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Withania somnifera root extract ameliorates hypobaric hypoxia induced memory impairment in rats

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ABSTRACT

Ethnopharmacological relevance: Withania somnifera (WS) root extract has been used traditionally in ayurvedic system of medicine as a memory enhancer and anti-stress agent.
Aim of the study: To evaluate the neuroprotective and prophylactic potential of WS root extract in ameliorating hypobaric hypoxia (HH) induced memory impairment and to explore the underlying molecular mechanism.
Materials and methods: WS root extract was administered to male Sprague Dawley rats during a period of 21 days pre-exposure and 07 days exposure to a simulated altitude of 25,000 ft. Spatial memory was assessed by Morris Water Maze. Neurodegeneration, corticosterone, acetylcholine (Ach) levels, acetylcholine esterase (AchE) activity, oxidative stress markers and nitric oxide (NO) concentration were assessed in the hippocampus. Synaptic and apoptotic markers were also investigated by immunoblotting. To study the role of NO in regulating corticosterone mediated signaling, the neuronal nitric oxide synthase (n-NOS) inhibitor, L-Nitro-arginine methyl ester (L-Name) and NO agonist sodium nitroprusside (SNP) were administered from 3rd to 7th day of hypoxic exposure.
Results: Administration of WS root extract prevented HH induced memory impairment and neurodegeneration along with decreased NO, corticosterone, oxidative stress and AchE activity in hippocampal region. Inhibition of NO synthesis by administration of L-Name reduced corticosterone levels in hippocampus during hypoxic exposure while co-administration of corticosterone increased neurodegeneration. Administration of sodium nitroprusside (SNP) along with WS root extract supplementation during hypoxic exposure increased corticosterone levels and increased the number of pyknotic cells.
Conclusion: WS root extract ameliorated HH induced memory impairment and neurodegeneration in hippocampus through NO mediated modulation of corticosterone levels.

1. Introduction

Exposure to hypobaric hypoxia (HH) at high altitude causes adaptive physiological changes that enable the organisms to cope up with the severity of oxygen deficiency in this environmental stress. However, mal-acclimatization to high altitude results in numerous pathological manifestations like High Altitude Pulmonary Edema (HAPE), High Altitude Cerebral Edema (HACE) and Acute Mountain Sickness (AMS) (Baily and Davies, 2001). Sensory–motor dysfunction and loss of memory functions have also been associated with prolonged exposure to HH (Li et al., 2000).

Previous findings from our laboratory have shown that chronic exposure to HH causes memory impairment which has been attributed to the occurrence of glutamate excitotoxicity (Hota et al., 2008), impaired cholinergic transmission (Muthuraju et al., 2009) and alterations in L-type calcium channel functions (Barhwal et al., 2009). Elevated NO levels and increased nNOS expression have also been reported to induce neurodegeneration in the hippocampal region (Maiti et al., 2007). In addition, the elevation in hippocampal corticosterone during hypoxic exposure resulted in increased oxidative stress and neurodegeneration (Baitharu et al., 2011). Inhibiting AchE activity using physostigmine and galantamine (Muthuraju et al., 2009), blocking NMDA (*N*-methyl-D-aspartate) receptors by MK-801 or accelerating clearance of synaptic glutamate by ceftriaxone decreased hypoxia mediated neurodegeneration (Hota et al., 2008). We have recently shown that modulation of hypoxia induced increase in corticosterone by administering metyrapone improved memory consolidation and retention (Baitharu et al., 2011).

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However, these blockers have several side effects and hence may not be used as prophylactics to prevent hypoxia induced cognitive impairment.

Withania somnifera (WS), a herb of Solanaceae family, finds mention in traditional Indian medication as an anti-stress and memory enhancer drug (Singh et al., 2003). The root extract of WS is rich in several components with strong antioxidant properties such as withaferin A, withanolides, withanone and other flavonoids (Bhattacharya et al., 1997; Dhuley, 1998; Chaurasia et al., 2000). WS root extract facilitates cognitive functions and augments mental retention capacity following diabetes, amyloid β, scopolamine and foot shock stress induced memory loss (Ramanathan et al., 2003; Parihar et al., 2004; Kuboyama et al., 2005; Konar et al., 2011). Despite several evidences proving the nootropic potential of WS root extract, its effects on HH induced memory impairment and corticosterone mediated signaling still remains an enigma. The present study therefore aimed at evaluating the efficacy of WS root extract in ameliorating the HH induced cognitive dysfunctions. Alterations in the expression of several proteins playing critical role in neuronal survivability and cognition were evaluated during the study. Possible NO mediated regulation of corticosterone and its role in manifesting neuroprotective effects of WS root extract have also been investigated in the present study.

2. Materials and methods

2.1. Chemicals and reagents

All the primary and secondary antibodies used in the experiments were procured from Santacruz (Santacruz Biotechnology Inc., California, USA). Corticosterone standard, withanolide A, withaferin A, withanone, withanolide D, sodium nitroprusside (SNP), L-Name (L-Nitro-arginine methyl ester) and other chemicals used for HPLC (High Performance Liquid Chromatography) were procured from Sigma chemicals (Sigma-Aldrich, USA). Kits for estimation of glutathione reductase and superoxide dismutase activity were purchased from RANDOX (Randox laboratory, UK). Quantichrom™ nitric oxide assay kit and acetylcholine assay kit were procured from BioAssay system (Hayward, CA, USA). *Withania somnifera* (WS) root powder was purchased from Ambe Phytochemicals (Delhi, India).

2.2. Animals

All the protocols followed in this experiment were approved by the ethical committee of the institute (IAEC No. IAEC/DIPAS/01/08/NB dtd 29/01/2008) following the guidelines of "Committee for the Purpose of Control and Supervision of Experiments on Animals" Govt. of India. Adult male Sprague Dawley rats weighing 220–230 g (3 months old) were taken. All animals were maintained at 12 h light and dark cycle (lights on from 8:00 a.m.–8:00 p.m.) in the animal house of the institute. Food pellets (Lipton Pvt. Ltd., India) and water was given ad libitum. The temperature and humidity of the animal house was maintained at 25 ± 2 °C and $55 \pm 5\%$ respectively. All animal handling was performed between the time window of 10.00 a.m. to 11.30 a.m. to avoid experimental deviations due to diurnal variations in corticosterone concentration.

2.3. Hypoxic exposure

Animals were exposed to a simulated altitude of 7600 m (25,000 ft, 282 mm Hg) in a specially designed animal decompression chamber where altitude could be maintained by reducing the ambient barometric pressure. Periodic evaluation of fluctuation in oxygen level arising from fresh air flush into the chamber was done using an oxygen sensor. The temperature and humidity in the chamber were maintained precisely at 25 ± 2 °C and $55 \pm 5\%$ respectively. The rate of ascent and descent to hypobaric conditions was maintained at 300 m/min as described previously (Hota et al., 2009; Barhwal et al., 2009). The hypobaric hypoxic exposure was continuous for the stipulated period except for a 10–15 min interval each day for replenishment of food and water, drug administration and changing the cages housing the animals.

2.4. Phytochemical analysis

WS was provided by Ambe Phytochemicals, authenticated by a taxonomist (Dr. O P Chaurasia) and submitted to herbarium of Defense Institute of High Altitude Research for future reference (VS No.: DIH/883/2008). Extraction of withanolides from WS root powder was performed according to the method described by Chaurasiya et al. (2008). In order to extract the withanolides, the finely powdered WS root (12.0 g) was extracted overnight with 60 ml of methanol–water (25:75, v/v) at room temperature on a rocking platform and then filtered. The filtrate was collected and the residue was extracted twice with the same volume of methanol–water at intervals of 4 h. The filtrates were pooled and extracted thrice with 180 ml of *n*-hexane. The *n*-hexane fraction was discarded and the methanol–water fraction was further extracted thrice with 180 ml of chloroform. The chloroform fractions were pooled and concentrated to a dry powder using a rotary evaporator. Percentage yield of WS root extract was found to be 3.42% (w/w). The glycowithanolides and withanones in the extract were estimated by HPLC using standards for withanolide A, withanolide D, withanone and withaferin A.

A sample (1 mg) of the dry powder was dissolved in HPLC-grade methanol (1.0 ml) and filtered through a Millipore sample clarification kit (Millex GV; 13 mm, 0.22 μm). The identification of withanolide A, withanolide D, withanone and withaferin A in WS root extract was done by HPLC using RP-C_{18} column (5 μm, 250 × 4.00 mm^2 ID, Waters). Methanolic water (MeOH: H_2O (4:1, v/v) was used as the mobile phase and flow rate was maintained at 2 ml/min. The components in the root extract were detected by UV detector at wavelength of 237 nm (Chaurasiya et al., 2008).

2.5. Dose response and toxicological evaluation

A dose response study was conducted by taking seven groups of rats ($n=3$/group) of which first two groups of rats served as normoxic group and hypoxic vehicle without any WS root extract administration. All other groups of rats received oral administration of WS root extract at doses of 50, 100, 150, 200 and 250 mg/kg body weight (BW) for a duration of 21 days prior to hypoxic exposure. The drug administration was continued during 7 days of exposure to HH simulating an altitude of 25,000 ft. Memory retention in Morris Water Maze and neurodegeneration (number of pyknotic cells) in hippocampus as assessed by Cresyl Violet staining following hypoxic exposure were considered as yardstick for dose optimization.

The LD_{50} of the standardized withanolides enriched fraction of WS root extract was determined by orally administering increasing doses of the extract. According to OECD-407 (OECD, 2008) guidelines, sub-chronic toxicity studies were conducted to a maximum dose of 1500 mg/kg BW (p.o.) for a period of 28 days and body weight, food and water intake and mortality were documented daily. Clinical pathology in liver, lungs, heart, stomach, duodenum, ileum, spleen, brain, kidneys was determined on 29th day.