ischemia-reperfusion induced excessive formation of ROS. The antioxidant activity of asiaticoside, an important constituent of C.A, protecting against beta amyloid induced neurotoxicity of Alzheimer's disease has been reported earlier.[49] Reduction in MDA concentration and an increase in glutathione were reported earlier by various investigators.[36,50] Streptozotocin and pentylenetetrazole induced oxidative stress were reported to be reversed by CA treatment.[29,35] The present finding of antioxidant activity of CA in cerebral ischemia reperfusion assault is in accordance with the similar finding in myocardial ischemia reperfusion injury reported earlier.[33]

C.A has been reported to have antidepressant activity in

rat model.[51] In our study, the results of Porsolts swim test show the same. In chronic hypoperfusion there was an increased propensity towards anxiety and listlessness, and C.A treatment decreased the anxiety in this model. It is evident from our study that C.A can alleviate the anxiety induced in chronically hypoperfused animals and is supporting the earlier studies that C.A possesses antianxiety effect.[36] In animal models, it has been shown that, the water soluble fraction of C.A possessed anxiolytic effect comparable to diazepam.[52] In human beings C.A exhibited anxiolytic activity as assessed by acoustic startle response.[53]

In Morris water maze test, hypoperfused animals showed deficits in spatial learning and memory, which was in accordance with previous reports of ischemia-reperfusion induced changes in learning and spatial memory.[12,13] Probe trial and new platform trials also showed deficits in reference memory and working memory respectively.[54] These changes were alleviated by C.A treatment. Even the earlier studies with C.A in Alzheimer's disease model of rat; and pentylenetetrazole induced kindling rat model showed that C.A can alter the cognitive deficits and memory impairments in these experimental models.[29,35]

In the past permanent occlusion of Bilateral common carotid artery was used as a model of neurodegenerative conditions, cerebrovascular insufficiency states and dementia.[22,55,56] Permanent Bilateral common carotid occlusion in rats causes reduction in cerebral blood flow by 30-45% to cortex and 20% to hippocampus after 7-10 days. Similarly, 20-30% reduction in cortical and 15% reduction in hippocampal glucose utilization is seen.[57,58] CA1 neurons in the hippocampus, a part of limbic system is most vulnerable to hypoxic and hypoglycaemic injury, which is responsible for memory and cognitive deficits.[38,59] Chronic reductions in cerebral blood flow and brain energy metabolism causes progressive dysfunction of neurons, resulting in cognitive deficits.[60] This ischemia is a vicious cycle, which operates through L-type Ca^{2++} channels to cause more and more microvascular constriction and reduction in blood flow as well as glucose and oxygen delivery.[61,62] Disruption of coupling of blood flow to neuronal activation, results in impairment of normal synaptic communication among neurons that underlie normal learning and memory process.[63,64] This reduced brain energy metabolism as a result of chronic hypoperfusion induces microglial activation and reactive astrocytosis.[65] Though microgliosis is evident from 15th day onwards, more pronounced changes are seen after 28 days. This microgliosis is an adaptive change to counteract the neuronal insult. These microglial cells elaborate various cytokines, which are responsible for mononuclear leukocyte cell accumulation and perivascular oedema seen here. Absence of neuronal loss is a conflict to explain the appearance of cognitive deficits, which can be explained on the basis, that impaired neuronal function, which is in fact, a result of altered brain energy metabolism, is the cause behind it.[65,66] C.A treatment reverses this gliosis perivascular oedema, mononuclear cell infiltrates and astrocytoses proving its adaptogenic property.

Long-term BCCAO is known to induce neuronal changes in the form of neuronal loss/cell death.[12,13,67] In the present study there were no microscopically demonstrable neuronal injuries. Absence of neuronal loss/cell death associated with conflicting data of behavioural analysis in the present study needs to be interpreted cautiously. Glial activation has consistently been shown to occur before any overt neuronal damage.[68-70] Since BCCAO causes only 30-45% reductions in blood supply to the forebrain areas, and collateral circulation through vertebral arteries are maintained, there is a theoretical possibility that the neurons are not seriously damaged leading to their death. Therefore, it may be postulated that neuronal damage may be evident if BCCAO has been continued for a prolonged period or end arteries like middle meningeal arteries are targeted. Alternatively, more sophisticated techniques of evaluation like immunohistochemistry or electron microscopy might have revealed any subtle neuronal changes, if any.[23,24]

Stroke being one of the important causes of mortality and morbidity over the world and considering the fact that there is no effective treatment for the post stroke ailment and morbidity, it could be propitious to go on with future studies with C.A in these patients as this particular herbal drug showed significant improvement in anxiety, depression and memory parameters in this animal model of chronic cerebral hypoperfusion and reductions in oxidative stress in ischemia–reperfusion model. It can be proposed for the treatment of combined depression and anxiety.

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