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Research Article

STABILIZATION OF PHYSIOLOGICAL PROCESSES AND PROMOTION OF HOMEOSTASIS OF *WITHANIA SOMNIFERA*

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Abstract

The present study investigates the effect of *Withania somnifera* (WS), a well-known adaptogenic agent in Indian system of Medicine, on acute cerebral reperfusion and long- term cerebral hypoperfusion in rats. Materials and methods: Acute ischemia-reperfusion (30 min occlusion of bilateral common carotid arteries followed by 45 min. reperfusion) and Long-term cerebral hypoperfusion (for 15 days) in C.F. strain rats were produced superoxide dismutase (SOD) activity, cyclic AMP level and histopathological changes in forebrain regions in acute ischemia - reperfusion and on long-term cerebral hypoperfusion induced behavioral and histopathological alterations were evaluated. Results: WS pre- treatment (50 mg/kg p.o. for 5 days) attenuated the reperfusion induced biochemical and histopathological alterations. Long term hypoperfusion induced anxiety and listlessness (open field paradigm) accompanied by deficits in learning and memory (Morris'water maze testing) along with histopathological changes in rat forebrains were attenuated with WS treatment. The results suggest that WS may be useful in cerebrovascular insufficiency conditions

Key words: *Withania somnifera*; physiologic process, homeostasis.

Introduction

Reperfusion injury is distinct entity from the primary ischemic injury because the oxygen arriving with blood circulation, in an attempt to alleviate the ischemic status, may worsen the damage. Excessive generation of reactive oxygen species (ROS) initiate lipid peroxidation and cause damage to macromolecular components of cell [6, 7]. Long-term cerebral hypoperfusion induced by bilateral common carotid artery occlusion (BCCAO) causes a reduction of blood flow from about 30-45%in cortex to 20% in hippocampus with resultant decrease in glucose utilization by 20-30% and15%, respectively [8,9]. Chronic reduction in blood flows and brain energy metabolism causes behavioral and cognitive deficits [10,11]. Extensive investigations reveals that rats subjected to permanent

occlusion of BCCA show impaired spatial learning/memory capabilities and/or structural alterations [12,13].Evidence has steadily accumulated to indicate that the rapid fluctuation in cyclic nucleotides during primary and secondary stroke is more than epiphenomena of the disease. This epiphenomenon leads to the suggestion that cyclic nucleotides and related enzymes might be used as target molecules to develop further therapeutic strategies for prevention or treatment of stroke [14]. In Indian system of Medicine (Ayurveda), WS is categorized as '*Rasayanas*', a group of plant derived drugs that are reputed to promote health and longevity by augmenting defense against disease, arresting the ageing process, revitalizing the body in debilitated conditions, increasing the capability of the individual to resist adverse environmental factors and by creating a sense of mental well being [15].Extracts of roots of WS are extensively used to reverse memory deficits and to normalize behavioural perturbations [15]. WS is known to possess additional neuro pharmacological effects like, enhancement of cognition, attenuation of cognitive deficits in animal models of Alzheimer's disease [16]. Although WS has been reported to have beneficial effects in diverse experimental models, it's possible role in cerebral ischemia-reperfusion injury and long-term hypoperfusion injury has not been investigated. Therefore, the present investigation was designed to assess the neuroprotective activity of WS on acute cerebral reperfusion and long-term hypoperfusion injuries in rats.

Materials and Methods

Animal and drug administration

After approval of Institutional Ethics Committee, the present study was conductedon inbred Charles-Foster (CF) male albino rats weighing 250-300 g. They were kept in the departmental animal house in colony cages at an ambient temperature of 25 ± 2°C and 45-55% relative humidity with 10 h: 14 h light: dark cycles. They had free access to standard rodent pellet diet and drinking water. The food was withdrawn 18 h prior to surgical procedure, however, water was allowed *adlibitum*. Principles of laboratory animal care (NIH Publication No. 86-23, revised 1985) guidelines were followed throughout WSG was dissolved in 0.9% saline and administered orally (p.o.) in the dose of 50 mg/kg, once daily. The dose of 50 mg/kg was selected on the basis of most effective dose observed earlier in our laboratory. For acute studies, the animals were divided into four groups of six animals each. First group served as sham operated control (underwent all surgical procedure except BCCAO). In the secondgroup, WS 50 mg/kg/day (p.o.) for 5 days was administered to sham-operated animals todetermine effect of drug *per se*. Third group animals underwent 30 min BCCAO and 45 min reperfusion. In the fourth group (treatment), WS 50 mg/kg, (p.o) was administered 60 min before subjecting animals to 30 min BCCAO and 45 min reperfusion and thereafter continued

for 5 days in the dose of 50 mg/kg/day, (p.o). For long-term hypoperfusion studies, again animals were divided into four groups of six animals each. First group served as sham operated control. In the second group, WS 50 mg/kg/day, (p.o.) for 15 days was administered for the entire experimental period in sham operated animals. Third group animals underwent permanent BCCAO and received vehicle only (hypoperfusion group). In the fourth group, WS was administered 60 min before BCCAO and WS was then continued up to 15th post surgical day. On day 15 (60 min after the last dose of WS in Group II and IV), animals of all groups were subjected to behavioural assessment paradigm and subsequently they were scarified by guillotine and brain samples were collected for histopathological studies.

Chemicals and reagents

1, 1, 3, 3-Tetraethoxypropane (TEP), (Merck, Germany), Thiobarbituric acid (TBA), NADH, nitrobluetetrazolium (NBT) and phenazine methosulfate (PMS), (Sigma, USA) were used.All other chemicals and reagents were of the highest analytical grades available locally.

Plant material

Systematic chemical analysis has ascertained several chemotypes of WS [17]. The Indian chemotype-1, rich in withanolide glycosides (= sitoindosides) [17] was used for the isolation of the test compounds. A herbarium specimen of the plant has been preserved at the R & D centre, Indian Herbs Ltd., Saharanpur, India. Freshly harvested 2-year-old thin roots of WS were dried, coarsely powdered and then exhaustively extracted with aqueous alcohol (1:1) at 55 ± 5°C. The extract was concentrated under reduced pressure to remove ethyl alcohol and the aqueous concentrate was exhaustively extracted with chloroform to remove fatty materials and free withanolides. The chloroform insoluble (water soluble) fraction was spray-dried to give a free flowing colourless powder (yield, 12-15%, W/W). It contained sitoindosides VII-X and withaferin-A as the major bioactive entities, the relative abundance of these compounds in the extract powder being 28-30%. Besides these, oligosaccharides (= polyglucans, molecular weight <2000 Da) with relative abundance of 12-15%, constituted the carriers of the bioactive compounds, in the form of inclusion complex [17]. The structure of sitoindosides was established by comprehensive spectroscopic analysis and crucial chemical transformations, as described earlier [2 & 17]. The composition of the sitoindosides and Withaferin-A in the standardized extract powder was determined by HPTLC [16]. A CAMAG assembly [Software © 1990, Scanner II.V, 3.14/PC/CTS version, 3.05 l amp deuterium, wave length 254nm, quenching mode] was used. The bioactive ingredients were detected and quantified in chloroform-methanol (90:10) and n-butyl alcohol-acetic acid-water

(4:1:2) solvent systems using authentic markers. The equimolar composition of the five was made by adding appropriate amounts of the deficient compounds. The combined formulation was freely soluble in normal saline.

Surgical procedure: Surgical technique for induction of cerebral ischemia by bilateral common carotid artery occlusion (BCCAO) was adapted from earlier published method of Iwasaki *et al* [18]. Rats were anaesthetized by an intra-peritoneal injection of ketamine (100 mg/kg). After a midline skin incision in the neck, both common carotid arteries were identified and isolated carefully from accompanying vagosympathetic nerve. Acute ischemia-reperfusion injury was produced by blocking bilateral common carotid arteries (BCCA) for 30 min (lifting arteries with the help of thread) and reperfusion for 45 min was allowed by just releasing the thread. Body temperature was maintained at about 37°C during the period with the help of a heating lamp. This protocol was adopted on the basis of earlier reports from our laboratory [19] and elsewhere [7]. At the end of reperfusion injury, the animals were sacrificed by decapitation and fronto-parietal part of cerebral cortex from both the hemispheres were transferred to appropriate homogenizing medium for biochemical estimation. For long-term hypoperfusion studies, BCCAs were doubly ligated with 3-0 silk sutures and cut in between [20]. The skin was then sutured and animals were returned to their home cage. On the day 15, 60 min after last dose of WS, all animals were subjected to behavioural testing and histopathological studies.

Biochemical estimation

All the procedures on the brain samples were performed on ice or ice-bath and samples were kept in the freezer compartment (-20°C) of refrigerator. Following biochemical parameters were studied in the forebrain of acute ischemia reperfusion injury rats.

Lipid peroxidation

Estimation of lipid peroxidation was done by measuring the lipid peroxidation product TBARS (thiobarbituric acid reactive substances) following the method of Ohkawa *et al* [21]. TEP was used as an external standard, and the level of lipid peroxidation was expressed as nanomoles of TBARS / mg of protein. Superoxide dismutase (SOD) activity Activity of superoxide dismutase was estimated by adopting the procedure of Kakkar *et al* [22]. The inhibition by superoxide dismutase of reduction of nitroblue tetrazolium to blue colored chromogen in the presence of phenazine methosulphate and NADH was measured at 560 nm. One unit of enzyme activity was defined as enzyme concentration required inhibiting the absorbance at 560 nm of chromogen production by 50% in one min under assay condition and expressed as specific activity in milli units/mg of protein.

Total tissue sulfhydryl groups (T-SH)

Total T-SH groups in brain were measured following the method of Sedlack and Lindsay[23]. The level of T-SH groups was expressed as moles of SH /100 g of wet tissue weight.

Estimation of brain protein: The protein content of brain tissue was estimated using the method of Lowry *et al* [24].

Cyclic AMP estimation

Cyclic AMP estimation of frontoparietal part of forebrain was done by ELISA by correlateEIATM cyclic AMP Kit (Assay Designs Inc., U.S.A.). This kit uses a polyclonal antibody tocyclic AMP to bind, in a competitive manner with the cyclic AMP in the standards or sample soran alkaline phosphatase molecule, which has cyclic AMP covalently, attached to it. Results were expressed as n mol of cyclic AMP per g (wet weight) of tissue.

Behavioural Testing Open Field Test

Locomotor activity was evaluated in an open field paradigm [25]. The open field is made ofPlywood and consisted of a floor (96 x 96 cm) with high walls (61 x 61 cm). Entire apparatusis painted black except for 6mm thick white lines that divide the floor into 16 squares. TheEntire room except the open field was kept dark during the experimentation. The open field was lighted by a 60-watt bulb focusing on to the field from a height of about 100 cm from thefloor. Each animal was placed individually at one corner of the apparatus and for next 5 min, it was observed for the ambutations (number of squares crossed), total period of immobility (in sec), number of rearing, grooming and fecal pellets.

Morris' water maze test

The maze consisted of a black circular pool[26] (diameter 2.14 m, height 80 cm) filled toa depth of 44 cm with water (25°C ± 1°C). Water was made opaque by adding Indian ink.On day 15 after surgery, spatial learning and memory was tested in water maze. On 14thDay, the rats received habituation (exposure in water maze for 1 min) in which there was noPlatform present. Then, on day 15, a circular platform (9 cm in diameter) was kept hidden 2cm below water level in the centre of one of the quadrants. The platform remained in the same position during training days (reference memory procedure) (escape trial). 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 20s 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 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 pool that initially contained platform was measured. On completion of the probe trial, a black platform that extended 1 cm above the surface of water was placed in a quadrant other than that chosen for the submerged platform. Each rat was then given four trials of 90 s to locate it. The latency to reach the platform was recorded (working memory procedure).

Statistical analysis

Statistical analysis of data was performed by applying one-way Analysis of Variance (ANOVA) followed by post hoc Tukey Test for biochemical parameters and behavioural observations. A p-value of <0.05 was considered statistically significant.

Results

Biochemical Observations

Acute BCCAO for 30 min followed by 45 min reperfusion induced increase in lipidperoxidation (TBARS), (2.2 fold), superoxidedismutase (SOD), (2.1 fold) activity and fall in T-SH levels (46% decrease). WS pretreatment attenuated enhanced TBARS level ($p <0.01$) and SOD activity ($p <0.01$) as wellas prevented the consumption of T-SH significantly ($p <0.01$) following cerebral ischemia-reperfusion injury. WS *per se* had no significant effect on any of these biochemical parameters (Table 1).

Ischemia followed by reperfusion increased cyclic AMP level significantly as compared to that in Sham operated animals ($p <0.05$). WS pretreatment of ischemia-reperfused animals led to a further significant rise in cyclic AMP level compared to ischemia-reperfusion group ($p <0.01$) (Table 2).

Behavioral observations Open Field Test

Animals with permanent BCCAO (hypoperfusion group) showed marked alterations in locomotor activities in open field paradigm. Table 3 shows that permanent BCCAO was associated with reduced number of ambulations ($p <0.01$), rearing ($p <0.01$) and grooming ($p <0.01$) along with increase in period of immobility ($p <0.01$). WS pretreatment prevented these alterations. In Sham-operated animals, WS *per se* did not have any effect on any of the parameters of open field test (Table 3).

Morris' water maze test

Table 4 shows that there was no difference between sham-operated control and WS *per se*groups. All rats located the hidden platform during the sessions of escape trial. The hypoperfused animals required more time than sham-operated control to find submerged platform that was significant in both second and third trials ($p < 0.01$).

WS prevented this delay in escape latencies in second and third sessions ($p < 0.01$) but not during the first session. The data of probe trial reveals that hypoperfused rats spent less time in quadrant of former platform than did sham-operated rats ($p < 0.05$). This change was significantly reversed by WS treatment ($p < 0.01$). The result of new platform trial shows that hypoperfused animals found the new platform slower than Sham-operated rats ($p < 0.01$). WS treated animals found the new visible platform quicker than the hypoperfused animals ($p < 0.01$). WS per se had no effect on these parameters.

Table 1. Effect of WS (50 mg/kg p.o.x 5 days) on biochemical parameters of oxidative stress in rat forebrain following cerebral ischemia–reperfusion injury (30 min BCCAO followed by 45 min reperfusion).

Groups	TBARS(nM/mg protein)	SOD(milliunits/mg protein)	T-SH(x10-5M/mg protein)
Sham-operated control	2.15 ± 0.39	320.15 ± 98.30	3.84 ± 1.27
Per se	2.20 ± 0.32	338.05 ± 99.50	3.96 ± 0.61
Ischemia–reperfusion	4.79 ± 0.83a	690.13 ± 182.02a	2.04 ± 0.44a
Treatment	3.10 ± 1.15a	424.97 ± 121.94a	3.14 ± 0.15a

All data are expressed as mean ± SD. Number of animals in each group = 6. Sham-operated Control and treatment groups are compared with ischemia-reperfusion group.

WS *per se* is compared with sham-operated control group. Superscripta indicate p value < 0.01. Statistical analysis was done by one–way ANOVA followed by Tukey test.

Table 2. Effect of WS (50 mg/kg p.o. x 5 days) on level of cyclic AMP in fronto-parietal region of rat brain following cerebral ischemia–reperfusion injury (30 min BCCAO followed by 45 min reperfusion).

Groups	Cyclic AMP (nmol/g)
Sham-operated control	9.15 ± 1.535
Ischemia – reperfusion	20.76 ± 7.29b
Treatment	42.96 ± 14.42a

All data are expressed as mean ± SD. Number of animals in each group = 6. Sham-operated control and treatment groups are compared with ischemia-reperfusion group. Superscriptsa and b indicate p value < 0.01 and < 0.05 respectively. Statistical analysis was done by one – way ANOVA followed by post hoc Tukey test.

Table 3. Effect of WS (50 mg / kg p.o. x 15 days) on open field parameter in long-term hypoperfused rats

Groups	Ambulation (n)	Immobility(S)	Rearing(n)	Grooming(n)	Fecal pellets(n)
Sham-operated control	57.50 ± 11.11	30.16 ± 7.0	21.00 ± 2.0	5.50 ± 1.05	3.33 ± 2.82
WS *per se*	58.16 ± 14.35	28.50 ± 4.58	21.66 ± 2.05	6.16 ± 1.47	3.16 ± 1.96
Hypoperfusion	28.00 ± 11.13a	46.66 ± 4.46a	15.50 ± 2.67a	1.66 ± 0.49a	2.33 ± 2.94
Treatment	59.16 ± 12.57a	33.16 ± 6.54a	24.83 ± 3.82a	5.50 ± 2.16a	2.49 ± 1.76

All data are expressed as mean ± SD. Number of animals in each group = 6. Sham–operated control and treatment groups are compared with hypoperfusion group. WS *per se* is compared with sham-operated control group. Superscript indicates p-value < 0.01. Statistical analysis was done by one-way ANOVA followed by post hoc Tukey test.

Table 4. Effect of WS (50mg/kg p.o.x15 days) on learning and memory in long-term hypoperfused rats in Morris' water maze.

Groups	Escape latency (sessions)			Probe trial	New platform trial
	I	II	III		
Sham-operated control	58.00 ± 15.26	25.50 ± 4.95	27.00 ± 5.76	29.00 ± 3.94	16.16 ± 3.06
WS *per se*	59.83 ± 11.46	26.83 ± 5.19	28.83 ± 3.97	30.16 ± 4.48	14.83 ± 4.53
Hypoperfusion	66.16 ± 10.78	46.16 ± 3.75a	42.83 ± 5.49a	22.00 ± 5.80b	29.00 ± 3.33a
Treatment	63.00 ± 12.88	29.00 ± 3.33a	20.66 ± 6.69a	33.16 ± 6.25a	15.83 ± 4.07a

All data (time in s) are expressed as mean ± SD. Number of animals in each group = 6. Sham–operated control and treatment groups are compared with hypoperfusion group. WS *per se* is compared with sham-operated control group. Superscripts a and b indicate p-value <0.01 and <0.05 respectively. Statistical analysis was done by one-way ANOVA followed by post hoc Tukey test.

Discussion

The results of biochemical parameters show that BCCAO for 30 min followed by 45 min reperfusion causes ischemia-reperfusion injury. Increased generation of free radicals initiate lipid peroxidation and is reflected as

increased level of TBARS. Increased SOD activity, a marker of oxidative stress, also suggests the same. These findings are in agreement with those reported earlier [7, 19]. Fall in T-SH reflects the consumption of tissue thiols: such a fall in GSH (a non protein sulfhydryl) during cerebral reperfusion injury is well reported [27]. Sulfhydryl compounds are among the most important endogenous antioxidants. They have role in maintenance of cellular proteins and lipids in their functional states. When these are consumed, the toxic effects of oxidative insult are exacerbated resulting in increased membrane and cell damage [28].

The data of present study show that WS could antagonize ischemia-reperfusion injury induced rise in TBARS level. Similarly, WS reversed ischemia-reperfusion induced changes in SOD and T-SH. These findings are in agreement with earlier reported antioxidant and antistress adaptogenic properties of WS [29, 30,3, 5, 31, 32].The data of the present ischemia-reperfusion study revealed significant increase in cyclic AMP level in brain (fronto-parietal region). Following ischemia-reperfusion injury, cyclic AMP is known to increase in striatum [33], neocortex and hippocampus [34] and in cerebral cortex [35]. The increase in cyclic AMP level following such injury has been implicated in reversing stroke induced vasospasm in central vessels [14].

Increased level of cyclic AMP is known to inhibit release of excitatory amino acid like glutamate through modulation of adenosine [36]. WS pretreatment enhanced cyclic AMP concentration in ischemia–reperfused animals. It is quite tempting to postulate that part of the beneficial effect of WS might be due to its effect on cyclic AMP. Permanent BCCAO in rats has been used as one of the animal models for cerebrovascular insufficiency states, white matter lesions, neurodegenerative conditions and dementia [37-39]. In present study, investigations on the open field behaviour, according to accepted tenets, showed that hypoperfused animals were anxiety prone. WS has significantly prevented hypoperfusion induced anxiety. This is in accordance with earlier reported anxiolytic–antidepressants activity of WS [4]. Results of Morris' water maze testing support the earlier reports of hypoperfusion induced deficits of spatial learning and memory [38]. Chronic reduction blood flow secondary to BCCAO has been reported to cause progressive dysfunction resulting in cognitive deficits [40, 41, 11].

Hypoperfused animals consistently took longer time to find the submerged platform reflecting a defective learning process. This when combined with the results of probe trial reflect the disturbances in reference and working memory. WS attenuated these alterations significantly suggesting its potentiality in improving learning and memory in hypoperfused rats. This finding is in agreement with cognition enhancing and memory improving effects of WS [42, 43, 16, 5, 17]. The data of the present study thus suggest protective role of WS in cerebral reperfusion and long-

term hypoperfusion induced injuries in rats. The present finding, apart from supporting earlier reported beneficial effects of WS in various animal models of cognitive disorders, is suggestive of its potentiality in the management of cerebral reperfusion and long term hypoperfusion disorders like stroke/post stroke syndromes.

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