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Shilajit attenuates behavioral symptoms of chronic fatigue syndrome by modulating the hypothalamic–pituitary–adrenal axis and mitochondrial bioenergetics in rats

Dinesh Kumar Surapaneni [a], Sree Rama Shiva Shanker Adapa [a], Kumari Preeti [a], Gangineni Ravi Teja [a], Muruganandam Veeraragavan [b], Sairam Krishnamurthy [a,*]

[a] Neurotherapeutics Lab, Department of Pharmaceutics, Indian Institute of Technology (Banaras Hindu University), Varanasi 221005 U.P., India
[b] Research and Development Centre, Natreon Inc. CL18A, Sector II, Salt Lake City, Kolkata 700091, India

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ABSTRACT

Ethnopharmacological relevance: Shilajit has been used as a rejuvenator for ages in Indian ancient traditional medicine and has been validated for a number of pharmacological activities.
Aim of the study: The effect of processed shilajit which was standardized to dibenzo-α-pyrones (DBPs;0.43% w/w), DBP-chromoproteins (DCPs; 20.45% w/w) and fulvic acids (56.75% w/w) was evaluated in a rat model of chronic fatigue syndrome (CFS). The mitochondrial bioenergetics and the activity of hypothalamus–pituitary–adrenal (HPA) axis were evaluated for the plausible mechanism of action of shilajit.
Materials and Methods: CFS was induced by forcing the rats to swim for 15 mins for 21 consecutive days. The rats were treated with shilajit (25, 50 and 100 mg/kg) for 21 days before exposure to stress procedure. The behavioral consequence of CFS was measured in terms of immobility and the climbing period. The post-CFS anxiety level was assessed by elevated plus maze (EPM) test. Plasma corticosterone and adrenal gland weight were estimated as indices of HPA axis activity. Analysis of mitochondrial complex chain enzymes (Complex I, II, IV and V) and mitochondrial membrane potential (MMP) in prefrontal cortex (PFC) were performed to evaluate the mitochondrial bioenergetics and integrity respectively.
Results: Shilajit reversed the CFS-induced increase in immobility period and decrease in climbing behavior as well as attenuated anxiety in the EPM test. Shilajit reversed CFS-induced decrease in plasma corticosterone level and loss of adrenal gland weight indicating modulation of HPA axis. Shilajit prevented CFS-induced mitochondrial dysfunction by stabilizing the complex enzyme activities and the loss of MMP. Shilajit reversed CFS-induced mitochondrial oxidative stress in terms of NO concentration and, LPO, SOD and catalase activities.
Conclusion: The results indicate that shilajit mitigates the effects of CFS in this model possibly through the modulation of HPA axis and preservation of mitochondrial function and integrity. The reversal of CFS-induced behavioral symptoms and mitochondrial bioenergetics by shilajit indicates mitochondria as a potential target for treatment of CFS.

1. Introduction

Fatigue can be clinically defined as a feeling of lack of energy resulting not exclusively from exertion. If fatigue is disabling and is accompanied by other constitutional and neuropsychiatric symptoms and lasts more than 6 months, a diagnosis of chronic fatigue syndrome (CFS) should be considered (Fukuda et al., 1994). The absence of concrete etiopathology makes CFS diagnosis difficult (Van Houdenhove and Luyten, 2007). Disturbances in the stress sensitive hypothalamic–pituitary–adrenal (HPA) axis as well as in brain neurotransmitter balances, particularly serotonin and norepinephrine have been reported due to exposure of chronic stress (Jerjes et al., 2007). The brain fMRI and morphometric studies have shown fatigue-related abnormalities in the frontal lobe in patients with CFS (Tanaka et al., 2006; Cook et al., 2007). Selective serotonin reuptake inhibitors have been widely prescribed, however chronic administration failed to show clinically significant effects in treatment of CFS (Maquet et al., 2006). Hence, there has been interest in alternative medicines for

* Corresponding author. Tel.: +91 9935509199.
E-mail addresses: saibliss@hotmail.com, ksairam.phe@itbhu.ac.in (S. Krishnamurthy).

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treatment of CFS. A clinical study based on biochemistry of the illness observed a remarkable correlation between the degree of mitochondrial dysfunction and the severity of CFS (Myhill et al., 2009). Of particular interest is the class of adaptogenic plants such as *Panax ginseng* and *Nardostachys Jatamansi* which have been reported to attenuate symptoms of CFS (Lylea et al., 2009). Shilajit has been used in traditional medicine for over 3000 years as a rejuvenator and an adaptogen (Sharma, 1978). Hence, we presume that shilajit may have beneficial effects in the treatment of CFS.

Shilajit is blackish brown exudate of variable consistency obtained from the rocky layers of mountain ranges (Kong et al., 1987). Shilajit comprises of 60–80% humus along with other organic components such as benzoic acid, hippuric acid, fatty acid, ichthyol, ellagic acid, resin, triterpenes, sterol, aromatic carboxylic acid, 3,4-benzocoumarins, amino acids and phenolic lipids (Srivastava et al., 1988). The major physiological action of shilajit has been reported to be due to the presence of bioactive dibenzo alpha pyrones along with humic and fulvic acids as carrier molecules for the active ingredients (Ghosal, 1990). Dibenzo alpha pyrones have been shown to protect mitochondrial function in hypoxic rats (Bhattacharyya et al., 2009a). Natural organic matter such as humic acid and fulvic acid acting as carrier molecules for the active ingredients can enhance the intestinal absorption and blood brain barrier penetration (Mirza et al., 2011). Processed shilajit has been reported to significantly modulate the central nervous system thereby showing learning augmentation, antistress activity, memory enhancement and anxiolytic activity (Agarwal et al., 2007). *Withania somnifera* (WS) has been used as prototype anti-stress agent (Bhattacharya and Muruganandam, 2003). Another factor in choosing WS, apart from its reported anti-CFS effect (Singh et al., 2002) is its effect on the mitochondrial function. Standardized extract WS dose-dependently attenuated ATP-depletion and other energy related indices during short and long-term FST (Bhattacharyya et al., 2009b).

In summary, the present study assesses the efficacy of shilajit in a stress-induced rat model of CFS. The effect of shilajit on the HPA axis was evaluated by estimating plasma corticosterone. Further, the PFC mitochondrial function and integrity was evaluated by measuring the activity of mitochondrial respiratory complex enzyme systems and mitochondrial membrane potential (MMP) respectively.

2. Materials and methods

2.1. Drugs and standardization

Processed and standardized shilajit was obtained from Natreon Inc, India. Standardization of shilajit with respect to bioactive contents (dibenzo-α-pyrones (DBPs), DBP-chromoproteins (DCPs) and fulvic acids) was done as reported earlier (Biswas et al., 2009). Briefly, high performance liquid chromatography (HPLC) was carried out in a WATERS (USA) HPLC system with PDA detector and isocratic mobile phase consisting of acetonitrile:orthophsophoric acid:water (32:1:67) with a flow rate of 0.6 ml/min using C-18 Novapak reverse phase column attached with a guard column for separation. The injection volume was 20 μl in water. The photodiode array detector wavelength was set at 240 nm. Quantification was done using the authentic markers. The shilajit contained DBPs (0.43% w/w); DCPs (20.45% w/w) and Fulvic acids (56.75% w/w).

Withania somnifera (WS) extract was obtained from Indian Herbs Ltd., India. WS was standardized with respect to their bioactives viz., withanolide glycosides, withaferin A and oligosaccharides content according to the published procedure (Bhattacharyya et al., 2009b). Briefly, HPLC analysis of withanolide glycosides and withaferin-A were performed using Waters HPLC system with PDA detector and Empower software with a Merck-HibarR pre-packed column (RT 250-4, LiChrosorbR RP-18, particle size 5 μm, 4×250 mm cartridge column) fitted with a reverse phase guard column and acetonitrile:water-1:1 (v/v) as the mobile phase, with a run time of 20 min and flow rate 0.6 ml/min in an isocratic mode, using withaferin A (isolated by multiple column chromatography) as an external standard. Oligosaccharides were determined using Waters HPLC system with a RI detector and Empower software with a carbohydrate analysis column [Waters] 300×3.9 mm; acetonitrile:water-80:20 (v/v) was used as the mobile phase; the run time was 10 min and flow rate was 2 ml/min in an isocratic mode. *Withania somnifera* extract was found to contain 14.56% w/w withanolide glycosides, 0.36% w/w Withaferin A and oligosaccharides 39.03% w/w.

2.2. Chemicals

Tetra methyl rhodamine methyl ester (TMRM) and Griess reagent were procured from Sigma Aldrich (St. Louis, MO, USA). Sodium succinate, sodium azide, Phenazine methane sulphonate (PMS) and Nitro blue tetrazolium were purchased from Merck (Daidrmstadt, Germany). All other chemicals and reagents were procured from local suppliers and were of analytical grade.

2.3. Animals

Charles Foster albino rats (140–150 g) were obtained from the Central Animal House, Institute of Medical Sciences; Banaras Hindu University (B.H.U). The animals were housed in polypropylene cages at an ambient temperature of 25 °C ± 1 °C and 45–55% RH, with a 12:12 h light/dark cycle. They had free access to commercial food pellets (Amrut Laboratory Animal feed, Sangli, India) and water. The experimental procedures were approved by Institutional animal ethical committee, B.H.U. The animals were cared in accordance with the Guide to the Care and Use of Experimental Animals (Vol. 1, 2nd ed., 1993, and Vol. 2, 1984). Animals were divided into six sets of 6 animals each. The sets represented the control group, stress control group, positive control (WS 100 mg/kg) and three doses of Shilajit (25, 50 and 100 mg/kg). The test and standard drugs were administered orally in 0.2% carboxy methyl cellulose (CMC) suspension. The dosing was done 1 h before the induction of stress procedure for 21 days. The stress control group received vehicle (0.2% CMC solution). On the 21st day, 30 mins after chronic swim stress the rats were tested in the elevated plus maze (EPM). The rats were then immediately decapitated and the prefrontal cortex was micro-dissected and stored immediately at −80 °C until further experimentation.

2.4. Chronic fatigue induced by forced swimming

Animals were forced to swim for a 15 min session every day for 21 days, in a fabricated metal cylinder (d=35 cm; h=45 cm) containing water up to 30-cm height at room temperature (24–28 °C). The total duration of immobility period and climbing period was measured in seconds using ANY-maze behavioral tracking system, USA (version—4.72) for the periods of 0–5, 5–10 and 10–15 min every day for 21 days (Porsolt, 1981; Lylea et al., 2009). Fig. 1.

2.5. Evaluation of anxiety

Elevated plus maze was used to analyze the anxiety in rats. The fabricated maze consists of two opposite open arms, 50 cm × 10 cm with 40-cm high walls and elevated to a height

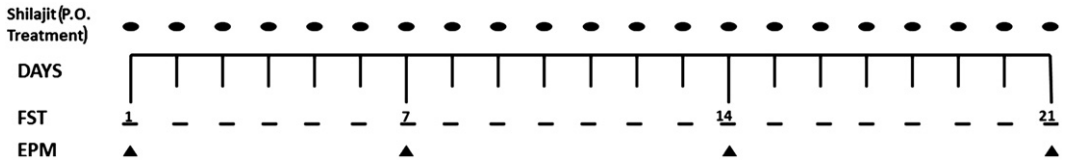

Fig. 1. Experimental protocol and dosing schedule. Shilajit (25, 50 and 100 mg/kg) was administered for 21 days. Forced swim test (FST) and elevated plus maze (EPM) test were done on days as depicted in the figure.

of 50 cm. The baseline anxiety levels of each animal was assessed before and after subjecting it to chronic forced swimming on day-1, day-7, day-14 and day-21. The test was carried out for 5-min time period and the presence in open arm and close arm along with number of head dips were noted using ANY-maze behavioral tracking system, USA (version—4.72).

2.6. Estimation of plasma corticosterone level

Estimation of plasma level of corticosterone was done by fluorimetry according to the method of Katyare and Pandya (2005).

2.7. Mitochondria respiratory chain enzymes

2.7.1. Isolation of rat brain mitochondria

Mitochondrion was isolated from PFC by the method as described by Puka-Sundvall et al. (2000) with minor modifications.

2.7.2. Evaluation of mitochondrial complex enzymes

2.7.2.1. Estimation of NADH dehydrogenase (complex-I) activity. Activity of NADH dehydrogenase was measured by catalytic oxidation of NADH with potassium ferricyanide as an artificial electron acceptor at excitation and emission wavelengths for NADH were 350 nm and 470 nm, respectively (Shapiro et al., 1979).

2.7.2.2. Estimation of succinate dehydrogenase (SDH) (complex-II) activity. The mitochondrial succinate: acceptor oxidoreductase was determined by the progressive reduction of nitro blue tetrazolium (NBT) to an insoluble colored compound, diformazan (dfz) was measured at 570 nm (Old and Johnson, 1989).

2.7.2.3. Estimation of cytochrome oxidase (complex-IV) activity. Cytochrome oxidase was assayed in mitochondrial preparation according to the method of Sottocasa et al. (1967) in presence of reduced cytochrome *c*. The decrease in absorbance was measured at 550 nm for 3 min.

2.7.2.4. Estimation of F_1-F_0 synthase (complex-V) activity. Mitochondrial F1-F0 synthase was measured by incubating mitochondrial suspension in 500 μl ATPase buffer (Griffiths and Houghton, 1974) and the phosphate produced was measured as per Fiske and Subbarow (1925).

2.7.2.5. Estimation of MMP. The Rhodamine dye taken up by healthy mitochondria was measured fluorimetrically at an excitation λ 535 ± 10 nm and emission λ of 580 ± 10 nm (Shu-Gui, 2002).

2.7.2.6. Estimation of MTT reduction. MTT reduction was measured by estimating formazan formed at 595 nm (Kamboj et al., 2008).

2.7.3. Mitochondrial oxidative stress

2.7.3.1. Estimation of mitochondrial nitrite level and malondialdehyde (MDA) formation. Nitrite levels were determined by a colorimetric assay using Greiss reagent (0.1%) at 540 nm (Green et al., 1982) and mitochondrial MDA using method of Sunderman et al. (1985).

2.7.3.2. Estimation of mitochondrial SOD and catalase. SOD was estimated by following reduction of NBT to blue colored formazan in presence of phenazine metha sulphate (PMS) and NADH was measured at 560 nm (Kakkar et al., 1984). Catalase activity was estimated by the addition of 50 μl H_2O_2 (6%) and decrease in the absorbance was measured at 240 nm for 3 min at 30 s interval (Beers and Sizer, 1952).

2.7.3.3. Estimation of protein. The protein content was estimated according to the method of Lowry et al. (1951).

2.8. Statistical analyses

Data are presented as means ± Standard deviation (SD). Data for immobility, climbing and anxiety behavior was analyzed by two-way ANOVA followed by Bonferroni test. Data of adrenal gland weight, plasma corticosterone and mitochondrial parameters were analyzed by one-way ANOVA followed by Student Newman Keuls test using Graph Pad prism version 5 (San Diego, CA). A level of $p < 0.05$ was accepted as statistically significant.

3. Results

3.1. Effect of shilajit on CFS-induced changes in immobility and climbing behavior

The immobility period is the behavioral parameter used to assess the precipitation of chronic fatigue in the experimental model for rodents. The immobility (Fig. 2A) and climbing (Fig. 2B) period in the last 5 min after 10 min of swimming was measured for 21 days. Analysis by two-way Anova of the results showed that there was significant interaction of treatment with time among groups [$F(12, 100) = 12.71$; $p < 0.05$]. Post-hoc analysis showed that stress significantly enhanced immobility on day 14 and day 21 as compared to day 1. Pretreatment with shilajit (25, 50 and 100 mg/kg) reversed the stress-induced increase in immobility period on days 14 and 21. The positive control group treated with WS (100 mg/kg) also reversed stress-induced increase in immobility on all days tested. Two- way Anova analysis of the climbing period revealed a significant interaction of groups over time [$F(12, 100) = 2.16$; $p < 0.05$]. Post-hoc analysis showed that stress significantly decreased climbing period on days 7, 14 and 21 compared to day 1. Shilajit in all doses tested significantly increased the climbing period as compared to stress control on day 21, but not on days 7 or 14. WS also significantly reversed stress-induced decrease in climbing period only on day 21 (Fig. 2).

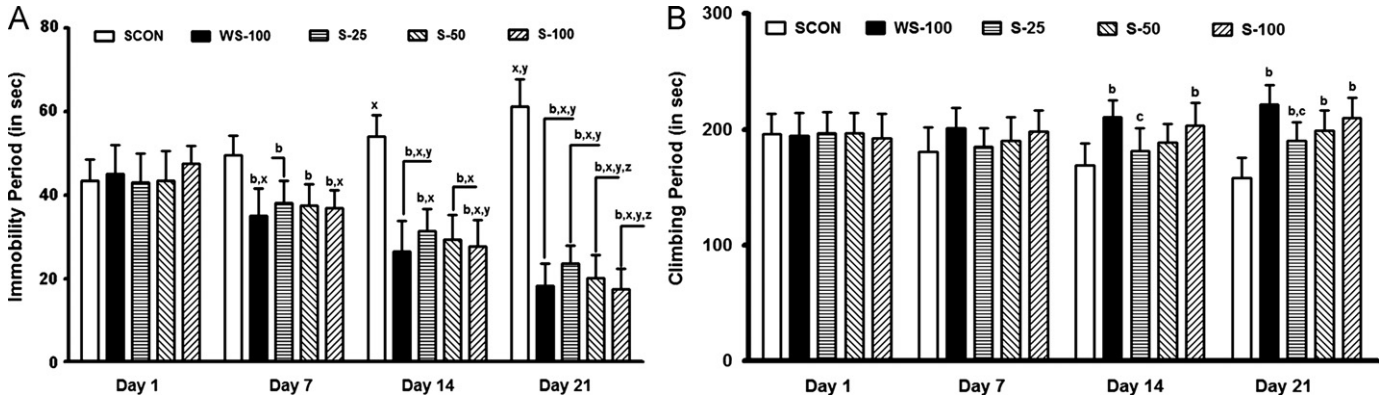

Fig. 2. Effect of chronic fatigue syndrome (CFS) due to forced swimming on immobility (panel A) and climbing behavior (panel B) on days 1, 7, 14 and 21. Bars represent data as Mean ± SD, $n=6$, $^{a}p<0.05$ compared to control (CON); $^{b}p<0.05$ compared to stress control (SCON); $^{c}p<0.05$ compared to WS-100 [WS (100 mg/kg)]; $^{d}p<0.05$ compared to S-25 [shilajit (25 mg/kg)] and $^{e}p<0.05$ compared to S-50 [shilajit (50 mg/kg)]. S-100 is shilajit (100 mg/kg). $^{x}p<0.05$ compared to day 1; $^{y}p<0.05$ compared to day 7; $^{z}p<0.05$ compared to day 14 (Two-way ANOVA followed by Bonferroni test).

Table 1
Effect of chronic fatigue syndrome (CFS) due to forced swimming on anxiety behavior on day 1, day 7, day 14 and day 21 in elevated plus maze.

Groups	*N*	Time in closed arm	Time in open arm	Entries in open arm	Head dips
Control					
Day 1	6	228.41 ± 16.72	46.82 ± 5.21	5.66 ± 0.51	7.66 ± 0.51
Day 7	6	223.02 ± 14.84	42.31 ± 6.83	5.50 ± 0.54	7.50 ± 0.54
Day 14	6	225.59 ± 17.01	41.02 ± 6.49	5.50 ± 0.54	7.50 ± 0.83
Day 21	6	232.65 ± 13.52	43.79 ± 7.31	5.83 ± 0.40	7.33 ± 0.81
Stress control					
Day 1	6	225.83 ± 25.41	45.98 ± 5.65	6.33 ± 0.81	7.50 ± 0.54
Day 7	6	250.61 ± 18.62^{a}	30.62 ± 5.79^{a}	3.33 ± 0.51^{a}	4.66 ± 0.51^{a}
Day 14	6	263.48 ± 17.36^{a}	22.64 ± 5.26^{a}	2.83 ± 0.75^{a}	3.33 ± 0.51^{a}
Day 21	6	273.31 ± 13.83^{a}	16.54 ± 5.34^{a}	2.66 ± 0.81^{a}	3.33 ± 1.03^{a}
WS 100 mg/kg					
Day 1	6	225.03 ± 15.61	50.97 ± 5.90	6.16 ± 0.41	7.33 ± 0.51
Day 7	6	218.61 ± 12.53^{b}	55.34 ± 5.27^{ab}	6.00 ± 0.63^{b}	7.00 ± 0.89^{b}
Day 14	6	208.99 ± 12.92^{b}	61.88 ± 5.65^{ab}	6.33 ± 0.81^{b}	7.33 ± 0.81^{b}
Day 21	6	202.31 ± 13.28^{ab}	66.42 ± 5.06^{ab}	6.33 ± 0.81^{b}	6.83 ± 0.98^{b}
Shilajit 25 mg/kg					
Day 1	6	226.37 ± 19.83	47.85 ± 6.35	6.16 ± 0.75	7.66 ± 0.51
Day 7	6	238.64 ± 18.32	39.88 ± 6.90^{bc}	3.83 ± 0.75^{ac}	5.83 ± 0.75^{abc}
Day 14	6	228.45 ± 13.79^{b}	46.50 ± 5.60^{bc}	4.00 ± 0.89^{abc}	5.83 ± 0.75^{abc}
Day 21	6	215.93 ± 16.28^{b}	54.64 ± 5.67^{abc}	4.33 ± 0.81^{abc}	4.66 ± 0.81^{abc}
Shilajit 50 mg/kg					
Day 1	6	223.57 ± 15.38	46.62 ± 5.82	6.00 ± 0.89	7.33 ± 0.51
Day 7	6	231.74 ± 14.28	41.63 ± 5.39^{bc}	4.66 ± 0.81^{bc}	6.00 ± 0.89^{ab}
Day 14	6	221.81 ± 15.68^{b}	47.69 ± 5.79^{bc}	4.83 ± 0.98^{bc}	6.33 ± 0.51^{ab}
Day 21	6	213.66 ± 12.35^{b}	52.66 ± 5.19^{abc}	5.16 ± 0.98^{bc}	6.33 ± 0.51^{bd}
Shilajit 100 mg/kg					
Day 1	6	224.60 ± 24.27	52.78 ± 5.40	5.83 ± 0.75	7.50 ± 1.04
Day 7	6	215.53 ± 16.65^{b}	59.12 ± 6.16^{abde}	6.16 ± 0.75^{bde}	6.66 ± 0.81^{b}
Day 14	6	206.21 ± 13.06^{b}	65.65 ± 5.07^{abde}	6.33 ± 0.81^{bde}	7.16 ± 0.75^{bd}
Day 21	6	198.98 ± 15.11^{ab}	70.71 ± 5.03^{abde}	6.50 ± 0.83^{bde}	8.16 ± 0.75^{bcde}

Results are mean ± S.D. $n=6$. $^{a}p<0.05$ compared to control; $^{b}p<0.05$ compared to stress control; $^{c}p<0.05$ compared to WS (100 mg/kg); $^{d}p<0.05$ compared to shilajit (25 mg/kg) and $^{e}p<0.05$ compared to shilajit (50 mg/kg) (Two-way ANOVA followed by Bonferroni test).

3.2. Effect of shilajit on CFS-induced changes in anxiety behavior

The CFS-induced anxiety behavior was measured using EPM test (Table 1). Two-way ANOVA of the time spent in open arm on day 1, 7, 14 and 21 showed that there was significant interaction of treatment with days among groups [F (15, 120)=10.84; $p<0.05$]. The post-hoc test showed that the stress control group spent significantly less time in exploring the open arm as compared to the control on day 7, 14 and 21. The drug treated groups significantly reversed the decrease in time spent in open arm as compared to stress control group on the above days. The analysis of the result of the time spent in closed arm by two-way ANOVA [F (15, 120)=3.36; $p<0.05$] showed significant treatment interaction with time among groups. Post-hoc test showed that the time spent in closed arm was significantly more in case of stress control as compared to the control group. Shilajit at all doses and WS significantly attenuated the time spent in closed arm on day 7, 14 and 21. Two-way ANOVA of the number of entries in open arm [F (15, 120)=5.99; $p<0.05$] showed a significant interaction of treatment with days among groups. Post-hoc analysis test showed that the numbers of entries in open arm were significantly less in stress control as compared to the control group and shilajit at all doses and WS significantly reversed this effect on day 7, 14 and 21. Two-way ANOVA of the

number of head dips [F (15, 120)=8.11; $p < 0.05$] and subsequent post-hoc test showed that the number of head dips were significantly decreased by stress and shilajit at all doses and WS significantly reversed this effect.

3.3. Effect of shilajit on adrenal gland weight

CFS is characterized by hypocortisolaemia as a result of chronic stress. One-way ANOVA of the results of the weight of adrenal gland (Fig. 3A) showed that there was significant interaction of treatment among groups [F (5, 30)=56.42; $p < 0.05$]. Further, post-hoc analysis by Student Newman Keuls test showed that CFS significantly decreased adrenal gland weight compared to the control group. Shilajit (25, 50 and 100 mg/kg) and WS (100 mg/kg) were found to significantly attenuate the loss in weight of adrenal gland. Shilajit showed a dose-dependent increase in adrenal gland weight.

3.4. Effect of shilajit on corticosterone level

The plasma corticosterone level is sensitive to stress responses. One-way ANOVA of the results showed that there was significant treatment interaction among groups [F (5, 30)=40.19, $p < 0.05$]. Further, post-hoc analysis by Student Newman Keuls test showed that CFS caused significant decrease in plasma corticosterone as compared to basal levels in controls (Fig. 3B). Shilajit dose-dependently reversed CFS-induced decrease in plasma corticosterone level. WS also significantly reversed the CFS-induced decrease in plasma corticosterone.

3.5. Effect of shilajit on mitochondrial electron transport chain enzymes

The activities of various mitochondrial electron transport chain enzymes are illustrated in Table 2. One-way ANOVA of the results showed that there was significant interaction of treatment among groups in the of activities of NADH [F (5, 30)=8.88; $p < 0.05$]; SDH [F (5, 30)=15.73; $p < 0.05$]; Cytochrome oxidase [F (5, 30)=16.96; $p < 0.05$] and ATP synthase [F (5, 30)=62.62; $p < 0.05$] as a measure of mitochondrial complex I, II, IV and V enzyme activities respectively. Further, post-hoc analysis by Student Newman Keuls test showed that chronic stress due to 21 days of forced swimming caused significant decrease in all enzyme activities representing different complex enzyme systems as compared to basal levels in controls. Shilajit (25, 50 and 100 mg/kg) significantly attenuated the decrease in NADH, SDH, Cytochrome oxidase and ATP synthase activities. High dose of shilajit (100 mg/kg) significantly increased the activities of NADH and SDH compared to the lowest dose (25 mg/kg). In addition, the effect of shilajit (100 mg/kg) in reversing stress-induced reduction in SDH activity was significantly different from shilajit (50 mg/kg). A dose-dependent reversal in stress-induced decrease in activities of Cytochrome oxidase and ATP synthase was observed with shilajit. Analysis of mitochondrial respiration in terms of MTT reduction showed a significant interaction among groups [F (5, 30)=29.95; $p < 0.05$]. Shilajit in all doses tested reversed the stress-induced decrease in MTT reduction. WS also reversed the stress-induced changes on the mitochondrial enzymes and respiration.

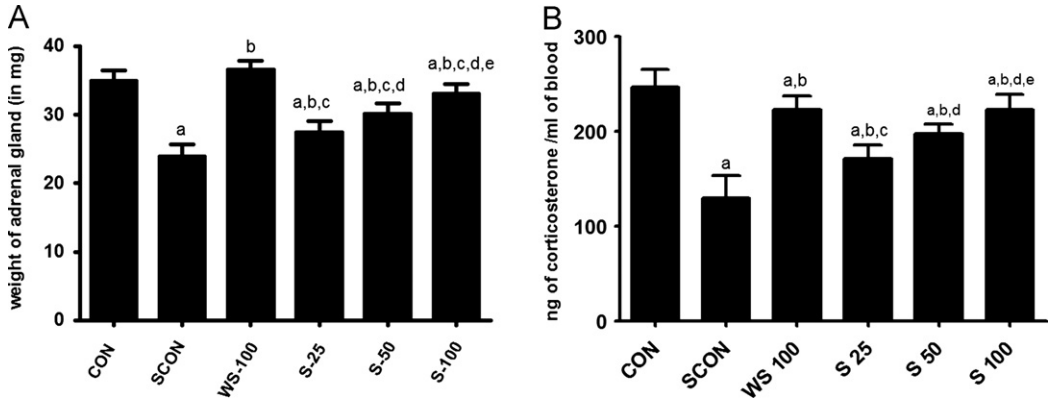

Fig. 3. Effect of CFS and treatment with shilajit 25, 50 and 100 mg/kg on weight of adrenal gland (panel A) and plasma corticosterone level (panel B). Bars represent data as Mean ± SD, $n=6$, $^{a}p < 0.05$ compared to CON; $^{b}p < 0.05$ compared to SCON; $^{c}p < 0.05$ compared to WS-100; $^{d}p < 0.05$ compared to S-25 and $^{e}p < 0.05$ compared to S-50. (One-way ANOVA followed by Student Newman Keuls test).

Table 2
Effect of CFS on activities of prefrontal cortex mitochondrial NADH dehydrogenase (Δfluoresence/min/mg of protein), Succinate dehydrogenase(formazan produced/min/mg of protein), Cytochrome oxidase(nmol of cytochrome c oxidized/min/mg of protein), ATP synthase(nmol of ATP hydrolyzed/min/mg of protein), mitochondrial respiration by MTT reduction(formazan produced/min/mg of protein), LPO(nmol of MDA/mg of protein), SOD(Units/min/mg of protein), NO(μM), and catalase(Units/mg of protein) levels in rats.

Mitochondrial Parameters	Control	Stress control	WS (100 mg/kg)	Shilajit (25 mg/kg)	Shilajit (50 mg/kg)	Shilajit (100 mg/kg)
NADH dehy.	38.07 ± 3.61	24.95 ± 4.7^{a}	36.15 ± 3.27^{b}	30.83 ± 5.14^{ab}	34.08 ± 4.25^{b}	37.74 ± 3.48^{bd}
SDH	1.93 ± 0.09	1.53 ± 0.09^{a}	1.75 ± 0.09^{ab}	1.63 ± 0.08^{a}	1.68 ± 0.09^{ab}	1.87 ± 0.10^{bcde}
Cyt c oxidase	145.42 ± 4.53	124.16 ± 4.69^{a}	143.94 ± 5.62^{b}	128.42 ± 5.14^{ac}	136.18 ± 5.21^{abcd}	144.01 ± 6.73^{bde}
ATP synthase	11.85 ± 0.20	7.31 ± 0.21^{a}	11.35 ± 0.78^{b}	8.47 ± 0.70^{abc}	9.87 ± 0.65^{abcd}	11.62 ± 0.62^{bde}
MTT	5.95 ± 0.49	3.29 ± 0.64^{a}	5.25 ± 0.30^{ab}	4.22 ± 0.39^{abc}	4.94 ± 0.26^{abd}	5.40 ± 0.32^{abd}
NO	6.7 ± 0.8	12.52 ± 1.03^{a}	7.69 ± 0.62^{b}	10.41 ± 0.66^{abc}	8.25 ± 0.66^{abd}	7.24 ± 0.56^{bd}
LPO	5.54 ± 1.04	18.06 ± 1.93^{a}	8.78 ± 0.56^{ab}	12.92 ± 1.13^{abc}	10.16 ± 1.41^{abd}	7.66 ± 0.67^{abde}
SOD	16.7 ± 1.2	21.4 ± 1.6^{a}	16.8 ± 1.3^{b}	19.6 ± 1.2^{abc}	18.1 ± 1.1^{bd}	16.7 ± 1.1^{bd}
Catalase	22.42 ± 1.27	13.18 ± 1.75^{a}	20.41 ± 0.89^{ab}	16.49 ± 1.19^{abc}	18.72 ± 1.07^{abcd}	20.57 ± 0.73^{abde}

Results are mean ± S.D. $n=6$. $^{a}p < 0.05$ compared to control; $^{b}p < 0.05$ compared to stress control; $^{c}p < 0.05$ compared to WS (100 mg/kg); $^{d}p < 0.05$ compared to shilajit (25 mg/kg) and $^{e}p < 0.05$ compared to shilajit (50 mg/kg) (One-way ANOVA followed by Student Newman Keuls test).

3.6. Effect of shilajit on mitochondrial membrane potential

Mitochondrial membrane potential (MMP) demonstrates the membrane integrity of the mitochondria. One-way ANOVA of MMP data showed that there was significant treatment interaction among groups [$F(5, 30)=7.95$, $p<0.05$]. Further, post-hoc analysis by Student Newman Keuls test showed that CFS due to 21 days of forced swimming caused significant decrement in fluorescence emission compared to basal levels in controls (Fig. 4). Shilajit in all doses tested significantly reversed the stress induced decrease in fluorescence emission indicating reversal of CFS-induced decrease in MMP. WS also significantly increased the CFS-induced decrease in MMP.

3.7. Mitochondrial oxidative stress

3.7.1. Effect of shilajit on mitochondrial NO levels and LPO

The results for NO levels and LPO are depicted in Table 2. One-way ANOVA of the results showed a significant interaction of treatment among groups in case of NO levels [$F(5, 30)=53.27$; $p<0.05$] and LPO [$F(5, 30)=80.36$; $p<0.05$]. Post-hoc analysis by Student Newman Keuls test showed that NO levels and lipid peroxidation product was significantly enhanced in stress as compared to control group. Shilajit at all doses significantly alleviated the stress induced increase in NO level and LPO product (MDA). The standard drug WS also reversed stress-induced increase in NO levels and lipid peroxidation.

3.7.2. Effect of shilajit on mitochondrial SOD activity

SOD is an enzyme that is considered as a first line of defense against ROS generation. One-way ANOVA of the results of SOD data showed a significant interaction of treatment among groups in case of SOD [$F(5, 30)=14.07$; $p<0.05$]. Post-hoc analysis by Student Newman Keuls test showed that SOD was significantly enhanced in stress as compared to control group (Table 2). Shilajit at all doses significantly attenuated the increase in SOD as compared to stress control group. WS also reversed stress-induced increase in SOD level.

3.7.3. Effect of shilajit on mitochondrial catalase activity

The results illustrating catalase activity are depicted in Table 2. One-way ANOVA of the results show a significant interaction of treatment among groups in case of catalase [$F(5, 30)=46.75$; $p<0.05$]. Post-hoc analysis by Student Newman Keuls test showed that stress significantly reduced catalase activity compared to control group. Shilajit showed a dose-dependent effect in reversal of stress induced decrease in catalase activity. The standard drug WS also reversed stress-induced decrease in catalase activity.

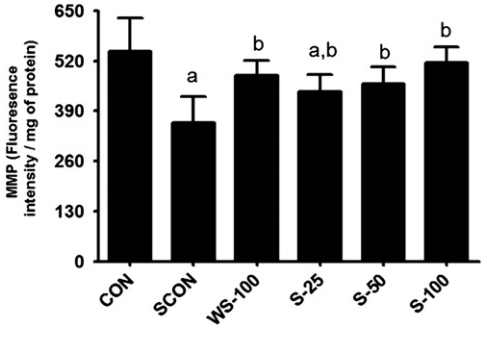

Fig. 4. Effect of CFS on mitochondrial membrane potential (MMP) in prefrontal cortex. Bars represent data as Mean ± SD, $n=6$, $^{a}p<0.05$ compared to CON; $^{b}p<0.05$ compared to SCON; $^{c}p<0.05$ compared to WS (100 mg/kg); $^{d}p<0.05$ compared to shilajit (25 mg/kg) and $^{e}p<0.05$ compared to shilajit (50 mg/kg). (One-way ANOVA followed by Student Newman Keuls test).

4. Discussion

The objective of present study was to evaluate the activity of shilajit in an experimental model of CFS. Salient findings of the study are that shilajit (25, 50, 100 mg/kg) protected against the behavioral symptoms and normalized the neuroendocrine perturbation in cortiscosterone level induced by CFS. Further, shilajit attenuated CFS-induced mitochondrial dysfunction and preserved its integrity.

Simple co-morbidity of CFS and depression does not address their temporal relationship; depressive symptoms could precede or occur in response to the illness (Afari and Buchwald, 2003). However, anxiety and depression are the most common emotional responses to CFS (Cassem, 1990). In the present model, forced swimming for 15 min everyday for 21 days caused significant changes in the behavioral parameters including chronic depression and anxiety in rats. Rats when forced to swim exhibit climbing behavior that indicates escape behavior which is followed by a typical immobile posture indicating behavioral depression (Porsolt, 1981). The experimental model is useful for estimating the behavioral changes due to chronic exposure to physical and mental stresses which produces CFS in rats (Lylea et al., 2009). Stress significantly increased immobility period over days 14 and 21 indicating development of behavioral despair in rats. Further, chronic swim stress significantly decreased climbing period indicating decrease in effort by rats to escape (escape behavior). Shilajit at all doses reversed the immobility and climbing behavior in CFS. Thus, shilajit ameliorated CFS-induced behavioral depressive symptoms in rats.

There is an overlap between CFS and generalized anxiety disorder, which includes overlap of some neurobiological similarities including decreased cerebral blood flow, sympathetic over activity and sleep abnormalities (Nutt, 2001). There has been previous speculation that CFS overtly resembles 'atypical depression' with its prominent anxiety and somatic symptoms, tendency to cortisol hyposecretion, and poor response to conventional antidepressant pharmacotherapy (Gold et al., 1995; Terman et al., 1998). However, the simple co-morbidity of CFS and anxiety disorders does not suggest that CFS is a physical manifestation of an anxiety disorder (Afari and Buchwald, 2003). In the present study, CFS significantly induced anxiety behavior from day 7 in terms of rats spending less time in open arm and more time in closed arm. However, stress did not induce anxiety like behavior on day 1 as observed by an earlier study (Lylea et al., 2009). These behavioral changes indicate increased level of anxiety in the EPM test (Fernandes and File, 1996). Treatment with shilajit in all doses decreased anxiety levels in rats; as such they spent more time in open arm and less time in closed arm. The number of head dips which represents exploratory behavior of rats (Rodgers and Johnson, 1995) was decreased in CFS. Shilajit reversed stress-induced decrease in head dips suggesting anxiolytic activity leading to spontaneous exploration of novel arena. Shilajit has earlier been reported to show anxiolytic activity in rodents (Jaiswal and Bhattacharya, 1992). Hence, the present experiment shows that shilajit is capable of attenuating CFS-induced anxiety in rats.

Mental stress plays an important role in pathophysiology of CFS. A retrospective study among people reporting CFS indicated fatigue and/or severe life events during the previous 3 months before the onset of the illness (Theorell et al., 1999). Studies have revealed intriguing abnormalities of the HPA axis in CFS sufferers

(Cleare, 2004). The change in plasma levels of glucocorticoids is a reliable index of HPA axis activity. In the present study, the chronic swimming significantly decreased plasma corticosterone levels indicating hypoactivity of the HPA axis. This was in accordance with hyposecretion of cortisol reported in CFS patients (Cleare et al., 1995). HPA axis may not have a direct effect but it might play a role in exacerbating symptoms in the course of CFS (Cleare, 2004). Shilajit dose-dependently reversed the CFS-induced attenuation of corticosterone level. To add credit to the normalizing effect of shilajit on the HPA axis, shilajit dose-dependently reversed the CFS induced decrease in weight of adrenal gland. The results suggest that shilajit may modulate the stress responses through the HPA axis.

A remarkable correlation is observed between severity of illness in CFS and mitochondrial dysfunction (Myhill et al., 2009). We study for the first time the modulation of mitochondrial complex enzymes in PFC due to chronic swim-induced CFS. NADH dehydrogenase is one of the two enzymes responsible for the transfer of electrons into the electron transport chain. It is the site of entry of NADH into the respiratory chain where it catalyzes the dehydrogenation of NADH generated through oxidation of numerous NAD^+-linked dehydrogenase reactions (Hatefi and Stiggall, 1976). The disorders in patients with impaired NADH dehydrogenase activity have been found to include: pure myopathy type with exercise intolerance and myalgia starting in childhood or adult life, encephalomyopathic type without stroke-like episodes, a syndrome comprising myopathy, encephalopathy and lactic acidosis (Morganhughes et al., 1988). NADH dehydrogenase activity in PFC was significantly impaired in CFS. This indicates a decrease in the activity of Complex I enzyme system in the mitochondrial electron transport chain (ETC) (Hatefi and Stiggall, 1976). Shilajit at all doses prevented the attenuation of active NADH dehydrogenase. SDH forms another pathway accountable for the initiation of transfer of electrons into the ETC. SDH forms a part of the complex II mitochondrial membrane bound enzymes playing a central role in neuronal energy metabolism as a part of respiratory chain and also in tricarboxylic acid (TCA) cycle (James and Timothy, 1995). Chronic swim stress significantly decreased SDH activity. This represents a decrease in the activity of Complex II in the mitochondrial electron transport chain. Decreased neuronal mitochondrial SDH activity has been reported with the use of excitotoxin, kainic acid. This action was reported to be mediated through kainic acid induced ROS production which inhibits the catalytic function of SDH (Federica et al., 2001). Shilajit in all the doses prevented the attenuation of mitochondrial SDH activity. The higher activity of NADH dehydrogenase and SDH ensures higher rate of transfer of electrons by complexes I and II into the electron transport chain. The electrons incorporated into the electron transport chain are transferred from ubiquinone to cytochrome oxidase via cytochrome c. Cytochrome oxidase in the presence of reduced cytochrome c and oxygen is responsible for the transfer of proton into the mitochondrial inner membrane. In living cells cytochrome oxidase represents the rate-limiting enzyme of the mitochondrial respiratory chain (Villani and Attardi, 2000). Defects in cytochrome oxidase or cytochrome reductase show a similar spectrum of diseases as in case of NADH dehydrogenase. Similar to other complex enzyme system, stress significantly decreased the activity of complex-IV enzyme cytochrome oxidase. Shilajit significantly reversed CFS-induced decrease in cytochrome oxidase activity. The complex V enzyme, ATP synthase utilizes the proton pump gradient for the synthesis of ATP from ADP (Diez et al., 2004). ATP synthase activity was decreased by chronic swim stress. This can be attributed to overall dysfunction in the ETC. Thus, this may result in the decreased availability of ATP for metabolic/homeostatic responses in CFS (Myhill et al., 2009). Shilajit reversed the CFS-induced decrease in ATP synthase activity, thereby providing the much needed ATP to meet the increased CFS-induced energy demands. MTT reduction to formazan blue has been used to assess mitochondrial respiration (Kamboj et al., 2008). MTT reduction studies showed that CFS-induced decrease in mitochondrial respiration. These findings suggest the existence of perturbations of mitochondrial bioenergetics in PFC due to CFS. Shilajit was found to be effective in normalizing the decrease in mitochondrial respiration due to CFS. This is perhaps due to reversal of CFS-induced changes in mitochondrial complex enzyme systems by shilajit.

The deleterious effect on the mitochondrial complex activity has been found to be exacerbated as we move up the electron transport chain which can result in decrease in MMP. Decrease in fluorescence intensity of TMRM dye was observed in PFC mitochondria in CFS indicating perturbations in MMP. This suggests loss of mitochondrial integrity in CFS. Maintenance of basal MMP and therefore mitochondrial integrity is essential for the activity of ATP synthase. Mitochondrial respiration and membrane potential are regulated by the allosteric ATP-inhibition of cytochrome c oxidase (Ramzan et al., 2010). Studies on cytochrome oxidase suggest that when the allosteric ATP-inhibition is switched off under stress and respiration is regulated by "respiratory control," based on transmembrane potential (Helling et al., 2008). The present study reveals that chronic stress as in CFS was found to attenuate the MMP and shilajit restored the loss of MMP in CFS. Thereby, shilajit has the potential to normalize mitochondrial perturbations in CFS.

A severe psychological stress in CFS has been reported to produce increases in NO (Pall, 2000). Nanomolar concentrations of NO reversibly inhibit synaptosomal respiration by competing with oxygen at cytochrome oxidase (Brown and Cooper, 1994). A direct role of NO in CFS was supported by the apparent role of the NO scavenger, the hydroxocobalamin form of vitamin B12, in the treatment of CFS (Pall, 2001). CFS in the present study increased NO levels. This is in accordance with earlier observations (Lylea et al., 2009). Shilajit at all doses effectively attenuated the superfluity of NO due to CFS. This was in concurrence with previous report of shilajit as a reversible NO captodative agent (Bhattacharya et al., 1995).

Mitochondrial NO forms peroxynitrite in combination with free radicals to cause brain mitochondrial oxidative damage by LPO and energy depletion by inhibiting mitochondrial complex II-III (Juan et al., 1997). In the present study there was an increase in the oxidative stress measured in terms of increased MDA levels in the mitochondria of PFC in CFS. Shilajit at all doses reversed the increase in MDA levels due to CFS. This was in line with previous reports which suggest anti-lipid peroxidative property of shilajit (Tripathi et al., 1996). The oxidative stress due to CFS led to mitochondrial adaptogenic responses which were witnessed by increase in activity of SOD. Shilajit neutralized the oxidative stress thereby attenuating the adaptogenic response to oxidative stress by excessive SOD levels. Catalase converts hydrogen peroxide into H_2O and O_2. Catalase activity was decreased in CFS and this decrease in activity was reversed by shilajit. Therefore by maintaining the balance between SOD and CAT, shilajit may improve the mitochondrial management of oxidative stress. These findings suggest that there exist an imbalance in oxidative stress management due to chronic stress in CFS and shilajit can minimize these alterations by acting on anti-oxidant enzymes system. The present finding is in accordance to the earlier anti-oxidant reports on shilajit (Bhattacharya et al., 1995).

In conclusion, shilajit reversed the CFS-induced behavioral symptoms of depression and anxiety. Shilajit stabilized the HPA axis stress response system by attenuating the CFS-induced decrease in corticosterone level and adrenal gland weight.

Further, shilajit reversed mitochondrial dysfunction induced by CFS and maintained the mitochondrial integrity. Hence, anti-CFS activity of shilajit may involve pathways related to neuroendocrine functions and mitochondrial bioenergetics or a conjunction of these pathways. The reversal of CFS-induced behavioral symptoms and mitochondrial bioenergetics by shilajit indicates mitochondria as a potential target for treatment of CFS.

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