EUROPEAN JOURNAL OF BIOMEDICAL AND PHARMACEUTICAL SCIENCES
http://www.ejbps.com

ISSN 2349-8870
Volume: 5
Issue: 4
518-526
Year: 2018

REM SLEEP DEPRIVATION-INDUCED OXIDATIVE STRESS AND ITS ATTENUATION BY *WITHANIA SOMNIFERA (L.)* IN DISCRETE REGIONS OF RAT BRAIN.

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Article Received on 29/01/2018 **Article Revised on 18/02/2018** **Article Accepted on 10/03/2018**

ABSTRACT
Currently, plants are used as therapeutic agents in the wide range of clinical applications. The aim of this study is to assess the Antioxidant activity of *Withania somnifera* in 72 hours REM sleep deprivation. The plant (root) sample collected and Ethanolic extraction was done by using Soxhlet apparatus. Five groups of Wistar strain male albino rats were used in this study. Each group comprises of 6 rats and multiple platform models used for REM sleep deprivation. The values were statistically analyzed using one-way analysis of variance (ANOVA) followed by Tukey's posthoc test for multiple comparison methods. The Significance level was kept at $P<0.05$. The activity of Superoxide dismutase, Lipid peroxidation, Catalase, Glutathione peroxidase, levels of reduced glutathione, Vitamin C, Vitamin E in discrete regions of the rat brain and plasma Corticosterone level were measured. *Withania somnifera* possesses remarkable antioxidant activity by normalizing all the altered enzymes and plasma corticosterone level as a result of 72 hours REM sleep deprivation exposure.

KEYWORDS: Antioxidants, *Withania somnifera*, REM sleep deprivation, oxidative stress and corticosterone.

INTRODUCTION
Sleep is essential for normal physiological functions in human and animals; it provides restorations of emotional and physical activity in a manner that is not well established.[1] The average sleep per 24 hours has declined by 1.5 hours over the past century.[2] Sleep occurs in two stages: Non rapid eye movement (NREM) and rapid eye movement (REM).[3] Evidence from many studies suggests that an important function of sleep is a consolidation of new information into long-term memory. Multiple studies in both humans and rodents reported memory impairments as a result of sleep deprivation (SD).[4] Human studies show that the total SD for a single night disturbs memory function.[5] Additionally, numerous animal studies also shown that 24 hours to 96 hours of SD lead to impairment of memory and behavioral changes. Moreover, SD was shown to impair long-term potentiation of the hippocampus, which is one of the main centers for learning and memory function.[6] SD also elevates hippocampal oxidative stress, which reflects on neuronal excitability, cognitive functions and molecular signaling.[7] SD reduces the expression of transcription and translation synaptic proteins in hippocampus and many regions of the brain.[8] During pregnancy SD increases the risk of preeclampsia, gestational diabetes, intrauterine growth restriction and the need for cesarean delivery.[9] Cytokines like Tumor necrosis factor-alpha and Interleukin 1- beta gene expression increases in the hypothalamus during REM sleep deprivation.[10] SD leads to stroke, obesity, diabetes, osteoporosis, cardiovascular disease, cancer and permanent cognitive deficits.[11]

Withania somnifera (WS) known commonly as ashwagandha and belongs to Solanaceaes family, the green shrub plant has been used in India as traditional medicine for the treatment of mental health, ageing process[12] and promote learning and memory in animal models.[13] It also has antioxidant activity, according to the Indian indigenous system of medicine. Hence this present study was focused to evaluate physiological antioxidant defense elements, like lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione

(GSH), Vitamin C and Vitamin E in discrete regions of rat brain.

MATERIALS AND METHODS

Animals

Wistar strain male albino rats (200- 250g) were maintained under standard laboratory conditions with water and food, the animals were handled according to the principles of laboratory care framed by the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Government of India. Animal experiments were carried out after getting clearance from the institutional animal ethical committee (IAEC NO: 55/12/2015).

Experimental protocol

Animals were divided into five equal groups; each group consists of six animals. The Ethanolic extract of *Withania somnifera* was used for this study.

Group - I Control animals were used for studying the baseline values.

Group - II Animals were administered 2% of Tween 80 as a vehicle.

Group - III Animals were exposed to 72 hours REM sleep deprivation.

Group - IV Animals were treated with only an Ethanolic extract of *Withania somnifera* (500 mg/kg) [14] for 18 days and

Group - V Animals pretreated with Ethanolic extract of *Withania somnifera* for 15 days +72 hours REM sleep deprivation with the same treatment.

Plant collection and extract preparation

The dried root powder of *Withania somnifera* was purchased from Government Irula Tribe Women's Welfare Society (ITWWS), Tandarai, Chengalpattu, Tamilnadu, India- 603001. Bill No: 130/05/2016. About 200 g of root powder was extracted with 1000 ml of 95% Ethanol under reflux by heating over a water bath at 60^0C. The extract was then vacuum dried. The yield of Ethanolic extract of *Withania somnifera* was 31.23 % (w/w). The suspension of the extracted drug was prepared by dissolving in 2% Tween 80 before administration to animals.[15]

REM sleep deprivation

REM sleep deprivation (RSD) for 72 hrs was induced by using the multiple platform models.[16] It was started and ended at the beginning of the light phase and the room was maintained at the controlled temperature (23±1^o C) and light-dark cycle (lights on between 07.00 and 19.00 hrs). In this experiment 6 rats from same group placed in a water tank (120 cm x 70cm x 50 cm) containing 10 round platforms (each round platform made up of 7 cm diameter and 10 cm height, it was raised 2 cm above the water level) arranged in two lines and 20 cm away from each other (edge to edge), in which the rats can move around freely from one platform to another one. Loss of muscle tone at the beginning of each REM (paradoxical) sleep episode causes rats to fell in the water, thus being awakened. During the SD period, the animals had free access to water bottles and chow pellets attaching from a grill located on the top of the chamber. As the animals can move freely within the multiplatform chamber, it has been reported that it has less immobilization stress compared to the single version of platform technique.[17]

Biochemical analysis

The activity of LPO was indirectly estimated by determining the accumulation of thiobarbituric acid reactive substances (TBARS) in the tissue homogenate by the method of Ohkawa et al.[18] According to Markland and Markland[19] the SOD activity was measured as the degree of inhibition of auto-oxidation of pyrogallol at alkaline pH. The activity of catalase was measured as the amount of hydrogen peroxide consumed per minute per milligram of protein by the method of Sinha.[20] Glutathione peroxidase level was estimated by measuring the amount of reduced glutathione consumed in the reaction mixture according to the method of Rotruck et al.[21] The reduced glutathione level was measured based on the development of relatively stable yellow color, when 200mM 5, 5' – dithiobis- (2-nitrobenzoic acid) (DTNB) solution was added, according to the method of Moron et al.[22] Vitamin C was estimated by the method of Omaye et al.[23] and Vitamin E was measured by the method of Desai.[24] The total protein was estimated by the method of Lowry et al.[25] using Bovine serum albumin (BSA) as standard. The corticosterone level was measured by using Elisa kit. (Cusabio, biotech Co Limited, China. C.No.CSB-E07014r).

Statistical analysis

All data were expressed as Mean ± Standard deviation (STDEV). The statistical significance was evaluated by one - way analysis of variance (ANOVA) using SPSS statistical package version 20.0 (SPSS, Cary, NC, USA). When there is a significant difference, Tukey's multiple comparison tests were performed by fixing the significance at level $P<0.05$.

RESULTS AND DISCUSSION

Plasma Corticosterone

The plasma corticosterone (d.f.=4, F=43.131) levels "Fig.1" were increased in Group-III compared to Group-I control. However, corticosterone levels in Group-V animals were significantly decreased when compared to Group-III after 72 hours REM sleep deprivation exposure.

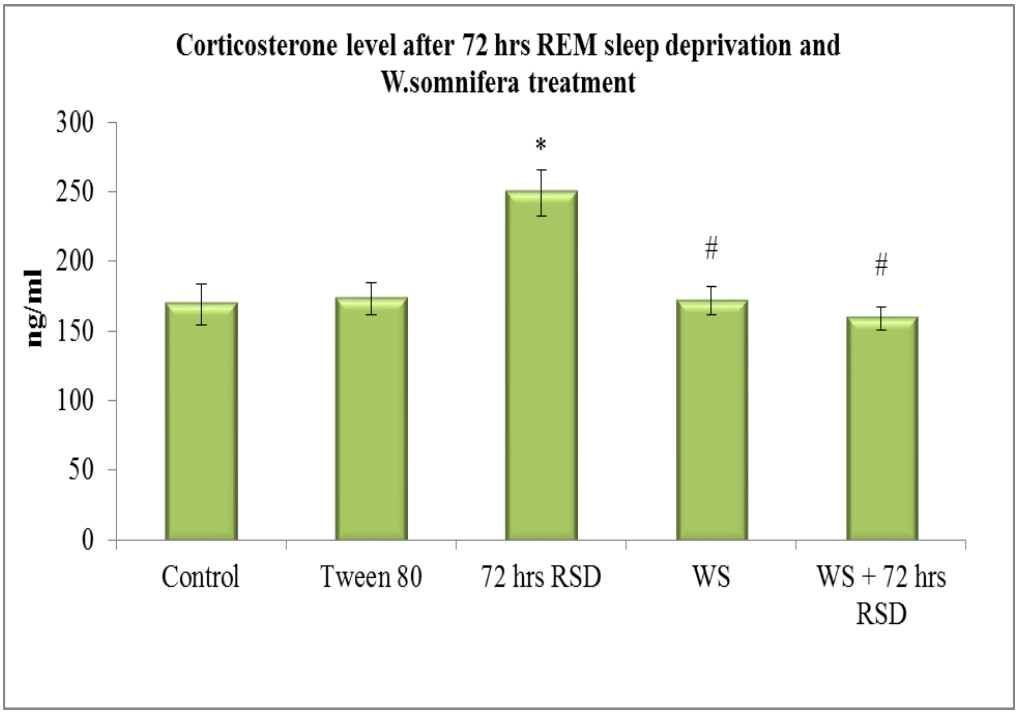

Fig. 1: Data are expressed as Mean ± STDEV. for six rats in each group. The values are expressed ng/ml. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <0.05.

Lipid peroxidation

The levels of LPO "Fig. 2" were significantly increased in Group-III after 72 hours REM sleep deprivation in discrete regions of the brain [Hypothalamus (d.f.=4, F=461.326), Hippocampus (d.f.=4, F=655.724), Brainstem (d.f.=4, F=667.603) and Prefrontal cortex(d.f.=4, F=1047.254)] when compared to Group-I control (animals not exposed to REM sleep deprivation). The LPO levels were lower in the drug-treated group (Group-V) compared to the Group-III animals. The values of decreased LPO level in Group-V were not statistically different from Group-I animals.

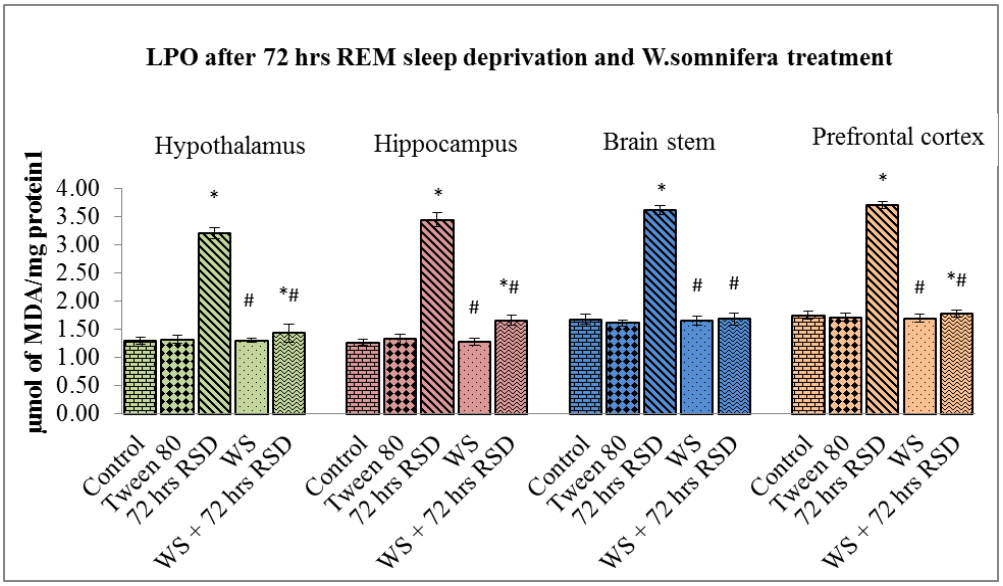

Fig. 2: Data are expressed as Mean ± STDEV for six rats in each group. The values are expressed µmol of MDA formed mg protein^{-1}. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <0.05.

Superoxide dismutase

The SOD is not evenly distributed in the regions studied as indicated by the control values. The activities of SOD "Fig. 3" were significantly increased in Group-III after 72 hours REM sleep deprivation in discrete regions of the brain [Hypothalamus (d.f.=4, F=108.284),

Hippocampus (d.f.=4, F=41.392), Brainstem (d.f.=4, F=86.756) and Prefrontal cortex(d.f.=4, F=17.374)] when compared to Group-I. The SOD activities were lower in the drug-treated group (Group-V) compared to the Group-III animals. The activities of SOD decreased in Group-V were not statistically different from Group-I animals.

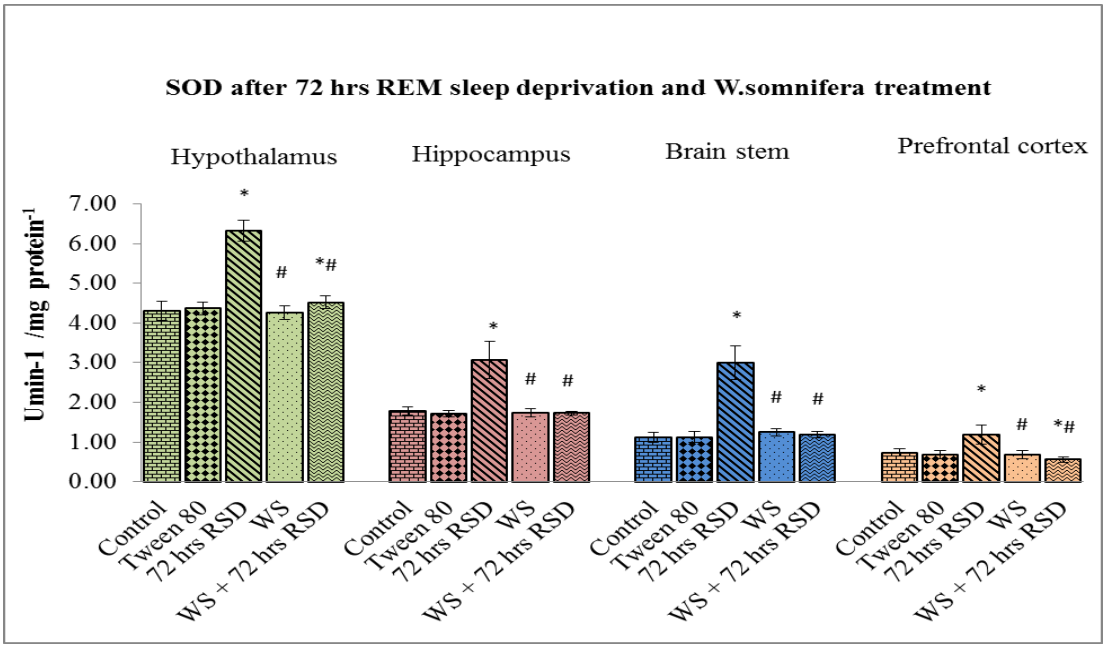

Fig. 3: Data are expressed as Mean ± STDEV for six rats in each group. The values are expressed Umin^{-1} mg protein^{-1}. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <*0.05*.

Catalase

The results are summarized in "Fig. 4". The catalase levels were increased in Group-III animals after 72 hours REM sleep deprivation in discrete regions of the brain [Hypothalamus (d.f.=4, F=35.736), Hippocampus (d.f.=4, F=52.934), Brainstem (d.f.=4, F=27.985) and Prefrontal cortex (d.f.=4, F=52.783)] when compared to Group-I. The catalase levels were lower in the drug-treated group (Group-V) compared to the Group III animals.

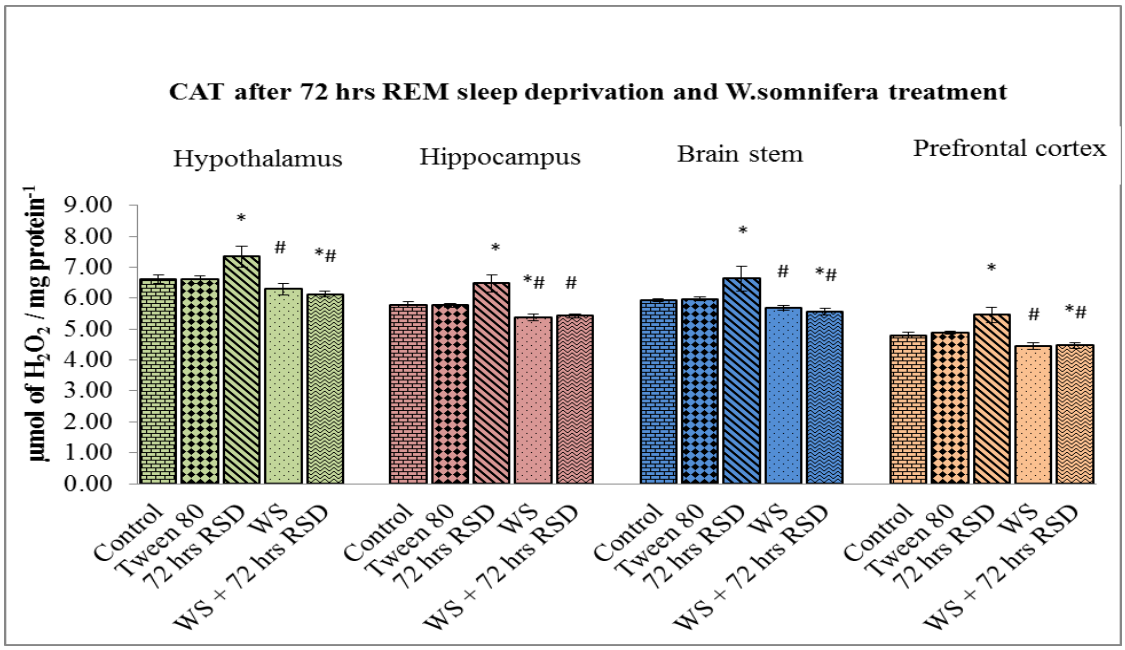

Fig. 4: Data are expressed as mean ± STDEV for six rats in each group. The values are expressed µmol of H_2O_2 consumed min^{-1} mg protein^{-1}. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <*0.05*.

Glutathione peroxidase

The results are summarized in "Fig. 5". The GPx also showed regional variations among the brain region studied. The GPx levels increased in Group-III after 72 hours REM sleep deprivation in discrete regions of the brain [Hypothalamus (d.f.=4, F=71.217), Hippocampus (d.f.=4, F=150.491), Brainstem (d.f.=4, F=7.066), and Prefrontal cortex(d.f.=4, F=66.037)] when compared to Group-I. The GPx levels were lower in the drug-treated group (Group-V) compared to the Group-III animals. The values of decreased GPx in Group- V were not statistically different from Group – I animals.

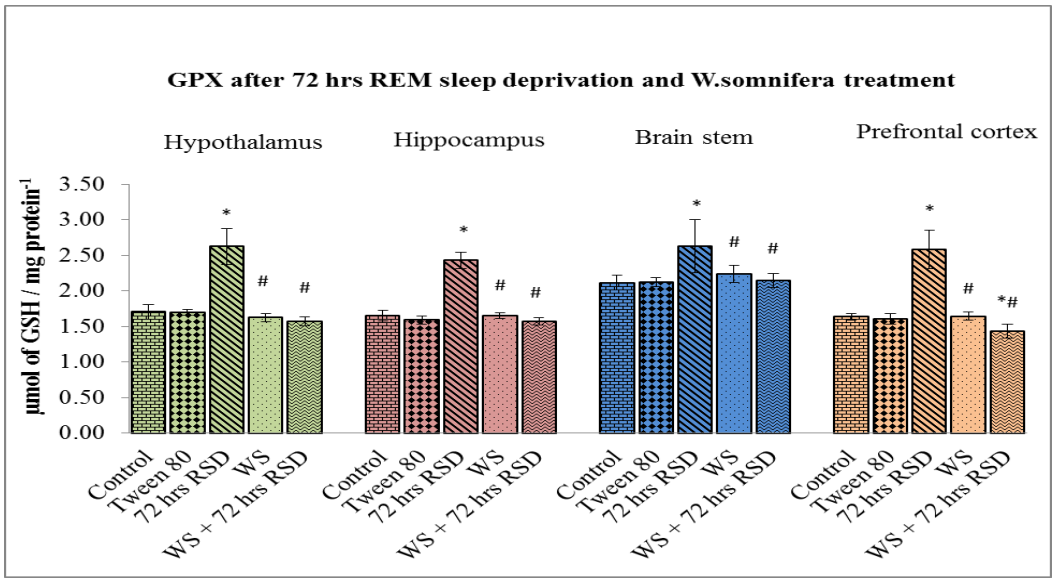

Fig. 5: Data are expressed as Mean ± STDEV for six rats in each group. The values are Expressed µmol of GSH oxidized min^{-1} mg $protein^{-1}$. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <0.05.

Reduced glutathione

The results are summarized in "Fig. 6". The GSH levels were significantly decreased in Group - III animals after 72 hours of REM sleep deprivation in discrete regions of the brain [Hypothalamus (d.f.=4, F=15.617), Hippocampus (d.f.=4, F=32.587), Brainstem (d.f.=4, F=51.122), and Prefrontal cortex (d.f.=4, F=27.979)] when compared to Group-I animals. The GSH levels were higher in the drug-treated group (Group-V) compared to the Group-III animals. The values of increased GSH in Group-V were not statistically different from Group-I animals.

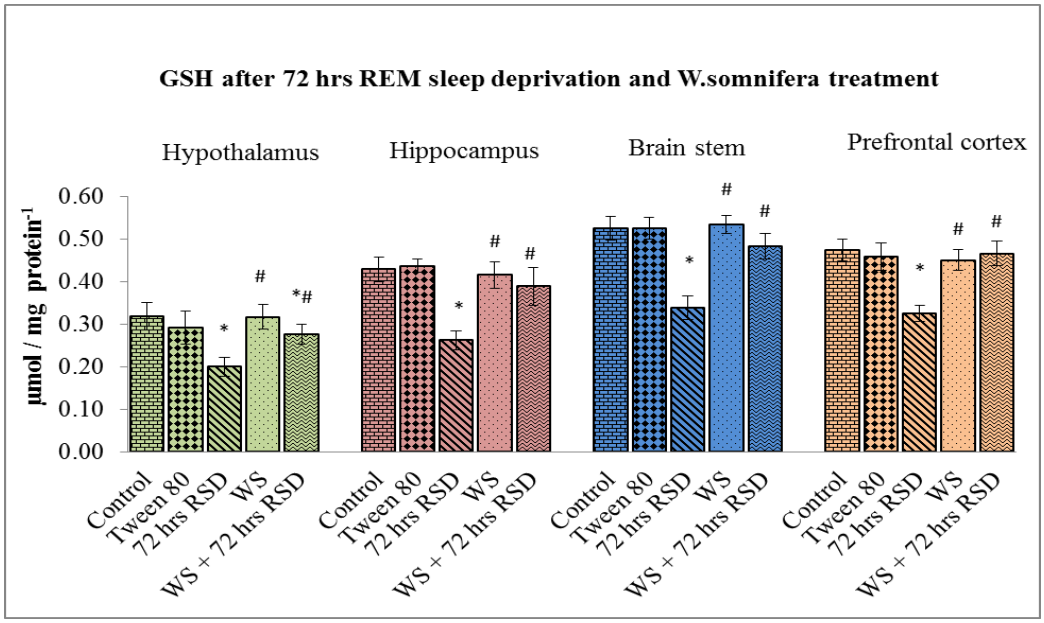

Fig. 6: Data are expressed as Mean ± STDEV for six rats in each group. The values are expressed µmol mg $protein^{-1}$. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <0.05.

Vitamin C and E

Vitamin C (Table.1) and Vitamin E (Table.2) levels were significantly decreased in Group - III animals after 72 hours REM sleep deprivation when compared to Group-I Control animals. On the other hand, Vitamin C and Vitamin E levels were higher in the drug-treated group (Group- V), compared to Group-III animals. The values of increased Vitamin C and Vitamin E in Group - V were not statistically different from Group - I animals.

Vitamin C Level after 72 hrs REM sleep deprivation and *W.somnifera* treatment

Brain regions	Group 1	Group 2	Group 3	Group 4	Group 5
Hypothalamus (d.f.=4, F=315.124)	339.50±8.30	338.88±5.12	232.56±6.48 *	345.70±5.88 #	333.61±7.05#
Hippocampus (d.f.=4,F=152.482)	394.50±4.02	389.30±5.82	287.25±11.06 *	381.50±10.17 #	370.98±10.57*#
Brain stem (d.f.=4,F=226.853)	292.31±4.98	291.90±5.39	220.48±5.68 *	286.25±4.99#	275.51±3.66*#
Pre frontal cortex (d.f.=4,F=284.817)	485.16±6.35	479.58±12.47	327.21±5.73 *	475.50±13.47#	460.95±7.47*#

Table 1. Data are expressed as Mean ± STDEV for six rats in each group. The values are expressed µg /g tissue^{-1}. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <*0.05*.

Vitamin E Level after 72 hrs REM sleep deprivation and *W.somnifera* treatment.

Brain regions	Group 1	Group 2	Group 3	Group 4	Group 5
Hypothalamus (d.f.=4,F=21.758)	11.85±1.16	11.72±0.82	7.79±0.70*	11.14±0.51#	12.25±0.95#
Hippocampus (d.f.=4,F=47.613)	16.52±0.73	17.36±0.68	12.25±0.72*	16.25±1.33#	16.12±0.87#
Brain stem (d.f.=4,F=79.482)	15.82±0.93	15.31±0.94	8.66±0.42*	13.33±0.60*#	12.82±0.53*#
Pre frontal cortex (d.f.=4,F=69.133)	17.90±0.88	18.73±0.68	12.44±0.70*	16.56±0.40*#	14.63±1.06*#

Table 2. Data are expressed as Mean ± STDEV for six rats in each group. The values are expressed µg /g tissue^{-1}. * Compared with Control; # Compared with 72 hrs REM sleep deprivation. The symbols represent statistical significance: *, # P <*0.05*.

In Group-II and IV animals, mostly all the parameters in all the regions of the brain were not significantly different from Group-I animals.

Many studies have shown that SD-induced oxidative damage in several types of tissues.[26] One of the studies that Sleep deprivation alters gene expression and antioxidant enzyme activity in mice.[27] Oxidative stress had linked with cognitive impairments.[28]

Antioxidants and free radicals scavenging system present in the cell protect against the damaging effects of free radicals.[29] In normal cells, there is a balance exists between oxidative products and antioxidants, like SOD, Catalase, GPx, GSH, Vitamin C and Vitamin E. Brain is more vulnerable to oxidative damage due to its high oxygen consumption.[30] For the transmission of impulses between the neuronal cells, it requires energy in the form of ATP, during the production of ATP using oxygen, a small percentage (less than 3%) of oxygen in mitochondria is mindfully converted to superoxide radical (SOR) This explains the reason for the increased production of free radicals in the brain. Also stress response results in the creation of some other reactive oxygen species such as hydrogen peroxide (H_2O_2), hydroxyl radicals (. OH) and superoxide anion radicals (O_2^-) that cause lipid peroxidation, especially in cell membrane leads to tissue damage.[31] The platform technique used for SD raises the possibility that long-term impairment of synaptic plasticity.[32]

In the present study, 72 hours REM sleep deprivation are producing marked and increased LPO, SOD, CAT, GPx activities and decreased in the levels of GSH, Vitamin C and Vitamin E in discrete regions of the brain that were tested (Hypothalamus, Hippocampus, Brainstem and Prefrontal cortex). Further, it also significantly increases the Corticosterone level in plasma.

Acute exposure to stress leads to an excessive generation of oxygen free radicals this might be the reason for the production of the excess amount of LPO.[33] SOD is a major intracellular enzyme, which protects the cell against oxygen free radicals by speeding up its dismutation of the superoxide anion (O2•−). In recent study states that the redox-sensitive transcription factor NF-E2-related factor 2 (Nrf2) plays an important role in activating antioxidant enzymes, the increased SOD activity could be due to the activation of Nrf2 induced by SD.[34] Catalase was responsible for the detoxification of

significant amounts of H_2O_2. So, catalase level was increased in 72 hour REM sleep deprivation.

GPx is a major antioxidant enzyme in many tissues, especially in the brain, which metabolizes peroxides, such as H_2O_2 and protects cell membranes from lipid peroxidation.[35] GPx activity in the brain is more important than CAT for the destruction of H_2O_2 because this enzyme was more in mitochondria and cytosol. The GSH levels were significantly reduced in SD animals; this might be due to its multiple activities in scavenging the free radicals produced by SD, which leads to its greater consumption.[36] In the present study also the GSH level was significantly reduced in the 72 hour SD.

Vitamin C acts as a powerful scavenger of superoxide – induced lipid peroxidation. Endogenous Vitamin C and Vitamin E levels have been reported to decline under stress conditions.[37] Plants have both enzymatic and non-enzymatic systems to scavenge active oxygen species. Vitamin C and E are non enzymatic compounds can protect the human body from free radicals.[38]

Vitamin E is a powerful antioxidant that inhibits free radical reaction and prevents oxidative stress.[39] Further, Vitamin E administration to an animal model of diabetes normalizes the GSH level and activities of GPx, Catalase and SOD.[40]

In the present study, *Withania somnifera* treated sleep-deprived animals showed a marked decrease in SOD, GPx and Catalase activity when compared with sleep-deprived animals. The further LPO level was also significantly decreased in the *Withania somnifera* treated sleep-deprived animals. GSH, Vitamin C and Vitamin E level were significantly increased in the *Withania somnifera* treated sleep-deprived animals when compared with the sleep-deprived animals.

The roots of *Withania somnifera* contain the Withanolides (withanolide A and 12-deoxy withastramonolide) and withaferin A.[41] Also, it has male infertility activity,[42] the neuroprotective effect,[43] hypoglycemic activity,[44] and an antibacterial and antimitotic activity.[45]

CONCLUSION

The observed data confirmed the presence of oxidative damage in the discrete regions of the brain when exposed to 72 hours REM sleep deprivation. The significant variations in SOD, LPO, CAT, GPx, GSH, Vitamin C, Vitamin E and corticosterone were normalized by an Ethanolic extract of *Withania somnifera* in the rat. Further, there was no adverse effect found in the rat model of treatment with *Withania somnifera* alone. In future, it is necessary to conduct more extensive studies with *Withania somnifera* to explicate their molecular mechanism in antioxidant activity.

ACKNOWLEDGEMENT

The authors are grateful to the University of Madras for providing the infrastructure to conduct the research.

Authors contributions

All the authors have contributed equally.

Disclosure of Interests

The authors declare that they have no conflicts of interest concerning this research article.

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