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Evaluation of antioxidant and neuroprotective effect of *Ocimum sanctum* on transient cerebral ischemia and long-term cerebral hypoperfusion

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Abstract

Free radicals are implicated in causation of cerebral reperfusion injury and chronic cerebral hypoperfusion in rats is associated with functional and histopathological disturbances. *Ocimum sanctum* (OS), a plant widely used in Ayurveda, has been shown to possess anti-inflammatory, antioxidant and cognition-enhancing properties. In the present study, we investigated the effect of methanolic extract of OS leaves in cerebral reperfusion injury as well as long-term hypoperfusion. Occlusion of bilateral common carotid arteries (BCCA) for 30 min followed by 45 min reperfusion caused increase in lipid peroxidation and up-regulation of superoxide dismutase (SOD) activity accompanied by fall in tissue total sulfhydryl groups (TSH) in rat forebrains. Ascorbic acid levels were unchanged, however. OS pretreatment (200 mg/kg/day for 7 days) prevented this reperfusion-induced rise in lipid peroxidation and SOD activity. OS pretreatment also stabilized the levels of TSH during reperfusion. Long-term cerebral hypoperfusion (a model of cerebrovascular insufficiency and dementia) induced by permanent occlusion of BCCA for 15 days demonstrated altered exploratory behavior in open-field testing and memory deficits as tested by Morris' water maze. Histopathological examination of hypoperfused animals revealed reactive changes, like cellular edema, gliosis and perivascular inflammatory infiltrate. OS treatment (200 mg/kg/day for 15 days) significantly prevented these hypoperfusion-induced functional and structural disturbances. The results suggest that OS may be useful in treatment of cerebral reperfusion injury and cerebrovascular insufficiency states.

Keywords: *Ocimum sanctum*; Reperfusion injury; Oxidative stress; Cerebral hypoperfusion; Learning and memory

1. Introduction

In recent times, focus on plant research has increased globally and the large body of evidence collected shows the immense potential of medicinal plants. *Ocimum sanctum* Linn (OS), commonly known as 'Holy Basil', is considered a sacred plant in India and grown in every rural household. Traditionally, fresh juice or decoction of OS leaves is used to promote health and in treatment of various disorders as advocated in Ayurveda, the Indian System of Medicine. Indian Materia Medica describes the use of aqueous, hydro-alcoholic and methanolic extract of OS leaves in a variety of disorders, like bronchitis, rheumatism and pyrexia (Nadkarni, 1976; Kritikar and Basu, 1935). Several recent investigations using these extracts have indicated that OS possesses significant anti-inflammatory (Singh et al., 1996), antioxidant (Maulik et al., 1997), immunomodulatory (Mediratta et al., 2002) and antistress (Sen et al., 1992) properties. In addition, it has been reported to have radioprotective and anticarcinogenic property (Devi, 2001). Likewise, the active principles of OS, comprising of phenols and flavones, have been shown to have significant antioxidant and anti-inflammatory activity, both in vivo and in vitro (Kelm et al., 2000; Devi et al., 2000).

Reperfusion injury is a distinct entity from the primary ischemic injury; the oxygen arriving with blood circulation, although necessary for alleviating the ischemic status, may be harmful and worsen the damage. Excessive generation of

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reactive oxygen species (ROS) is believed to be the main culprit in the causation of reperfusion injury (Gringo, 1997; Nakashima et al., 1999). Chronic cerebral hypoperfusion induced by permanent occlusion of bilateral common carotid arteries (BCCA) in rats induces a state of chronic low-grade ischemia in rat brains over an extended period of at least several months (Ni et al., 1994; Davidson et al., 2000). Extensive investigations report that rats subjected to permanent occlusion of BCCA show impaired spatial learning/memory capabilities and/or structural alterations (Sarti et al., 2002).

In Ayurveda, OS is described as 'rasayana' (plants having adaptogen like properties). These Ayurvedic rasayanas and adaptogens, like *Panax ginseng*, have been reported in literature to improve physical and mental health, increase nonspecific resistance of body, promote physiological functions and augment cognition (Kritikar and Basu, 1935; Brekhman and Dardymox, 1969; Bhattacharya, 1993; Rege et al., 1999). Extensive recent investigations have validated these reports. It is interesting to note that nearly all these rasayanas have surprisingly similar spectrum of pharmacological activity. *Withania somnifera* (also a rasayana plant), for example, has been shown to have antistress, antioxidant, anticarcinogenic, radioprotective and immunomodulatory actions like OS (Bhattacharya and Muruganandam, 2003). However, *W. somnifera* and other rasayanas possess additional neuropharmacological effects, like enhancement of cognition, attenuation of cognitive deficits in animal models of Alzheimer's disease, and anxiolytic and antidepressant actions (Bhattacharya and Muruganandam, 2003). Moreover, *W. somnifera* has demonstrated the neuroprotective effect in the animal model of stroke (Chaudhary et al., 2003). Although OS shares almost all of its medicinal properties with other rasayanas in the group, its neuropharmacological profile has not been investigated before. Therefore, we decided to evaluate the effect of OS on transient partial cerebral ischemia induced by BCCA occlusion for 30 min followed by reperfusion and on chronic cerebral hypoperfusion.

2. Materials and methods

2.1. Animals

After approval from Institutional Ethics Committee, the experiments were conducted on in bred male Charles–Foster rats (250–300 g). The animals were maintained in colony cages under an ambient temperature of 25±2 °C and 45–55% relative humidity, with a 10-h light/14-h dark cycle. They were allowed food and water ad libitum; food was withdrawn 18 h prior to surgery, however. Principles of Laboratory Animals Care and Use (NIH Publication No. 85-23, revised 1985) guidelines were followed throughout.

2.2. Chemicals and reagents

1,1,3,3-tetraethoxypropane has been obtained from Merck (Germany). Thiobarbituric acid, eugenol, NADH, nitroblue tetrazolium (NBT) and phenazine methosulphate (PMS) were procured from Sigma (USA). All other chemicals and reagents were of the highest analytical grades available locally. Measurement of lactate was done using the ready-to-use kit (Zydus Pathline, India).

2.3. Plant material and standardization of extract

Methanolic extract of OS leaves was provided kindly by Dr. R.K. Goel (Head, Pharmacology, Banaras Hindu University, Varanasi, India). In brief, the procedure for preparation and standardization of extract was as follows—Fresh, tender leaves of OS were collected in the month of December from the Ayurvedic garden of our Institute. The leaves were size reduced and macerated with methanol for 7 days. The extract was filtered, vacuum dried and stored in a refrigerator until further use. The yield was 6.04%. The methanolic extract of OS was quantified for the essential oil, eugenol by HPTLC using a CAMAG assembly (evaluation software© 1990 TLC system; Scanner II. V, 3.14/PC/CTS version), Toluene:ethyl acetate (93:7) as developing solvent, eugenol as standard and quenching at 260 nm. The percentage of eugenol was 5.1%. The animals received this extract orally in dosages of 200 mg/kg/day suspended in 0.3% carboxymethyl cellulose (CMC). This particular dose was selected on the basis of our preliminary experiments that assessed the ability of OS extract to attenuate the reperfusion-induced lipid peroxidation (at dosages of 50–1000 mg/kg/day) and earlier reports (Bhattacharya et al., 2001; Sairam, 2001).

2.4. Experimental procedure

2.4.1. Induction of transient cerebral ischemia and chronic hypoperfusion

Transient cerebral ischemia was produced by following the method of Iwasaki et al. (1989). Under pentobarbitone anesthesia (35 mg/kg i.p.), a midline skin incision in neck was given, common carotid arteries were identified and isolated carefully from accompanying nerves. Rat brains were made ischemic by occluding BCCA for 30 min and then reperfusion was allowed by releasing block for 45 min. Body temperature was maintained at about the 37 °C during the period with the help of heating lamp. Induction of sufficient degrees of ischemia was confirmed by estimating the forebrain lactate levels during occlusion period (lactate levels 34.57±2.58 nM/mg protein in sham-operated controls versus 64.13±3.39 nM/mg protein after 30 min BCCA occlusion, $P<0.001$ $n=6$ in each group). Sham-operated control animals underwent all the surgical procedure except occlusion of BCCA.

Chronic cerebral hypoperfusion was induced by adapting the method of Pappas et al. (1996). After their isolation from accompanying nerves, carotid arteries were doubly ligated with 3-0 silk sutures and cut in between. The skin was then sutured and animals were returned to their home cage.

2.4.2. Steady state experiment

For transient cerebral ischemia studies, the animals were divided into four groups of six animals each. The first group served as sham-operated control and received 0.3% CMC (vehicle) for 7 days in double-distilled water. In the second group, methanolic extract of OS leaves (200 mg/kg/day p.o. suspended in 0.3% CMC, once in a day for 7 days) was administered to determine the effects of OS per se. In third-group animals, which received only vehicle for 7 days, transient cerebral ischemia was induced by subjecting them to 30 min BCCA occlusion and 45 min reperfusion. Group 4 animals were pretreated with OS (200 mg/kg/day p.o. once a day for 7 days) before subjecting them to ischemia–reperfusion. Immediately after the reperfusion, animals were sacrificed by decapitation, brains were taken out and forebrain regions were transferred to appropriate homogenizing medium for biochemical estimation.

Similarly, for chronic hypoperfusion experiments, animals were divided into four groups (six animals each). The first group served as sham-operated control. In the second group, OS (200 mg/kg/day p.o. once in a day) for entire experimental period was administered in sham-operated animals (OS per se). Animals in the third group were subjected to permanent BCCA occlusion for 15 days and received vehicle only (hypoperfusion group). In the fourth group, OS (200 mg/kg orally) was administered 60 min before permanent BCCA occlusion. OS (200 mg/kg/day p.o. once a day) was then continued up to the 15th postsurgical day. On day 15 (60 min after last dose of OS), all animals were subjected to behavioral assessment in open-field paradigm and Morris water maze. Then, under overdose of pentobarbitone, animals were sacrificed by decapitation and brain samples were collected for histopathological analysis.

2.5. Biochemical analysis

Assessment of oxidant–antioxidant status of the rat forebrains subjected to transient cerebral ischemia was done by measuring the levels of lipid peroxidation, superoxide dismutase (SOD) activity, TSH and ascorbic acid. Estimation of lipid peroxidation was done by measuring the levels of malondialdehyde (MDA), a by-product of lipid peroxidation. The rat forebrain regions were rinsed with ice-cold normal saline. To 1 g of wet tissue, 9 ml of 1.12% KCl was added. The mixture was then homogenized with the help of Teflon homogenizer and 200 μl of whole homogenate was taken for the assay. The concentration of MDA in brain homogenates was expressed in terms of nM MDA/mg protein. 1,1′,3,3′,-tetraethoxypropane was used as reference compound (Ohkawa et al., 1979). For evaluation of SOD activity, the rat brains were homogenized in ice-cold sodium pyrophosphate buffer (pH 8.3) in a ratio of 50 mg/ml, and 200 μl of this homogenate was used for the assay. The inhibition by SOD of reduction of NBT to blue-colored chromogen in the presence of PMS and NADH was measured at 560 nm. One unit of enzyme activity was defined as enzyme concentration required to inhibit the absorbance at 560 nm of chromogen production by 50% in 1 min under assay condition and expressed as specific activity in milliunits per milligram of protein (Kakkar et al., 1984). For estimation of TSH, a 200-mg sample of brain tissue was homogenized in 8 ml of 0.02 M EDTA and 500 μl of this was used for the assay. Measurement of TSH was done by analyzing the reaction of SH groups in tissue with Ellman's reagent and levels are expressed as mmol SH/mg of proteins (Sedlack and Lindsay, 1968). Measurement of lactate levels was done using the ready-to-use kit following manufacturer's instructions (Zydus Pathline). The assay was based on the principle—lactate oxidase cleaves the lactate into pyruvate and H_2O_2 that in the presence of peroxidase reacts with 4-amino antipyrine and phenol to a red chinonimin dye. The increase in color is proportional to lactate concentration (Noll, 1984). The protein content of brain tissue was estimated by following the method of Lowry et al. (1951). Ascorbic acid levels were determined by the method of Omaye et al. (1979).

2.6. Behavioral procedures

2.6.1. Open-field testing

Exploratory behavior was evaluated in an open-field paradigm (Lister, 1990). The open field was made of plywood and consisted of a floor (96×96 cm) with high walls. The entire apparatus was painted black except for 6-mm-thick white lines that divided the floor into 16 squares. Each animal was placed at one corner of the apparatus and for next 5 min, it was observed for the ambulations (number of squares crossed), total period of immobility (in seconds), number of rearings, groomings and fecal pellets.

2.6.2. Morris' water maze test

Spatial learning and memory was tested in water maze (Morris, 1984) following the method of Pappas et al. (1996). The maze consisted of a black circular pool (diameter 2.14 m, height 80 cm) filled to a depth of 44 cm with water (25 °C). On postsurgical day 14, rats received habituation (exposure in water maze for 1 min) in which there was no platform present. Then, on day 15, a circular platform (9 cm in diameter) was kept hidden 2 cm below water level in the center of one of the quadrants. The platform remained in the same position during all the sessions. At the beginning of each session, a random sequence of four starting poles along the perimeter of the 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 s to find the hidden platform. The animal was allowed a 20-s 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 s. The procedure was repeated for all the four start locations. Two 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; swimming path was observed and time spent in the quadrant of pool, which initially contained platform, was measured.

2.7. Histopathological examination

At the end of behavioral testing, the animals were deeply anaesthetized with pentobarbitone. Following decapitation, the brains were taken out and fixed in 10% formalin. Multiple, paraffin-embedded, coronal sections (5-μm thick) were taken from each brain (spanning through striatum to caudal hippocampus). Serial sections (spaced apart by 250 μm, 15 in total for each brain) were selected for histopathological analysis of neuronal damage. After staining with hematoxylin and eosin, the slides were examined using light microscopy by an observer blinded to experimental groups.

2.8. Statistical analysis

Statistical analysis of data (using GraphPad InStat, Version 3.05, 32 bit for Win95/NT, GraphPad Software, San Diego, CA, USA) was performed by applying one-way analysis of variance (ANOVA) followed by Tukey test for biochemical parameters and Mann–Whitney *U* test for behavioral observations. A P value <0.05 was considered statistically significant.

3. Results

3.1. OS pretreatment blocks transient cerebral ischemia-induced oxidative stress

Table 1 shows the effect of OS pretreatment (200 mg/kg/day for 7 days) on oxidant–antioxidant status of rat brain measured after ischemia–reperfusion. BCCA occlusion for 30 min followed by 45 min reperfusion produced a 2.2-fold increase in MDA concentration. OS pretreatment attenuated this increase by about 27%. These findings were supported by the statistical analysis of the data. An ANOVA revealed significant effect between the groups [$F(3,20)=70.71$, $P<0.001$]. A comparison of groups with the post hoc Tukey test indicated that ischemia–reperfusion injury induced a significant increase in lipid peroxidation byproduct MDA ($P<0.001$) and this increase was significantly reduced in the animals pretreated with OS ($P<0.01$). When control animals were compared with OS per se group, there was no significant difference ($P>0.05$). Thus, OS on its own did not alter MDA levels.

Ischemia–reperfusion produced a robust (64%) increase in SOD activity. OS pretreatment reduced this up-regulation by approximately 25%. An ANOVA indicated a significant difference between the groups [$F(3,20)=10.77$, $P<0.01$]. The post hoc Tukey test revealed that SOD activity in ischemia–reperfusion group was significantly higher than control or OS pretreated animals (control vs. reperfusion $P<0.001$; reperfusion vs. OS pretreated animals $P<0.05$). As with MDA, OS per se had no effect on SOD levels ($P>0.05$).

The levels of TSH groups fell substantially in rat brains subjected to reperfusion injury (a 24% decrease). This ischemia–reperfusion-induced consumption of TSH was prevented effectively by OS pretreatment [group effect by ANOVA, $F(3,20)=11.19$, $P<0.001$]. The post hoc Tukey test demonstrated that reperfusion injury causes a significant consumption of tissue thiols as compared to sham-operated controls ($P<0.01$) and this decrease was prevented effectively by OS pretreatment ($P<0.05$). Here, too, OS did not show any effect per se ($P>0.05$).

Ascorbic acid levels, however, did not show any change after reperfusion injury and/or OS pretreatment [group effect, $F(3,20)=0.36$, $P>0.05$]. Thus, total ascorbic acid levels appear unaffected during reperfusion injury.

3.2. Hypoperfusion-induced behavioral deficits are attenuated by OS treatment

Table 2 summarizes the effect of OS on open-field behavior measured after subjecting the animals to hypo-

Table 1
Effect of *O. sanctum* pretreatment (200 mg/kg/day for 7 days) on oxidant–antioxidant status of rat brains subjected to transient cerebral ischemia (30 min BCCA occlusion followed by 45 min reperfusion)

Group	MDA (nM/mg protein)	SOD (milliunits/mg protein)	TSH ($\times 10^{-5}$ M/mg protein)	Ascorbic acid (mg/100 g)
Control	2.58±0.10	450.98±53.68	3.22±0.12	11.25±0.67
OS per se	2.27±0.28	427.82±44.82	3.74±0.18	11.15±0.66
Ischemia/reperfusion	5.87±0.22***	739.83±27.25***	2.46±0.07*	10.84±0.97
OS+ischemia/reperfusion	4.60±0.13**	557.52±43.51*	3.63±0.25***	10.13±0.97

Values are the mean±S.E. Sample size: $n=6$ in each group. Control and treatment (OS+ischemia/reperfusion) groups were compared with the ischemia/reperfusion group. OS per se is compared with the control group. Asterisks indicate statistical significance by one-way ANOVA with post hoc Tukey test.

* $P<0.05$.

** $P<0.01$.

*** $P<0.001$.

Table 2
Effect of *O. sanctum* (200 mg/kg/day for 15 days) on open-field behavior in animals subjected to chronic hypoperfusion

Groups	Ambulations (number)	Immobility (s)	Rearings (number)	Groomings (number)	Fecal pellets (number)
Control	51.83±6.37	29.16±3.71	26.50±2.10	10.16±1.69	2.33±0.76
OS per se	62.50±8.65	34.16±2.91	20.50±1.54	9±1.21	2.83±0.7
Hypoperfusion	34.16±4.67*	47.16±3.72*	15.50±1.25**	10.5±2.04	2.50±0.76
OS+hypoperfusion	61.50±4.12*	34.50±2.93*	20.50±0.76*	7.66±0.49	2.66±0.61

Values are the mean±S.E. Sample size: n=6 in each group. Control and OS+hypoperfusion groups are compared with the hypoperfusion group. OS per se group is compared with control.

* Statistically significant as $P<0.05$ (ANOVA followed by post hoc Mann–Whitney test).

** Statistically significant as $P<0.01$ (ANOVA followed by post hoc Mann–Whitney test).

perfusion for 15 days. In open-field paradigm, animals with permanent BCCA occlusion showed marked alterations in exploratory behavior [ANOVA, $F(3,20)=4.46$, $P<0.05$]. The number of ambulations were decreased by about 35% in hypoperfused animals as compared against control (post hoc Mann–Whitney test, $P<0.05$). This alteration in exploratory behavior was ameliorated by OS treatment ($P<0.05$). Accompanying this alteration in exploratory activities, the period of immobility, too, differed significantly between the groups [ANOVA, $F(3,20)=4.76$, $P<0.05$]. Hypoperfused animals exhibited more freezing behavior when compared with control. This hypoperfusion-induced increased immobility was attenuated by OS treatment. Again, the significant difference was demonstrated by post hoc Mann–Whitney test ($P<0.05$, control vs. hypoperfusion and hypoperfusion vs. treatment group). Furthermore, number of rearings also differed significantly between the groups [group effect by ANOVA, $F(3,20)=9.00$, $P<0.01$]. A post hoc analysis of the data by Mann–Whitney test revealed that the hypoperfused animals exhibited a decrease in rearing behavior when compared to control ($P<0.01$) and this decrease was attenuated effectively by OS treatment ($P<0.05$). However, no difference between the groups was observed for number of groomings and fecal pellets [ANOVA, $F(3,20)=0.76$ and 0.09 for grooming and fecal pellets, respectively, $P>0.05$]. Likewise, OS per se did not show any effect on any of these parameters ($P>0.05$).

Fig. 1A shows the effect of OS treatment on learning memory performance of rats measured after subjecting the animals to chronic hypoperfusion. In the first session of escape latency trial, wherein the latency to locate the submerged platform was assessed, ANOVA did not demonstrate any significant difference between the groups [$F(3,20)=2.29$, $P>0.05$]. In the second session, although ANOVA demonstrated a significant group effect [$F(3,20)=3.89$, $P<0.05$], the post hoc Mann–Whitney analysis did not show any significant difference when the latency to reach the submerged platform in hypoperfused animals (43.37±3.57 s) was compared with the control (34.79±5.14 s) and OS-treated (33.87±3.89 s) animals ($P>0.05$). OS per se (24.91±2.01 s) animals also did not differ from the control animals ($P>0.05$). The difference in

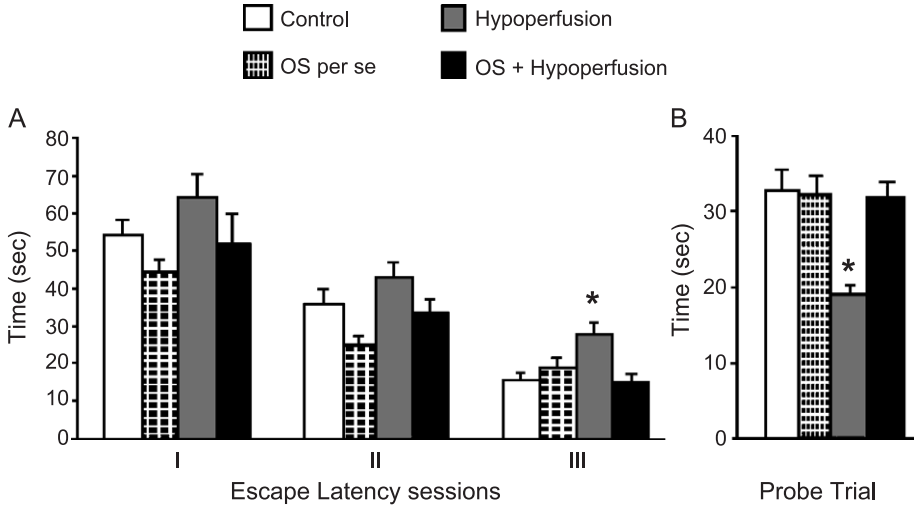

Fig. 1. The effect of OS (200 mg/kg/day p.o. for 15 days) on the hypoperfusion-induced (BCCA occlusion for 15 days) learning and memory deficits in the Morris water maze. (A) In escape latency sessions, the mean of values from four trials in each session was used to express the escape latency to assess acquisition and retention. Significant difference between the groups was observed in the third session. (B) In the probe trial (conducted 4 h after third escape latency session), a bias towards the quadrant of initial platform position was measured. The groups differed significantly. Values are mean±S.E.; number of animals in each group=6. Statistical analysis was done by one-way ANOVA with post hoc Mann–Whitney U test (*Statistical significance as $P<0.01$ for the control vs. hypoperfusion and hypoperfusion vs. OS+hypoperfusion groups).

escape latencies between the groups reached significant levels during the third session conducted on the next day [ANOVA, $F(3,20)=7.50$, $P<0.01$]. Here, hypoperfused animals took longer time to find the submerged platform (28.16±2.64 s) as compared to control (15.62±1.23 s) animals ($P<0.01$), and this increase in latency was attenuated significantly by OS treatment (14.70±2.27 s, $P<0.01$).

In the probe trial (Fig. 1B), a significant difference in the spatial bias of animals towards the quadrant of pool that contained the submerged platform during the escape latency sessions was observed [ANOVA, $F(3,20)=8.29$, $P<0.01$]. The hypoperfused animals spent a significantly less time (19.12±1.02 s) in the quadrant of the initial platform position than control (32.75±2.39 s) and OS-treated (31.25±2.42 s) animals ($P<0.01$, Mann–Whitney test).

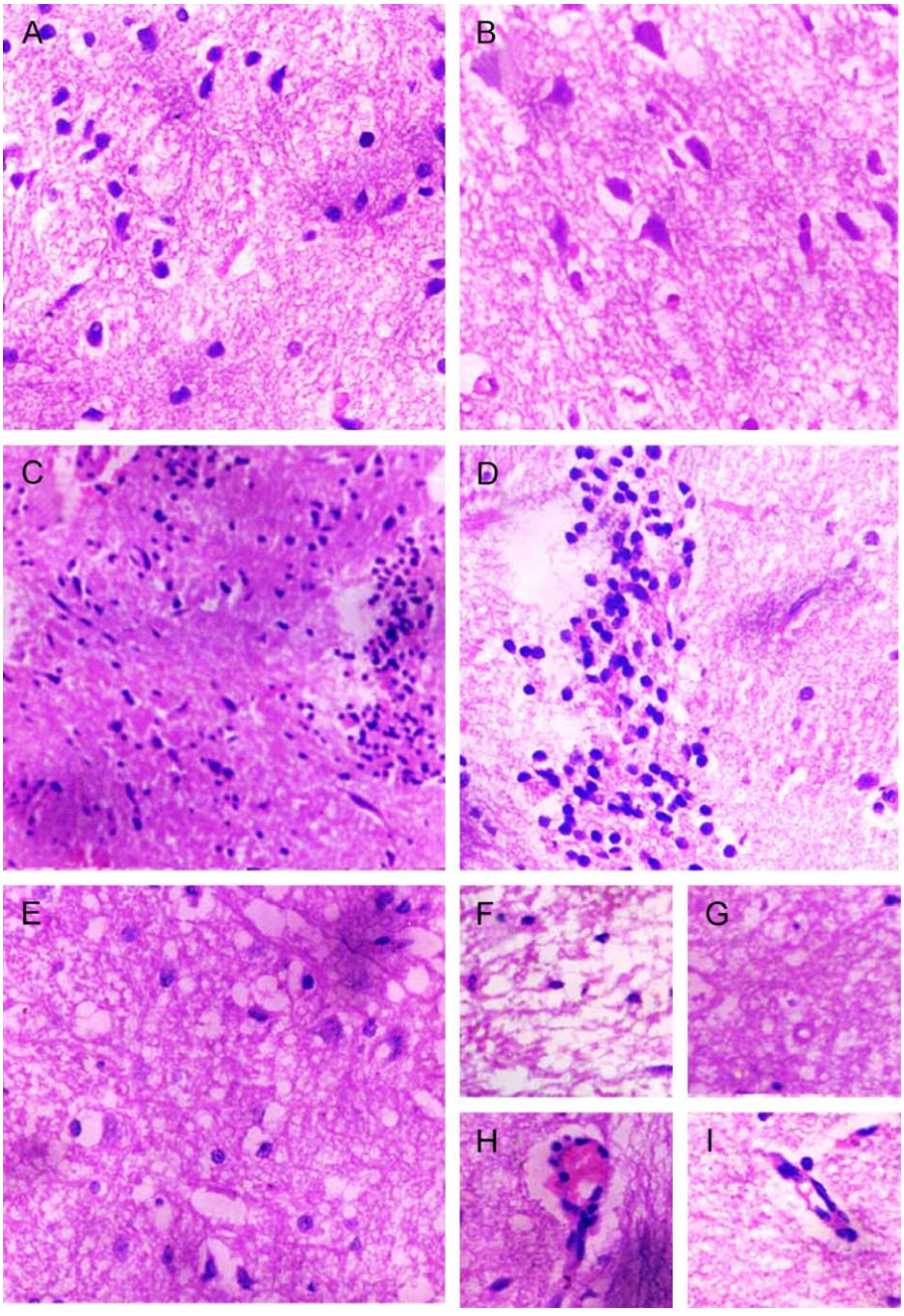

Fig. 2. Representative photomicrographs (H & E stain) of neocortex at the level of striatum in the animals subjected to sham operation (A) or BCCA occlusion for 15 days (C, D, F and H) and the effect of OS treatment on hypoperfusion induced morphological changes (E, G and I). Animals subjected to hypoperfusion showed morphological alterations suggestive of reactive microgliosis and astrocytosis (C—10× and D—40×). This astroglial reaction was accompanied by cellular edema (F) and perivascular inflammatory infiltration (H). OS treatment attenuated this astroglial proliferation (E). Moreover, severity of cellular edema and perivascular infiltrate was also reduced (G and I). OS per se did not show any change in normal histological appearance of rat brains (B).

OS per se did not show any change (31.95±2.70 s) as compared to control (P>0.05).

3.3. OS treatment attenuates the hypoperfusion-induced morphological changes

Fig. 2 illustrates the effect of OS on hypoperfusion-induced histological changes in the rat brains. In OS per se group (Fig. 2B), the histological appearance of rat brains was similar to that of sham-operated animals (Fig. 2A). Occlusion of BCCA for 15 days caused histological alterations suggestive of reactive astrocytosis and microgliosis (Fig. 2C and D) accompanied by cellular edema (Fig. 2F) and perivascular inflammatory infiltrate (Fig. 2H). When hypoperfused animals received OS treatment, no/minimal reactive astrocytosis and microgliosis was observed (Fig. 2E). Moreover, OS treatment also significantly attenuated the severity of cellular edema (Fig. 2G) and perivascular inflammatory infiltrate (Fig. 2I).

4. Discussion

This study aimed at investigating the potential beneficial effects of OS on ischemia-induced oxidative stress as well as functional and anatomical integrity following long-term cerebral hypoperfusion in rats.

In the present study, occlusion of BCCA for 30 min followed by 45 min reperfusion caused up-regulation of SOD activity. Polymorphonuclear leukocytes are known to be involved in cerebral reperfusion injury. Leukocyte accumulation has been noted in the brain after cerebral ischemia (Barone et al., 1991). These activated neutrophils are a source of free radicals, especially superoxide anions (Gringo, 1997). The increased SOD activity is, therefore, an indication that the brain's antioxidant machinery is activated in response to excessive generation of free radicals (Bannister et al., 1987). However, in the setting of overwhelming oxidative stress, this activation of SOD is not necessarily protective. Enhanced SOD activity catalyzes the conversion of superoxide anions to hydrogen peroxide and molecular oxygen. Hydrogen peroxide, the product of this reaction, is more toxic than the oxygen-derived free radicals and requires to be scavenged further by tissue thiols (glutathione redox pathway) and catalase (reviewed in Bannister et al., 1987; Fridovich, 1995). Apart from its own toxicity, hydrogen peroxide in the presence of iron leads to generation of toxic hydroxyl radicals (Blake et al., 1987). The deleterious effect of enhanced SOD activity in the settings of oxidative stress is a recognized phenomenon since long ago. This is evidenced by a number of studies that address this issue both in vivo and in vitro (Ratych et al., 1987; Omar and McCord, 1990). Indeed, the best antioxidant protection was obtained only when exogenous SOD was coadministered with catalase (Reilly et al., 1991). Thus, excess SOD in relation to other hydrogen peroxide removing antioxidant defense mechanisms is in fact detrimental to the tissues (Blake et al., 1987). This is supported by the concomitant increase in MDA and fall in TSH levels observed in the present study. Excessive generation of reactive oxygen species (ROS) results in the lipid peroxidation of the cell membrane and subsequent damage is reflected by accumulation of MDA, a by-product of lipid peroxidation (Halliwell, 1991). Sulfhydryl groups represent an important endogenous antioxidant mechanism. Glutathione and other protein/nonprotein sulfhydryls are scavengers of ROS and help to maintain macromolecular components of the cell in their functional status. Overwhelming oxidative stress secondary to reperfusion injury initiates a chain of protein-*S*-thiolation/dethiolation and results in consumption of TSH (Thomas et al., 1995). Thus, the data of reperfusion insult clearly confirm the earlier reports of generation of ROS after cerebral postischemic reperfusion (Sorrenti et al., 1994; Nakashima et al., 1999). Indeed, in an earlier investigation similar to the present experiment, reperfusion for 1 h following 30 min BCCA occlusion resulted in significant increase in lipid peroxidation and decrease in GSH (Shivakumar et al., 1992).

OS pretreatment significantly prevented the rise in MDA levels and up-regulation of SOD activity, suggesting that it attenuates the excessive formation of ROS secondary to reperfusion injury. This is in agreement with the observations that OS possesses significant anti-inflammatory and antioxidant activity (Maulik et al., 1997; Devi, 2000). Moreover, OS has been able to decrease inflammation-induced leukocyte migration and vascular permeability (Singh and Majumdar, 1999). OS, on its own, has been shown to increase endogenous GSH levels (Devi and Ganasoundari, 1999). Although, in present study, OS per se did not show any effect on endogenous TSH levels, it prevented their consumption secondary to reperfusion injury, nevertheless. Ascorbic acid levels were, however, unchanged during the reperfusion injury. Possibly, reperfusion insult decreases the ascorbate (reduced form of ascorbic acid) levels without affecting the total ascorbic acid levels. This observation receives direct support from an earlier experiment that also demonstrated no change in total ascorbic acid levels with a decrease in ascorbate levels secondary to cerebral reperfusion injury (Cooper et al., 1980).

Phytochemical investigations on OS leaf extract have resulted in isolation of many active principles, like phenols (eugenol, cirsilineol, isothymucin, isothymonin, apigenin and vosamarinic acid) and flavonoids (orientin and vicenin). These pharmacophores have been shown to possess potent antioxidant and anti-inflammatory (cyclooxygenase inhibitory) activity (Kelm et al., 2000; Devi et al., 2000). We have quantified our methanolic leaf extract of OS for major component eugenol and the yield was 5.1%. Therefore, it appears that eugenol and other contents of OS are responsible for the presently observed attenuation of

oxidative damage during reperfusion injury. Moreover, eugenol, a compound that, although biologically very active, has a very low toxicity, enjoys status as a lead for search of new neuroprotective pharmaceuticals (Stoof et al., 1999).

In evaluations of the potential neuroprotective drug, the need for assessing the drug effects on the histological and/or functional measures of neuronal damage has always been emphasized (Hunter et al., 1998; STAIR, 1999). However, transient occlusion of BCCA results in transient metabolic abnormalities in rat brains without any long-term behavioral/histological changes (Sontag et al., 1992; Plaschke et al., 2001). Therefore, in order to assess the potential neuroprotective effect of OS, we tested the OS extract on hypoperfusion-induced behavioral and structural alterations. Permanent occlusion of BCCA has been used as a model for neurodegenerative condition and cerebrovascular insufficiency states (for a review, see Sarti et al., 2002). Chronic cerebral hypoperfusion with accompanying compromised brain homeostasis induced progressive behavioral deficits and histological changes (Tsuchiya et al., 1992, 1993; Beal et al., 1993).

In the present study, hypoperfused animals, when tested in open-field paradigm, exhibited decreased ambulations, rearings and groomings with an increase in period of immobility. These findings, according to accepted tenets, indicate that the hypoperfused animals were more susceptible to develop anxiety when exposed to novel environment. (Lister, 1990) OS has significantly attenuated these alterations in exploratory behavior, suggesting that OS protects against hypoperfusion-induced anxiety. In the Morris water maze, a test for spatial learning and memory, results of escape latency testing (third session) and probe trail suggest deficits of reference and working memory. This observation is in accordance with earlier reports of hypoperfusion-induced spatial learning and memory deficits (Ni et al., 1994; Sarti et al., 2002). OS effectively attenuates these deficits.

The presently observed reactive morphological changes point towards the occurrence of ‘reactive microgliosis’ (Streit et al., 1999). Experimentally induced cerebral hypoperfusion was previously shown to result in astroglial activation and/or proliferation (Abraham and Lazar, 2000; Ihara et al., 2001). Microglial activation in response to pathological stimuli results in release of proinflammatory cytokines (Streit et al., 1999). Release of cytokines then can cause secondary neuronal damage. This is evidenced here in the form of perivascular inflammatory infiltration and cellular edema. The absence of neuronal loss is in conflict with the present behavioral observations. It has been suggested earlier that such behavioral disturbances may be due to subcellular alterations, purely functional and/or transmission failure (Plaschke et al., 2001). Indeed, spatial learning and memory disturbances without structural neuronal damage have already been reported (Pappas et al., 1996; Block and Schwartz, 1997; Plaschke et al., 2001).

In the present study, OS has significantly attenuated these hypoperfusion-induced changes. Eugenol and its derivatives, like eugenedilol and isoeugenolol, have been shown to possess potent vasodilatory properties, an effect attributed to blockade of voltage-dependant Ca^{+2} channels (Huang et al., 1999; Lin et al., 1999; Nishijima et al., 1999). Therefore, it is likely that, by virtue of its eugenol content, OS helps in the establishment of collaterals, thus improving cerebral perfusion and microcirculation in the setting of permanent BCCA occlusion.

Summarizing the effects of OS, we demonstrated that OS prevents the oxidative stress during reperfusion injury as well as attenuates the behavioral deficits and histopathological alterations secondary to hypoperfusion. In conclusion, the present study provides experimental evidence for OS as a neuroprotective agent and emphasizes the need to understand more fully the neuropharmacological effects of OS.

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