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Research Article

SCREENING OF ECLIPTA ALBA EXTRACTS FOR ANTICANCER ACTIVITY

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ABSTRACT

Sleep deprivation can lead to various alterations at physiological and psychological levels such as EEG changes, metabolic changes, irritation, blurred vision, memory lapses, hallucinations, psychosis and can even lead to death. Treatment still remains a challenge as hypnotics are associated with side effects. The objective of the present study was to investigate the protective effects of melatonin and buspirone and their combinations against the biochemical and behavioral alterations induced by 48 hrs sleep deprivation in mice. Pretreatment with melatonin (2.5 mg/kg and 5 mg/kg), buspirone (5 mg/kg and 10 mg/kg), and melatonin (2.5, 5 mg/kg) in combination with buspirone (5 mg/kg) showed significant improvement in behavioral parameters such as increase in body weight, increase in locomotor activity, and reduction in anxiety like behavior. Biochemical parameters estimation also revealed similar results such as significant attenuation of lipid peroxidation and nitrite concentration and significant elevation of glutathione and catalase levels following treatment with melatonin (2.5, 5 mg/kg) and buspirone (5,10 mg/kg) and melatonin (2.5, 5 mg/kg) in combination with buspirone (5 mg/kg) as compared to their effect *per se*. Thus, preliminary findings suggest the protective effect of melatonin and buspirone and their combinations against sleep deprivation and associated alterations.

Keywords: Anxiety; Buspirone; Melatonin; Oxidative stress; Sleep deprivation.

INTRODUCTION

Sleep plays a pivotal role in normal biological functions[1]. There is a large body of evidence showing a strong correlation between sleep deprivation and anxiogenic behavior in humans and animal[2,3]. An extensive literature has documented the substantial behavioral effects in animal models of sleep deprivation. These effects include decrease in locomotor activity [3, 4], genital reflexes[5], stereotyped and aggressive behavior[6], anxiety-like behavior[2,7,8], impaired cognitive performance[9], and changes in body temperature[10]. These behavioral changes are attributed to changes in several neurotransmitter pathways, including dopaminergic and noradrenergic neurotransmission[6,11,12]. Further, sleep deprivation has been reported to induce free radical production and decreased anti-oxidative defense[13,14]. Melatonin (N-acetyl-5-methoxytryptamine) is a hormone secreted by pineal gland and is responsible for maintaining the biological clock[15]. Melatonin is synthesized and secreted during the dark phase in all species[16]. Several studies have confirmed the need of 7-8 hrs of sleep per night[17] and even a short period of sleep deprivation can result into abnormal endocrine responses[18]. Persistent sleep deprivation can lead to insomnia[19] and further complications.

As per earlier reports, various sedative and hypnotics have their own side effects (as daytime sleepiness and dependence) therefore treatment of sleep related problems still remains a problem[20]. Further melatonin being an endogenous hormone has also been known to improve the quality of the sleep and reduce the formation of free radicals and allows the recovery of antioxidant enzymes[21]. Buspirone is a standard anxiolytic, partial agonist with high affinity for 5-HT1A receptors[22]. Buspirone presents anxiolytic and rapidly penetrates the brain to interact with central 5-HT1A receptors and have higher affinity for serotonin (5-HT1A)[22]. With this back ground the present study was designed to evaluate the anti-anxiety potential of the melatonin in animal model of sleep deprivation.

MATERIALS AND METHODS

Animals

Male Laca mice bred in central animal house of Panjab University, Chandigarh and weighing between 25-30 g were used. The animals were kept under standard laboratory conditions, maintained on 12 hrs light/dark cycle and had free access to food and water. The animals were acclimatized to laboratory conditions before the test. Each animal was used once in the experiments and the experiments were conducted. All the experiments were conducted between 0900 and 1700 hrs. The experimental protocols were approved by Institutional Animals Ethics Committee and were conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals.

Sleep deprivation

Animals were sleep deprived for 48 hrs by placing the grid suspended over water method as described by Shinomiya et al.[23]. Animals were placed on a grid floor (29×15×7 cm) inside the plastic cage filled with water to 1 cm below the grid surface for 48 hrs. The stainless steel rods of the grid (3 mm wide) were set 2 cm apart from each other. Food and water were provided ad libitum.

Drug and treatment schedule

Following drugs were used in the study-Melatonin (2.5 mg/kg and 5 mg/kg, p.o., Dabur India Ltd, Sahibabad), Buspirone (5 mg/kg and 10 mg/kg, p.o., Ranbaxy Laboratories Ltd., Gurgaon). Buspirone was dissolved in double distilled water and melatonin was dissolved in a few drops of dimethylsulfoxide (DMSO) and then volume made-up with water and administered by per oral (p.o.) route in constant volume of 1 ml per 100 g of body weight. Animals were divided into ten groups (six animals in each group) as per the study design as depicted in the Fig. 1. Groups 1 and 2 were treated as naive and sleep deprived (Grid suspended over water). Groups 3 and 4 were treated with buspirone (5 and 10 mg/kg, p.o.) and groups 5 and 6 were treated with Melatonin (2.5 and 5 mg/kg, p.o.). Groups 7 and 8 were treated with Melatonin (2.5 and 5 mg/kg, p.o.) in combination with lower dose of buspirone (5 mg/kg, p.o.), while groups 9 and 10 were treated as melatonin (5 mg/kg, p.o.) and buspirone (10 mg/kg, p.o.) *per se*.

Behavioral assessmentsBody weight

The body weights of animals were recorded before the start of the experiment and thereafter prior to each behavioral quantification.

Measurement of anxiety levels

Elevated plus maze

It is a test for assessing anxiogenic and anxiolytic drugs effect in rodents[24]. The plus maze apparatus consist of two open (16×5 cm) and two closed arm (16×5×12 cm) and placed at a height of 25 cm for mice. The animals are placed individually at the centre of the elevated plus maze with their head facing toward an open arm. During the 5-min test, average time spent in the open arm of the maze was recorded.

Mirror chamber test

The mirror chamber consisted of a wooden chamber having a mirror chamber enclosed within it. During the 5 min test session, following parameters were noted: (a) latency to enter the mirror chamber, (b) average time spent in mirror chamber. Animal was placed individually at the outside of the distal corner of mirror chamber at the beginning of the test. An anxiogenic response was defined as decreased average time spent in the mirror chamber[24].

Measurement of ambulatory activity

The ambulatory activity was recorded by using actophotometer (IMCORP, Ambala, India). Before locomotor task, animal was placed individually in the activity meter for 3 min. The locomotor activity was recorded using actophotometer for a period of 5 min. Ambulatory activity

was recorded and expressed in terms of total photo beam counts for 5 min per animal[24].

Biochemical tests

Dissection and homogenization

On day 7, after behavioral quantification, the animals were sacrificed by decapitation immediately. The whole brains were removed and 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenate were centrifuged for 20 min at 15000 rpm and supernatant was used for estimation of lipid peroxidation and reduced glutathione levels. The post-nuclear fractions for catalase assay were obtained by centrifugation of the homogenate at 1000 g for 20 min, at 4°C and for other enzyme assays centrifuged at 12,000 g for 60 min at 4°C.

Lipid peroxidation assay

The quantitative measurement of lipid peroxidation was performed[25]. The amount of malondialdehyde (MDA), a measure of lipid peroxidation was measured by reaction with thiobarbituric acid (532 nm) using Shimadzu Spectrophotometer. The values were calculated using molar extinction coefficient of choursomophore ($1.56\times105M^{-1}$ cm^{-1}) and expressed as percentage of control.

Estimation of reduced glutathione

Reduced glutathione in brain was estimated according to the method described by Ellman[26]. Supernatant (1 ml) was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4°C for 1 h. The sample was centrifuged at 1200 g for 15 min at 4°C. To 1 ml of this supernatant, 2.7 ml of phosphate buffer (0.1 M, pH 8) and 0.2 ml of 5,5-dithiobis (2-nitrobenzoic acid) (DTNB) were added. The yellow color developed was read immediately at 412 nm using Shimadzu spectrophotometer. Results were calculated using molar extinction coefficient of choursomophore (1.36×104 M^{-1} cm^{-1}) and expressed as percentage of control.

Estimation of catalase

Catalase activity was assayed by method of Luck[27], wherein the breakdown of H2O2 was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 phosphate buffer (1.25×10^{-2} M H_2O_2) and 0.05 ml of supernatant of the brain homogenate (10%), and the change in absorbance was recorded at 240 nm using the Shimadzu Spectrophotometer. Enzyme activity was calculated using the millimolar extinction coefficient of H_2O_2 (0.07). The result was expressed as micromoles of H_2O_2 decomposed/min/mg protein.

Estimation of nitrite

The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent (0.1% N-(1-napthyl) ethylenediamine dihyrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as per Green et al.[28]. Equal volumes of supernatant and Greiss reagent were mixed. The mixture was incubated for 10 min at room temperature. The absorbance was measured at 540 nm using Shimadzu spectrophotometer. The concentration of nitrite in the supernatant was determined from a standard curve and expressed as percentage of control.

Protein estimation

Protein estimation was done by biuret method using bovine serum albumin as standard[29].

Statistical analysis

All the values were expressed as MEAN±SEM. The data was analyzed using analysis of variance (ANOVA) followed by Tukey's's test. In all the tests the criterion for statistical significance was $P<0.05$.

RESULTS

Effect of melatonin on the body weight and locomotor activity of sleep-disturbed mice

48 hour of sleep deprivation significantly reduced the body weight and locomotor activity as compared to naive mice (Table 1). Pre-treatment with melatonin (2.5 mg/kg and 5 mg/kg) and buspirone (5 mg/kg and 10 mg/kg) for five days significantly attenuated decrease in body weight and improved the locomotor activity. Co-administration of sub effective doses of melatonin (2.5 mg/kg and 5 mg/kg) with buspirone (5 mg/kg) further significantly attenuated the decrease in body weight and improved the locomotor activity as compared to sleep deprived mice, which is also significant as compared to their effect alone. Additionally, the effect of combination is comparable to the effect of higher dose of buspirone (10 mg/kg) alone.

Effect of melatonin on anxiety levels in sleep-disturbed mice on elevated plus maze and in mirror chamber

Sleep deprivation for 48 hrs significantly increased the anxiety levels in mice both in mirror chamber test (increase in

Table 1. Effect of Melatonin on the body weight and locomotor activity of sleep-deprived mice

Group (mg/kg)	Locomotor counts/5 min	% change in body weight
Naïve	190.33±6.11	4.16
SD	75.90±5.15[a]	-25.00[a]
BUS(5)+SD	126.00±4.34[b]	-18.18[b]
BUS(10)+SD	153.80±5.18[b,c]	-10.41[b,c]
MEL(2.5)+SD	93.00±5.50	-20.45[b]
MEL(5)+SD	119.60±5.24[b,d]	-12.50[b,d]
MEL(2.5)+BUS(5)+SD	152.40±3.35[b,c,d]	-9.09[b,c,d]
MEL(5)+BUS(5)+SD	181.80±3.69[b,c,d,e,f]	-4.54[b,c,d,e,f]
BUS(10)PER SE	197.60±5.73 [NS]	4.34[NS]
MEL(5)PER SE	187.20±3.90 [NS]	4.00 [NS]

Values are expressed Mean + SEM (% age of Naive). [a]P<0.05 as compared to Naive.

[b]P<0.05 as compared to SD. [c]P<0.05 as compared to Bus (5). [d]P<0.05 as compared to Mel (2.5), [e]P<0.05 as compared to Mel (2.5)+ Bus (5), [f]P<0.05 as compared to Mel (5). NS Not significant (ANOVA followed by Tukey's test).

first transfer latency and decrease in number of entries) (Table 2) and elevated plus maze test (increase in number of entries and duration of time spent in closed arm and decreased number of entries as well as duration of time spent in closed arm) (Table 3). Pretreatment with melatonin (5 mg/kg) and buspirone (5 mg/kg and 10 mg/kg) for five days showed significant improvement and dose dependent effect in reducing anxiety levels by decreasing the first transfer latency and increasing the number of entries in mirrored chamber test and decrease in number of entries and duration of time spent in closed arm and increased number of entries and duration of time spent in open arm in elevated plus maze test. Co-administration of melatonin (2.5 mg/kg and 5 mg/kg) with buspirone (5 mg/kg) further significantly improved the reduction in anxiety levels and the effect was comparable to higher dose of buspirone (10 mg/kg) alone.

Effect of melatonin and buspirone on antioxidant parameters of sleep-disturbed mice

Lipid peroxidation activity and nitrite concentration: Sleep deprivation for 48 hrs significantly elevated the lipid peroxidation (LPO) (Fig. 2) and nitrite concentration (Fig. 3) as compared to naive (Not sleep deprived) group of mice. Pretreatment with melatonin (5 mg/kg) and buspirone (5 mg/kg and 10 mg/kg) significantly attenuated the increased levels of these parameters as compared to sleep deprived mice. Co-administration of melatonin (2.5 mg/kg and 5 mg/kg) with buspirone (5 mg/kg) further significantly reversed the increased levels of LPO and nitrite. glutathione and catalase activity: Sleep deprivation for 48 hrs significantly reduced glutathione (Fig. 4) and catalase levels (Fig. 5) as compared to naive (Not sleep deprived) group of mice. Pretreatment with melatonin (5 mg/kg) and buspirone (5 mg/kg and 10 mg/kg) reversed the depleted glutathione and catalase levels. Co-administration of melatonin (2.5

Table 2. Effect of Melatonin on anxiety levels in sleep-deprived mice in mirror chamber test

Treatment Group (mg/kg)	Latency to enter Mirror Chamber (Sec)	Time spent in Mirror Chamber (Sec)
NAIVE	67.66±6.33	66.33±3.33
CONTROL (SD)	116.00±6.65[a]	22.48±4.09[a]
BUS(5)+SD	88.00±5.74[b]	40.10±3.35[b]
BUS(10)+SD	73.50±5.07[b,c]	60.70±3.63[b,c]
MEL(2.5)+SD	102.40±2.06[NS]	35.80±2.69[b]
MEL(5)+SD	90.80±5.23[b,e]	48.80±4.46[b,e]
MEL(2.5)+BUS(5)+SD	71.40±2.99[b,c,e]	64.20±3.89[b,c,e]
MEL(5) +BUS(5) +SD	68.80±3.73[b,c,d,e,f]	62.80±3.55[b,c,d,e,f]
BUS(10) PER SE	68.83±3.96[NS]	66.33±3.68 [NS]
MEL(5) PER SE	67.00±3.78 [NS]	67.20±3.96 [NS]

Values are expressed Mean + SEM (% age of Naive). [a]P<0.05 as compared to Naive.

[b]P<0.05 as compared to SD. [c]P<0.05 as compared to Bus (5). [d]P<0.05 as compared to Mel (2.5), [e]P<0.05 as compared to Mel (2.5)+ Bus (5), [f]P<0.05 as compared to Mel (5). NS Not significant (ANOVA followed by Tukey's test).

mg/kg and 5 mg/kg) with buspirone (5 mg/kg) further potentiated the glutathione and catalase activity.

DISCUSSION

Sleep is important for maintenance of proper physiological and psychological functions of the body. Marked behavioral changes can be the consequence of pathological changes in the regions of brain involved in sleep regulation[19]. Sleep deprivation is considered as a risk for several pathologies such as insomnia and obstructive sleep apnoea[30]. Several lines of experimental evidence suggest that sleep particularly paradoxical sleep plays a role in learning/memory processes[31] and plays a vital role in increasing the antioxidant activity of the brain[32]. Several lines of earlier studies from animals and humans suggest that sleep deprivation adversely affects functions of the central nervous system; it particularly impairs the ability to retain new information and altered anxiety levels. Present study revealed that 48 hrs sleep deprivation interfered with anxiety levels in the elevated plus maze and mirror chamber test. In agreement with our results, the majority of reports demonstrated anxiogenic effect of sleep deprivation thorough using a variety of anxiety models. Further, findings of the present study demonstrated the anti-anxiety potential of the melatonin. Also, co-administration of melatonin with buspirone was found to potentiate the anti-anxiety effect of the buspirone, providing an indication towards the modulatory effect of melatonin on serotonergic neurotransmission. As per the literature melatonin is synthesized from tryptophan within the pinealocytes. Most synthetic activity occurs during the dark phase, with a major increase (7-150 fold) in the activity of serotonin-N-acetyltransferase (arylalkylamine N-acetyl transferase, AA-NAT). Serotonin is the intermediate product in the melatonin synthesis and in the presence of serotonin-N-transferase gets converted into N-acetyl serotonin which further with the help of hydroxyl indole o-methyl transferase gets converted into melatonin. These findings further provide an indication toward the involvement serotonergic neurotransmission in the anxiolytic effect of melatonin. Furthermore, the other possible explanation behind this phenomenon may be the de novo synthesis of the melatonin, where serotonin is an intermediate product as show in the Fig. 6. Some reports show that sleep

Table 3. Effect of Melatonin on anxiety levels in sleep-deprived mice on elevated plus maze

Group (mg/kg)	Entries in open arm of EPM	Entries in closed arm of EPM	Time spent in open arm of EPM (Sec)	Time spent in closed arm of EPM (Sec)
CONTROL (SD)	0.50±0.22[a]	6.10±0.52[a]	10.00±2.92[a]	290.00±2.92[a]
BUS(5)+SD	2.28±0.42[b]	4.00±0.48[b]	20.57±2.63[b]	279.40±2.63[b]
BUS(10)+SD	4.00±0.30[b,c]	3.85±0.50[b]	32.00±3.30[b,c]	268.00±3.30[b,c]
MEL(2.5)+SD	2.50±0.31[b,d]	5.21±0.37[b]	18.40±1.50[b]	281.60±1.50[b]
MEL(5)+SD	4.80±0.37[b,e]	4.60±0.50[b]	23.40±3.67[b,e]	276.60±3.67[b,e]
MEL(2.5)+BUS(5)+SD	4.20±0.42[b,c,e]	2.40±0.50[b,c,e]	36.60±2.50[b,c,e]	263.40±2.50[b,c,e]
MEL(5)+ BUS(5)+SD	4.80±0.37[b,c,d,e,f]	34.90±2.61 [b,c,d,e,f]	1.60±0.24 [b,c,d,e,f]	266.40±2.61 [b,c,d,e,f]
BUS(10)PER SE	4.70±0.58[NS]	36.80±4.39 [NS]	3.60±0.24 [NS]	263.20±4.39 [NS]
MEL(5) PER SE	4.60±0.67 [NS]	35.00±3.36 [NS]	3.20±0.37 [NS]	265.00±3.36 [NS]

Values are expressed Mean + SEM (% age of Naive). [a]P<0.05 as compared to Naive.

[b]P<0.05 as compared to SD. [c]P<0.05 as compared to Bus (5). [d]P<0.05 as compared to Mel (2.5), [e]P<0.05 as compared to Mel (2.5)+ Bus (5), [f]P<0.05 as compared to Mel (5). NS Not significant (ANOVA followed by Tukey's test).

deprivation had no effects on anxiety levels which may be attributed to differences in the type of sleep deprivation and the experimental protocols. The harmful effect of sleep loss on anxiety has been demonstrated regardless of the model of sleep deprivation[33,34,3]. In our study, rats were sleep deprived by using the grid suspended over water method[23], which depends on the loss of muscle tone during REM sleep. This model produces a marked sleep (90% to 95%) deprivation, which mimics the electrophysiological changes similar to the subjects. The grid suspended over water method has obvious advantages over other models of sleep deprivation[23]. This paradigm allows rats from the same cage to be sleep deprived together as a group to maintain established social hierarchy and remove possible isolation stress associated with the single and multiple flowerpot techniques. In this study, sleep deprivation for 48 hrs by placing animals on grid suspended over water[23] resulted in several behavioral changes such as reduced motor activity, increase in anxiety levels, and decrease in body weight inspite of increase in food intake. Further, 48 hrs of sleep deprivation lead to a significant imbalance between the free radical generation (increase in lipid peroxidation and nitrite) and antioxidant enzymes (glutathione and catalase). In another set of findings melatonin treatment significantly attenuated the oxidative burden, and there was potentiation of the antioxidant effect when administered in combination with buspirone. These findings are in line with earlier reports demonstrating the free radicals scavenging action of the sleep and melatonin. Therefore, sleep deprivation will result in oxidative stress[14]. Changes in biochemical parameters were also observed such as increased lipid peroxidation and nitrite levels whereas, glutathione and catalase activity were found to be reduced. Present study suggest that pretreatment with Melatonin and Buspirone provides a protective effect such as decrease in anxiety like behavior, increase in motor activity and improved levels of antioxidant parameters in the brain which were altered with 48 hrs sleep deprivation. Melatonin administration most probably contributed towards enhancing antioxidant parameters because of its already reported properties in activation of

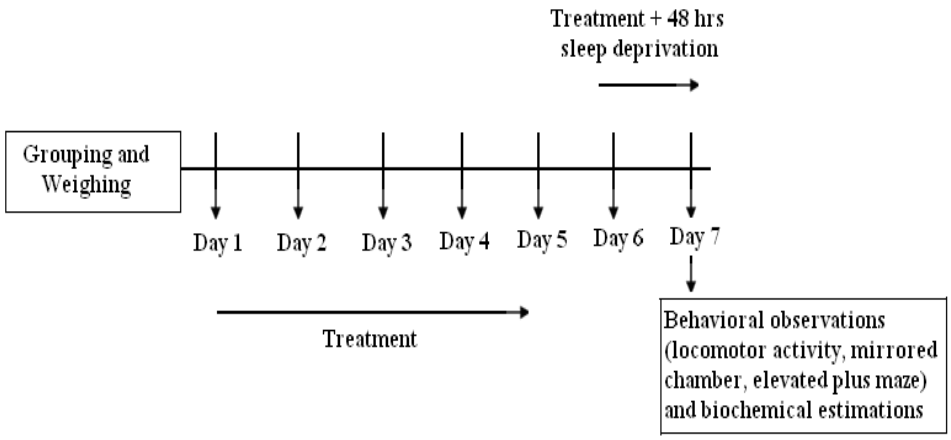

Fig. 1. Diagrammatic representation of study design

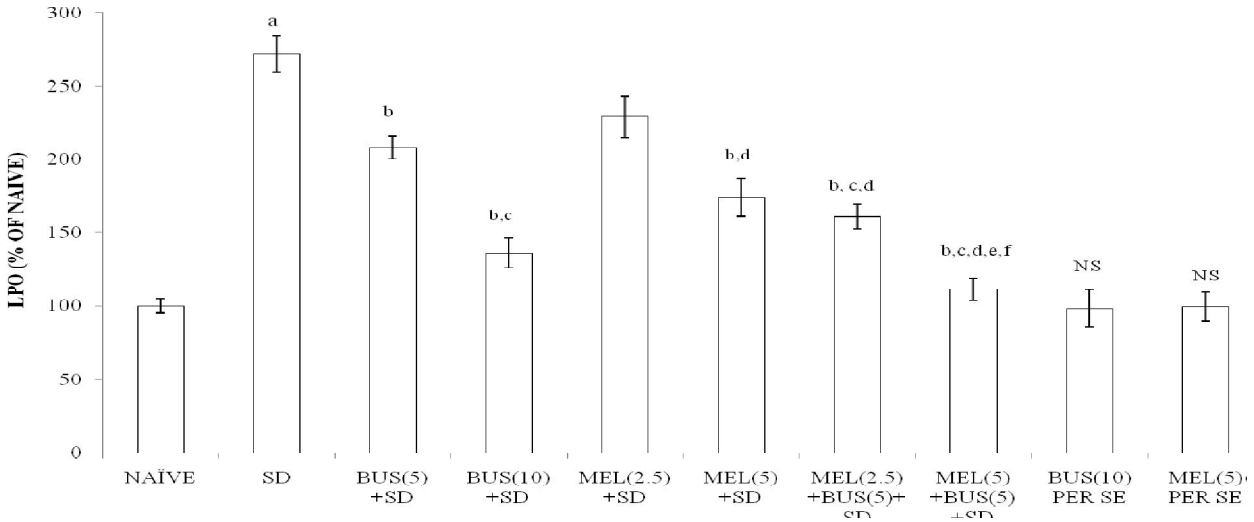

Fig. 2. Effect of melatonin on the lipid peroxidation levels

Values are expressed Mean + SEM (% age of Naive). [a]P<0.05 as compared to Naive.

[b]P<0.05 as compared to SD. [c]P<0.05 as compared to Bus (5). [d]P<0.05 as compared to Mel (2.5), [e]P<0.05 as compared to Mel (2.5)+ Bus (5), [f]P<0.05 as compared to Mel (5). NS Not significant (ANOVA followed by Tukey's test).

antioxidant enzymes[32]. Melatonin is also a well known hypnotic drug as well as an anti-anxiety agent and the possible mechanism of action is thorough binding to BZD receptor binding site in modulating its behavior as well as its effect on oxidative stress[35]. Melatonin is also an anabolic hormone and could be the reason for improvement of motor activity and weight gain. Whereas, the rationale of using buspirone in combination with melatonin was that sleep deprivation was prominently increasing the anxiety levels in addition to the decrease in antioxidant parameters. Treatment with hypnotics is associated with side effects like dependence and daytime sleepiness and these effects are the consequence of their action on benzodiazepine receptors[36]. Buspirone is having anxiolytic effects[37]. It is having higher affinity for serotonin (5-HT1A)[38] receptors rather than BZD's receptors thus virtually no effect on GABA binding. Therefore, decrease in anxiety like behavior without further complications can be attributed to buspirone and was found to exert a synergistic anxiolytic like effect when given in combination with melatonin.

CONCLUSION

Thus, findings of the present study provides an evidence that the combination of two drugs i.e. melatonin and buspirone is exerting a protective synergistic action against biochemical

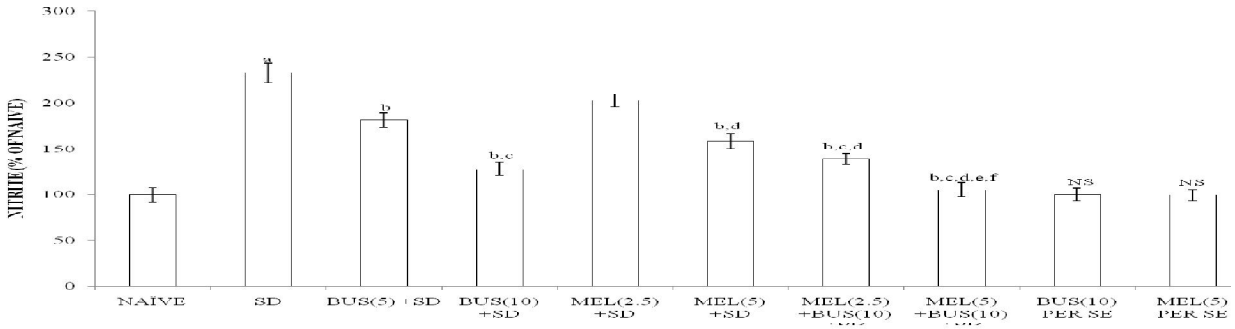

Fig. 3. Effect of melatonin on the nitrite concentration

Values are expressed Mean + SEM (% age of Naive). ᵃP<0.05 as compared to Naive.

ᵇP<0.05 as compared to SD. ᶜP<0.05 as compared to Bus (5). ᵈP<0.05 as compared to Mel (2.5), ᵉP<0.05 as compared to Mel (2.5)+ Bus (5), ᶠP<0.05 as compared to Mel (5). NS Not significant (ANOVA followed by Tukey's test).

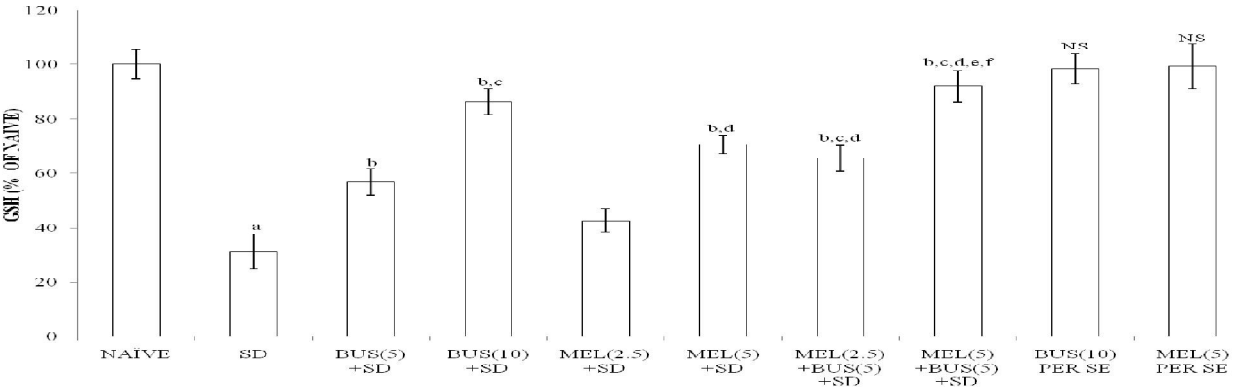

Fig. 4. Effect of melatonin on the reduced glutathione (GSH) levels

Values are expressed Mean + SEM (% age of Naive). ᵃP<0.05 as compared to Naive.

ᵇP<0.05 as compared to SD. ᶜP<0.05 as compared to Bus (5). ᵈP<0.05 as compared to Mel (2.5), ᵉP<0.05 as compared to Mel (2.5)+ Bus (5), ᶠP<0.05 as compared to Mel (5). NS Not significant (ANOVA followed by Tukey's test).

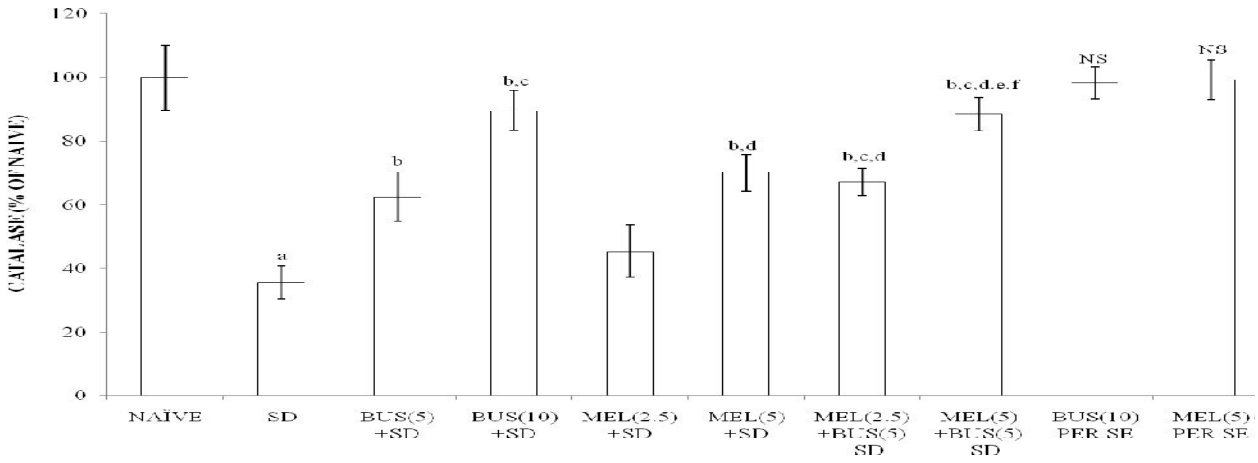

Fig. 5. Effect of melatonin on the catalase levels

Values are expressed Mean + SEM (% age of Naive). ᵃP<0.05 as compared to Naive.

ᵇP<0.05 as compared to SD. ᶜP<0.05 as compared to Bus (5). ᵈP<0.05 as compared to Mel (2.5), ᵉP<0.05 as compared to Mel (2.5)+ Bus (5), ᶠP<0.05 as compared to Mel (5). NS Not significant (ANOVA followed by Tukey's test).

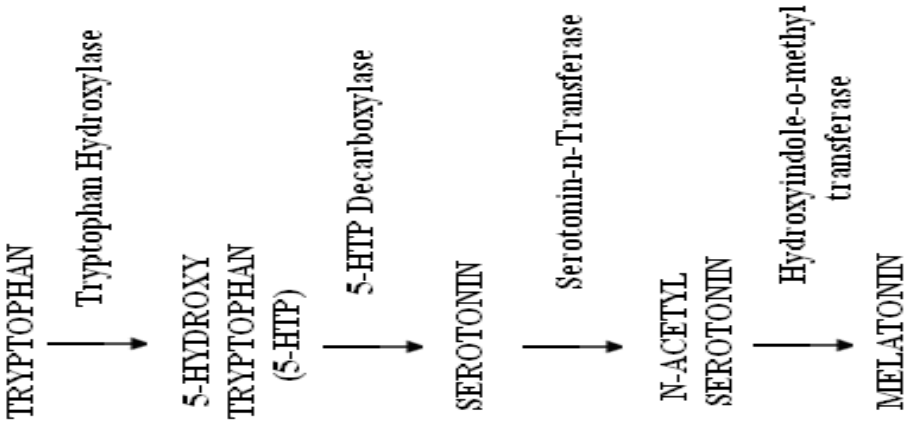

Fig. 6. De novo synthesis pathway of melatonin

and behavioral alterations induced by 48 hrs sleep deprivation in mice and can be considered as an effective therapy in management of sleep related disorders.

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