Original Article

Effect of N-Acetyl cysteine on some behavioral and oxidative Stress parameters in sleep deprived mice

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Abstract

Purpose: The present study was designed to investigate the effect of sleep deprivation on anxiety like behaviour, cognitive functions and oxidative stress in mice and whether co-administration of N-acetylcysteine (NAC) can modulate the effect of sleep deprivation on these parameters. **Methods:** Mice were sleep deprived for 72 hours using grid suspended over water method. NAC was administered orally in the dose of 50 mg/kg and 100 mg/kg, respectively for 5 days starting 2 days before sleep deprivation. Morris water maze and passive avoidance apparatus were used to test cognitive functions and elevated plus maze was used to assess the anxiety like behaviour. Oxidative stress was assessed by estimation of malondialdehyde (MDA) and reduced glutathione (GSH) levels in the brain. **Results:** A significant reduction in step down latency (SDL) in passive avoidance and prolongation of escape latency in Morris water maze test, and increase in anxiety like behaviour were observed in sleep deprived mice as compared to naïve mice. Treatment with NAC significantly antagonized the effect of sleep deprivation on SDL in passive avoidance test and escape latency in Morris water maze test. In addition, anxiolytic effect was also observed when compared with sleep deprived animals. NAC treatment also attenuated the sleep deprivation - induced increased MDA levels and decreased GSH levels in the brain. **Conclusion:** Results indicate that NAC has protective effect against sleep deprivation - induced cognitive dysfunctions, anxiety like behaviour and oxidative stress.

Keywords: Elevated plus maze, Morris water maze, oxidative stress, sleep deprivation, step down latency.

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INTRODUCTION

It is well established that adequate sleep plays an important role in the maintenance of general and mental health. Sleep is one of the vital physiological functions required for thermoregulation and maintenance of autonomic, neuroendocrine and immune functions (Rechtschaffen 1998; Graves *et al*. 2003). Sleep deprivation is one of the most common problems encountered in modern society as a consequence of change in lifestyle. The disturbance of normal sleep due to various reasons may represent a serious health risk factor leading to various disease processes (Kalonia and Kumar 2007;Kumar *et al*.2011). Studies indicate that sleep loss is associated with alterations in behavioral, hormonal and neurochemical processes in the body. It is also associated with irritability, fatigue, increase in anxiety levels, mood changes, depression and poor performance (Steiger, 2007; Singh A and Kumar A 2008; Kalonia *et al* 2008; Kumar *et al*.2011).

Sleep is thought to influence cognitive functions by facilitating neuronal and synaptic plasticity. Evidence indicates that sleep deprivation impairs hippocampus dependent cognitive functions and also impairs long term potentiation in the hippocampus (Singh *et al*. 2008; Hagewoud *et al*. 2010; Zhang *et al*. 2013). Sleep deprivation model is a frequently used model to demonstrate the role of sleep in learning and memory in both clinical and experimental studies. There is a large body of evidence showing memory deficits in sleep deprived experimental animals in various behavioral models including Morris water maze, avoidance tasks and radial maze tasks (Smith and Rose 1996; Smith *et al*.1998; Silva *et al*. 2004, Alhaider *et al*. 2010; Alzoubi *et al*.2013). The exact mechanism responsible for the occurrence of learning and memory impairment following sleep deprivation is not known. It has been proposed that sleep deprivation is associated with increased oxidative stress and decrease in antioxidant defenses. Studies have

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shown that sleep deprivation induced memory deficits possibly occurred due to increase in oxidative stress in the brain (Ramanathan *et al*. 2002; Everson *et al*. 2005; Singh *et al*.2008;Silva *et al*. 2004; Kalonia *et al*. 2008; Alzoubi *et al*.2012) and administration of compounds with antioxidative properties has been found to improve such sleep deprivation induced memory deficits (Zhang *et al*. 2013; Alzoubi *et al*.2012; Alzoubi *et al*.2013b).

N-acetylcysteine (NAC) is the N-acetyl derivative of amino acid l- cysteine and has been used in the treatment of paracetamol poisoning for many years. It supplies the cysteine required for glutathione (GSH) synthesis, thus enhances endogenous antioxidant levels by increasing the intracellular stores of glutathione (Jayalakshmi *et al*.2007). Studies indicate that administration of NAC combat oxidative stress induced damage in peripheral tissues and central nervous system due to its effective free radical scavenger and antioxidant property (Martinez *et al*.2000; Farr *et al*. 2003).Hence the present study was taken in order to explore the effect of NAC on anxiety like behaviour, cognitive functions and oxidative stress markers in sleep deprived mice.

MATERIAL AND METHODS

Animals

Swiss albino mice weighing between 25 to 30 g were used in the study. The animals were procured from the Central Animal House, University College of Medical Sciences, University of Delhi, Delhi. The animals were housed in standard laboratory conditions with pellet diet and water available *ad libitum*. The experimental protocol was approved by Institutional Animal Experimentation Ethics Committee and care of the animals was done as per "CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), India guidelines for laboratory animal facilities".

Drugs and treatment schedule

Animals were divided in to four groups having 6 animals for each experimental procedure. First group was treated as naïve (vehicle), i.e. not sleep deprived. The second group served as control: animals were sleep deprived for 72 hours. Groups three and four were administered NAC orally in the dose of 50 and 100 mg/kg, respectively for 5 days starting 2 days before sleep deprivation. NAC (SRL, Mumbai, purity of 99.0%) was dissolved in distilled water and administered in the volume of 10 ml/kg/dose. All other reagents used in the experiments were of the highest available grade.

Sleep Deprivation Procedure

Mice were sleep deprived using the grid suspended over water method (Shinomiya *et al*. 2003; Kalonia and Kumar 2007; Kumar *et al*. 2011). Sleep deprivation was induced by placing mice on a grid floor (29x15x7 cm) inside the plastic cage filled with water to 1 cm below the grid surface. The rods of the grid (3 mm wide) were fixed 2 cm apart from each other. Sleep-deprived animals were subjected to sleep deprivation for 72 h.

Assessment of anxiety

Elevated plus maze

Elevated plus maze is commonly used test to demonstrate anxiogenic and anxiolytic effect of drugs in animals. The maze contained a central platform (8 × 8 cm) from which four symmetrical arms radiated (16 × 5 × 10 cm). It was elevated to a height of 25 cm. The animals were placed individually in the center of the maze, facing toward the open arm. The number of entries in open and closed arms, and time spent in each arms were recorded for a period of 5 min (Pellow *et al.*, 1985).

Assessment of cognition

Step down latency in passive avoidance apparatus

Passive avoidance apparatus was used, consist of a grid floor on the center of which a wooden block was placed which served as shock free zone (SFZ). Mice were placed on SFZ and on stepping down from the SFZ were given an electric shock through the grid floor. Animals were given 3 trainings at an interval of 1h and the acquisition step-down latency (SDL) in sec was recorded after 1h of third training session without giving shock. The retention test was performed 24 h after training (on day 2), when each mouse was again placed on the platform and the step-down latency was measured. A cut-off time of 600 s was taken and for the animal which did not step down during this period, SDL was taken as 600 s (Joshi *et al*. 2007).

Morris water maze test

Water maze consisted of a large circular pool divided into four imaginary quadrants. A submerged platform (10cm × 10cm) was placed 2 cm below the surface of water in the center of one of the quadrants. Animals received a training session consisting of 4 trials per day for 4 days. In all 4 trials, the starting position was different. Mice were allowed to locate the platform for maximum of 120s during acquisition period with an inter-trial interval of 10 minutes. The time taken to find the hidden platform (escape latency) was recorded during trial. If the mouse failed to find the platform within 120 s, it was guided gently onto platform and allowed to remain there for 20 s. On 5th day of the test, retention trial was conducted in which the platform was removed and the mice were given 120s to search the previous location of the platform. The time taken to find the hidden platform during retention trial was recorded by using Any-Maze software (Stoelting, USA) (Morris 1984).

Biochemical tests

After behavioral assessment, animals were sacrificed and brains were quickly dissected out for biochemical

estimation, washed with ice-cold 0.9% saline, weighed and stored at -80°C until processing.

Tissue preparation

The isolated whole brain tissue was homogenized with 10 times (w/v) sodium phosphate buffer. The homogenate was centrifuged at 3000 rpm for 15 min, and the supernatant was used for estimation of MDA and GSH.

Malondialdehyde (MDA) estimation

Estimation of brain lipid peroxidation was done by measuring MDA levels, as described by Ohkawa *et al*. Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). The reagents acetic acid 1.5 ml (20%) pH 3.5, 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulfate (8.1%) were added to 0.1ml of processed tissue sample. The mixture was then heated at 100 ºC for 60 mins. The mixture was cooled with tap water and 5 ml of n-butanol: pyridine (15:1% v/v), and 1 ml of distilled water were added. The mixture was shaken vigorously. After centrifugation at 4000 rpm for 10 mins, the organic layer was withdrawn and absorbance was measured at 532 nm using spectrophotometer.

Reduced glutathione (GSH) estimation

Reduced glutathione was estimated by the method as described by Ellman. Briefly, brain tissues were homogenized with 10 times (w/v) 0.1 sodium phosphate buffer (pH 7.4). This homogenate was then centrifuged with 5% trichloroacetic acid to centrifuge out the proteins. To 0.1 ml of this homogenate, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and 0.4 ml of double distilled water was added. The mixture was vortexed and the absorbance read at 412 nm within 15 mins.

Statistical analysis

Data were expressed as the mean ± standard error of mean (SEM). The data were analyzed by one-way analysis of variance followed by Tukeys's post hoc test. P-value <0.05 was considered to be statistically significant.

RESULTS

Table 1

Tt (mg/kg)	Closed arm		Open arm	
	NOE	Duration	NOE	Duration
Naïve	5.34± 0.49	259.6±7.35	5.2±0.30 [0.001]	32.2±2.28
Control	8.83 ± 0.40 [0.001]	280.8±4.37 [0.05]	2.5 ± 0.42	15.8 ±1.22 [0.001]
NAC50	7.34 ±0.62	274.4±3.44	4.16± 0.47[0.05]	25.4± 0.76[0.001]
NAC100	5.67 ± 0.49[0.01]	260.2± 4.02[0.05]	4.83± 0.30 [0.01]	26.7± 1.14[0.001]

Table 2

Tt (mg/kg)	GSH	MDA
Naïve	156.5± 4.34[0.001]	183.4 ± 3.57[0.001]
Control	128.6±2.45	278.8±7.07
NAC50	135.2±3.52	256.8± 2.54[0.05]
NAC100	144.2± 2.78[0.05]	241± 5.2[0.001]

Table 3

Tt (mg/kg)	Acquisition	retention
Naïve	214.8±8.38	245.8±8.8
Control	166±6.7 [0.01]	176.2±9.2 [0.001]
NAC50	168.2±6.5	196.8±9.6
NAC100	192.5±8.76	234.4±9.72 [0.01]

Table 4

Tt (mg/kg)	acquisition Escape latency (4th day)	Retention escape latency (5th day)
Naïve	17.8±2.0	13.6±1.2
Control	46±1.84 [0.001]	44.2±1.49 [0.001]
NAC50	37.5.2±1.86 [0.05]	37.4±1.8 [0.05]
NAC100	33.6±2.23 [0.01]	29.2±0.87 [0.001]

Effect of NAC administration on anxiety behaviour in sleep deprived mice

Sleep deprivation in mice for 72 hrs caused significant increased the number of entries and time spent in closed arms as compared to naïve mice. Treatment with NAC (50 mg/kg) showed increase in time spent in open arms however this difference was not significant when compared with sleep deprived group. NAC at higher dose (100 mg/kg) revealed a significant increase in the time spent in the open arms compared with the sleep deprived group.

Effect of NAC administration on Step down latency in passive avoidance apparatus in sleep deprived mice

Sleep deprivation for 72 hrs in mice significantly decreased the acquisition latency as well as retention latency compared to naïve mice, indicating impairment of learning and memory. Co-treatment with NAC (100 mg/kg) significantly reversed the sleep deprivation induced retention deficit as a significant difference in SDL was found in NAC (100 mg/kg) treated mice as compared to control mice.

Effect of NAC treatment on escape latency in Morris water maze in sleep deprived mice

Sleep deprived mice showed significant increase in acquisition and retention escape latency to find platform on day 4 and day 5, respectively as compared to naïve mice. Co-treatment with NAC (50 mg/kg and 100 mg/kg) significantly attenuated sleep deprived induced impairment of memory.

Effect of NAC treatment on MDA and GSH levels in sleep deprived mice

Sleep deprivation for 72 hrs in mice caused significant increase in MDA levels and depletion of GSH levels compared to naïve (vehicle) mice. In NAC treated sleep deprived mice, a significant increase in GSH levels was found when compared with control (sleep deprived) group. NAC pretreatment significantly attenuated elevated lipid peroxidation in sleep deprived mice as evident by decrease in MDA levels when compared with control mice.

DISCUSSION

The current study was performed to observe the effect of 72 hr sleep deprivation on anxiety and learning and memory behavior in mice using elevated plus maze, step down passive avoidance and the Morris water maze tests. The biochemical estimations of MDA and GSH levels in the brain were also performed to assess the role of altered oxidative stress on these behavioral parameters. NAC, a well known antioxidant was also administered to investigate the effect of NAC on sleep deprivation induced modulation of anxiety like behavior and cognitive functions.

Sleep has an essential role in human's life as it is required for maintenance of physiological homeostasis and psychological balance in body (Berger 1975; Kalonia *et al*. 2008). Now a days, sleep disorders are increasingly affecting a large part of general population resulting in deleterious effects on health. Sleep deprivation has been found to be associated with development of various neurological and neuropsychiatric disorders in humans. It is considered as a risk factor for aggravation of various health diseases like depression, increased anxiety, decreased motor activity, psychosis and impaired cognition (Kumar *et al* 2011;Singh and Kumar 2008)

Anxiety like behavior has been described as one of the important consequences of sleep deprivation. Many studies have demonstrated relationship between sleep deprivation and development of anxiety (Singh and Kumar 2008; Kumar *et al*. 2011; Pires *et al*.2012).A number of evidence indicates that sleep also plays a major role in modulation of memory consolidation and sleep loss is known to cause hindrance of various cognitive processes in humans. Also, experimental studies showed that sleep loss affects cognitive functions as deficits in cognitive functions particularly those associated with learning and memory has been observed in sleep deprived animals (Smith *et al*. 1998; Mograss *et al*.2009; Lim and Dinges 2010; Aleisa *et al*. 2011).

In the present study, sleep deprivation induced stress significantly caused anxiety like behavior as indicated by increase in the number of entries and percentage of time spent in closed arms as compared to naïve mice in elevated plus maze. Moreover, 72 hrs sleep deprivation period was associated with significant reduction in SDL in passive avoidance apparatus and increase in the time to reach platform in Morris water maze test.

The exact mechanism responsible for anxiety like behavior and cognitive dysfunction induced by SD is still unknown. However,, oxidative stress has been implicated in the pathogenesis of various diseases and may be a common pathogenic mechanism underlying many neurodegenerative disorders and psychiatric disorders . The brain being rich in polyunsaturated fatty acids, due to high oxygen consumption and modest antioxidant defenses is highly vulnerable to oxidative damage (Ng *et al*. 2008; Salim 2014; Siwek *et al*. 2013). It has been suggested that normal sleep is very essential to revert oxidative stress by removing the free radicals that were produced during the wake period (Silva *et al* 2004;Everson *et al*. 2005). In addition, sleep act as an antistressor by increasing the efficiency of antioxidant mechanisms in the CNS (Reimund 1994; Kalonia and Kumar 2007). Accumulating evidences have shown that there is strong association between sleep deprivation and oxidative stress . Indeed, reduced antioxidant defenses are found in sleep deprived rodents (Gopalakrishnan *et al*. 2004;Silva *et al*. 2007 ;Zhang *et al*.2013). Studies demonstrate that sleep deprivation caused a significant decrease in superoxide dismutase, glutathione and catalase activity in the brain (Ramanathan *et al*. 2002; Kalonia and Kumar 2007). In our study, sleep deprivation for 72 hrs resulted in marked oxidative stress as indicated by an increase in lipid peroxidation and decrease in reduced glutathione levels.

There is growing evidence that oxidative stress might be also a plausible pathogenic factor for anxiety disorders. Both animals and human studies have revealed a strong correlation between anxiety and oxidative imbalance (Krolow *et al*. 2014; Salim 2014). In fact, oxidative stress has long been linked with pathogenesis of various diseases in which cognitive processes are diminished such as normal aging, alzheimer's disease, ischemic injury, aluminum toxicity, parkinson's disease (Poeggeler *et al*. 1993; Čížová *et al*. 2004; Jain *et al*. 2011). It is well known that antioxidative stress mechanisms are important for cognitive functions and increased oxidative stress is associated with neuronal damage in the brain leading to impaired spatial learning and memory (Reimund 1994; Silva *et al*. 2004; Alzoubi *et al*.2013).Many studies proved that sleep deprivation caused production of reactive oxygen species and hence increased oxidative stress (Ramanathan *et al*. 2002; Zhang *et al*. 2013). It is believed that oxidative damage caused by sleep deprivation in different region of brain especially the hippocampus affects the long term potentiation and results in cognitive impairment. (Romcy-Pereira and Pavlides 2004; Singh *et al*. 2008; Zhang *et al*. 2013). In this respect, administration of antioxidant compounds has been shown to improve such impairments (Alzoubi *et al*.2012; Zhang *et al*. 2013; Alzoubi *et al*.2013b).

NAC is a well known antioxidant and acts by increasing the intracellular stores of glutathione. Its free radical scavenging action is considered due to presence of thiol group that interacts directly with reactive oxygen species. Many studies have been carried out to evaluate the effect of NAC on cognitive deficits induced by different conditions. Treatment with NAC has shown protective effect in aluminum, cadmium and bisphenol-A induced cognitive deficits and oxidative stress in rats (Prakash and Kumar 2009; Goncalves *et al*.2010; Jain *et al*. 2011). Furthermore, NAC supplementation ameliorated learning and memory deficits caused by hyperglycemia induced oxidative stress in diabetic animals (Kamboj *et al*.2008). In this study we showed that administration of NAC prevented memory impairment and anxiety like behavior in mice induced by sleep deprivation. In addition, NAC also normalized levels of oxidative markers that were altered during sleep deprivation. This indicates the beneficial effect of NAC in sleep deprivation associated oxidative stress and this beneficial effect may be due to antioxidative effect of NAC.

In conclusion, the present study suggests that sleep deprivation is associated with learning and memory impairment and anxiety like behavior, which could possibly be due to the generation of free radicals because of altered antioxidant defense system. NAC administration protects against sleep deprivation induced memory impairment and anxiety like behavior probably through antagonizing oxidative stress in brain.

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