



# $\alpha$ -Asarone in management of sleep deprivation induced memory deficits and anxiety in rat model

Arathi Radhakrishnan<sup>1</sup> · N. Jayakumari<sup>2</sup> · Velayudhan Mohan Kumar<sup>3,4</sup> · Kamalesh K. Gulia<sup>1</sup>

Received: 5 March 2018 / Accepted: 24 July 2018 / Published online: 30 July 2018  
© Japanese Society of Sleep Research 2018

## Abstract

Insomnia is associated with anxiety and memory deficits.  $\alpha$ -Asarone, an active principle of *Acorus* species, which has anxiolytic and memory-restoring properties, was recently identified as a potential hypnotic with the least side effects. The current study was conducted in rats to find out whether the hypnotic dose of  $\alpha$ -asarone can produce anxiolytic and memory restoring effects.  $\alpha$ -Asarone (10 mg/kg, i.p.) and its vehicle were administered to rats, deprived of sleep by gentle handling for 5 h for 5 days. Spatial memory was assessed in the radial arm maze. Anxiety was tested using elevated plus maze and open field test. In addition to using the vehicle of the drug as a control, midazolam (2 mg/kg, i.p.), a known anxiolytic, was also used as a positive control. Increased anxiety levels produced after sleep deprivation were reduced after  $\alpha$ -asarone administration. The anxiolytic effect was comparable to that produced by midazolam. Increase in the reference and working memory errors, resulting from sleep deprivation, was reduced after  $\alpha$ -asarone injection. It also produced an improvement in the percentage of correct choices. Decreased lipid peroxidation and increased activities of catalase and glutathione reductase facilitated the improvement in cognitive functioning.  $\alpha$ -Asarone, is not only a good therapeutic agent for insomnia and associated anxiety, it can also reduce insomnia-associated memory deficits. This is the first report on the anxiolytic and cognition enhancing property of  $\alpha$ -asarone in sleep-deprived rats.

**Keywords**  $\alpha$ -Asarone · Sleep deprivation · Anxiety · Memory · RAM · EPM

## Abbreviations

SD Sleep deprivation  
EPM Elevated plus maze  
OFT Open field test  
RAM Radial arm maze  
MDA Malondialdehyde

GSH-R Glutathione reductase  
CAT Catalase  
SOD Superoxide dismutase  
GSH-Px Glutathione peroxide

## Introduction

Insomnia or lack of sleep results in a wide range of behavioral and cognitive deficits [1, 2], out of which anxiety and memory problems are two major manifestations [3]. Patients with co-morbid anxiety and insomnia have a poor quality of life [4] and those with high anxiety have increased sleep disturbance [5]. Anxiety-like traits are also observed in animal models of sleep deprivation (SD) [6, 7]. In addition, the importance of sleep in learning and memory has been shown in several studies [8, 9]. Though it is extremely difficult to have an ideal animal model for insomnia, sleep deprivation (SD) procedure in rats can be considered as an acceptable procedure for studying experimentally the various aspects of insomnia [10, 11].

✉ Kamalesh K. Gulia  
kkguliak@hotmail.com

Velayudhan Mohan Kumar  
wfsrs2005@rediffmail.com

<sup>1</sup> Division of Sleep Research, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala 695012, India

<sup>2</sup> Department of Biochemistry, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala 695011, India

<sup>3</sup> Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala 695012, India

<sup>4</sup> Present Address: 8A, Heera Gate Apartments, DPI Junction, Jagathy, Trivandrum, Kerala 695014, India

Insomnia-associated anxiety and cognitive deficits is associated with increased oxidative stress in several regions of brain [6, 12, 13]. Sleep loss results in an increased metabolism in brain which in turn facilitates oxidative stress [14]. Removal of free radicals and restoration and conservation of energy are considered as important functions of sleep [14].

Widely used hypnotic drugs like benzodiazepines, non-benzodiazepines and their analogs [15] do reduce anxiety that is associated with insomnia [16]. However, chronic usage of these hypnotics brings about a dose-dependent impairment in the acquisition of new information and impairment of long-term episodic memory [17]. Increased risk of Alzheimer's disease and dementia in old age has been shown to be associated with chronic intake of these hypnotics [18]. In addition, hypnotic drugs produce several undesirable side effects [19] and are notable for producing amnesia and hallucinations, especially when used in large doses [20]. Consequently, efforts are on to find some sedative substances from the herbs that are commonly suggested for treatment of insomnia and associated problems in traditional medicines [21].

$\alpha$ -Asarone, an active principle extracted from the *Acorus* species, has hypnotic property at low dose (10 mg/kg), with no withdrawal effect in rats. At this dose, it improved the quality of sleep even under SD conditions [22].  $\alpha$ -Asarone has also been reported to have anxiolytic and cognition enhancing properties in various animal models [23–29]. In rodents,  $\alpha$ -asarone reversed the cognitive deficits induced by scopolamine [24], lipopolysaccharide [25] and amyloid- $\beta$  (25–35) [26] and reduced anxiety under normal conditions [27] and after corticosterone administration [28]. However, the effect of  $\alpha$ -asarone on changes in anxiety and cognition associated with SD is not well-studied. Therefore, the present study was undertaken to investigate the effect of  $\alpha$ -asarone on anxiety and spatial memory deficits in rats subjected to SD.

$\alpha$ -Asarone produces a reduction in the oxidative stress and an improvement in the antioxidant levels in the brain [24, 26, 29]. This can probably reverse anxiety and cognitive deficits produced by SD. Antioxidant levels in the cortex, subcortex (including the thalamus, hypothalamus, limbic structures and basal fore-brain) and brainstem (including the midbrain, pons and medulla) were studied to have a gross idea about the magnitude of change, and extend of areas involved in their actions [30]. Hypnotic effect of 10 mg/kg  $\alpha$ -asarone was observed after 5 h of SD for 5 consecutive days [22]. So the antioxidant status in brain was studied after 5 h of SD for 5 consecutive days.

## Materials and methods

### Animals

A total of 60 adult male Wistar rats (14–16 weeks; 250–350 g) housed in polystyrene cages, kept in controlled

temperature ( $26 \pm 1$  °C) and light–dark schedule of 12 h each (lights on at 6:00 h), with food and water provided ad libitum, were used in the study. All the procedures employed in this study were approved by the Institutional Animal Ethics Committee of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala.

### Study design

#### Elevated plus maze (EPM) and the open field maze test (OFT) for anxiety testing

After testing baseline anxiety levels, the rats ( $N=20$ ) were randomly distributed into three groups. First group received vehicle ( $N=7$ ), second ( $N=8$ ) received 10 mg/kg  $\alpha$ -asarone and the third group ( $N=5$ ) received 2 mg/kg midazolam (positive control) intra-peritoneally at 9:00 h for 5 days, before 5 h of SD. EPM test was conducted on days 1 and 4, and OFT was performed on days 2 and 5 from 13:30 to 14:00 h (Fig. 1a).

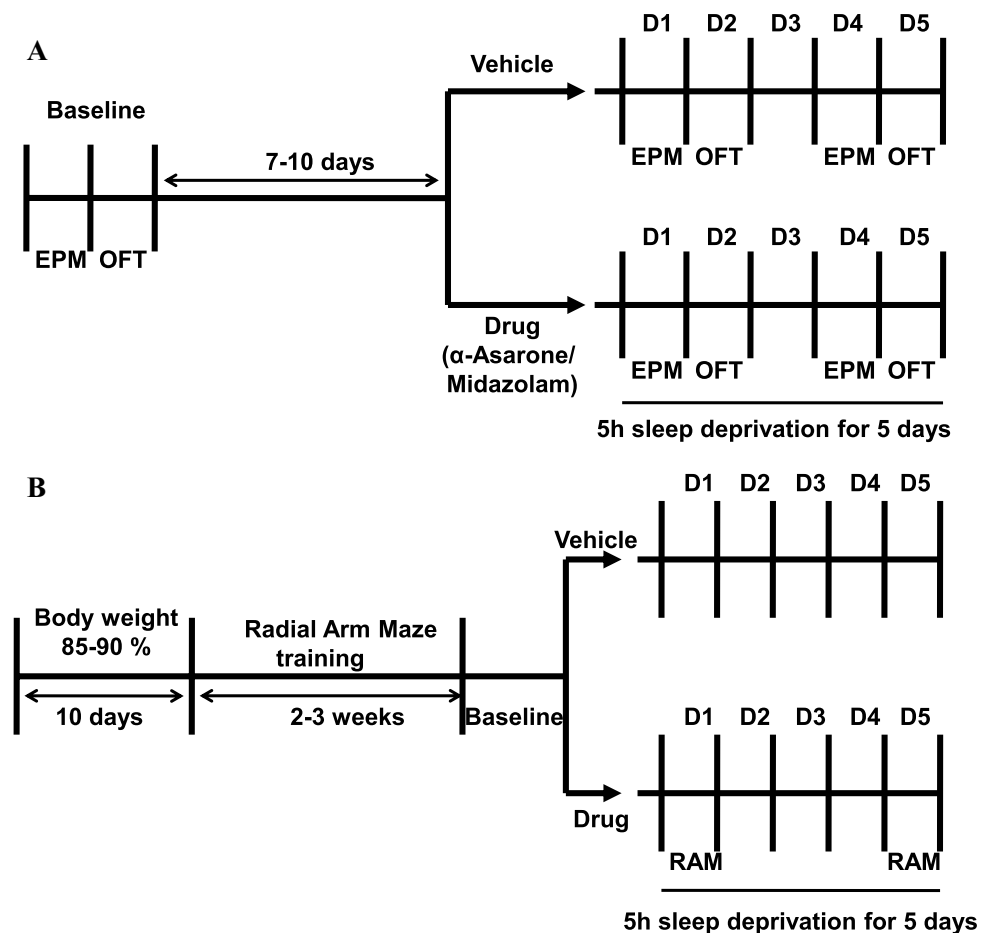
#### Radial arm maze (RAM) test for spatial memory

Spatial memory was tested using RAM in another set of rats ( $N=12$ ) that underwent training and food restriction. On completion of training, the baseline memory levels were checked and all the animals ( $N=10$ ) were randomly divided into two groups (Fig. 1b) The animals ( $N=2$ ) which were not complying with the test requirement during the training sessions were excluded. The first group received vehicle ( $N=5$ ) and the second group received 10 mg/kg  $\alpha$ -asarone ( $N=5$ ) intra-peritoneally at 9:00 h for 5 days before starting the SD procedure for 5 h. The RAM test was conducted from 13:30 to 14:00 h on days 1 and 5 of SD (Fig. 1b).

#### Antioxidant analysis

The animals were randomly distributed into five groups. Group I ( $N=7$ ) was taken as control without any treatment. Group II ( $N=6$ ) and III ( $N=7$ ) received vehicle and 10 mg/kg  $\alpha$ -asarone, respectively, at 9:00 h for 1 day before starting the SD procedure for 5 h. Group IV ( $N=5$ ) and V ( $N=5$ ) received vehicle and 10 mg/kg  $\alpha$ -asarone, respectively, at 9:00 h for 5 consecutive days before starting the SD procedure for 5 h. Animals in Group II and III were decapitated after 5 h of SD and animals in Group IV and V were decapitated after 5 days of SD using guillotine at 14:00 h. Different brain regions (Cortex, Subcortex and Brainstem) were dissected out in ice-cold saline and stored at  $-80$  °C for oxidative stress markers analysis.

**Fig. 1** Diagrammatic representation of the experimental schedule. **a** EPM and OFT were performed on different days, before sleep deprivation. After a gap of 7–10 days rats were sleep deprived for 5 h for 5 days (D1–D5). On days D1 and D4 EPM test and on days D2 and D5 OFT were performed. **b** Rats were prepared for RAM test by restricting their food intake so that their body weight was maintained at 85–90% of the original. They were then trained in the radial arm maze for 2–3 weeks. On completion of training, a baseline memory test was conducted. After that they were sleep deprived for 5 h for 5 days (D1–D5). On days D1 and D5 RAM tests were performed



## Drug administration

α-Asarone (*trans*-1,2,4-trimethoxy-5-(1-propenyl) benzene) and Tween80 (Polyoxyethylene (20) sorbitan monooleate) were obtained from Sigma-Aldrich Co. LLC. and midazolam was procured from the Neon Laboratories Ltd. α-Asarone was freshly dissolved in the base containing normal saline and 5% Tween80. The base was taken as the vehicle.

## Procedures and parameters measured

### Sleep deprivation

All the animals were sleep deprived for 5 h (9:00–14:00 h), by gentle handling for 5 days [22]. The rats were kept awake by introducing new objects into the cage, tapping the cage and mildly touching the animal with soft bristled brush. The method of gentle handling used for SD does not involve forced locomotion, and it gives minimum stress to the animals [31].

## Anxiety tests

The animals were tested for anxiety in EPM and OFT using ANY-maze video-tracking system (version 4.82) from Stoelting Co. (U.S.A.). The plexiglass EPM having two open arms (50×10 cm) and two closed arms (50×10×50 cm), arranged in plus shape, was kept at a height of 45 cm. The arms of same type faced each other and were connected through an open center zone (10×10 cm). Experiment was conducted for 5 min. The parameters such as entries to open arm and the closed arm, average speed, time spent in the open arm and the closed arm, total distance traveled, time spent in mobility and ethologically derived parameters like head dipping, rearing, grooming and stretch-attend posture were assessed [22].

The plexiglass OFT maze chamber of dimension 100×100×40 cm was divided into three concentric zones, namely, outer, middle and inner zone. Experiment was conducted for 5 min. Entries and time spent in the inner and outer zones was noted. Parameters including average speed, time spent in moving around, total distance traveled and ethologically derived parameters like rearing and grooming were also measured.

## Spatial memory test

RAM apparatus had eight black painted radial arms ( $45 \times 10 \times 10$  cm), placed at a height of 35 cm above the floor, in a room with fixed extra-maze visual cues. Food was placed at the end of each arm. The signal acquisition was done using ANY-Maze video-tracking system (version 4.82) from Stoelting Co. (USA) and scored manually.

Rats were subjected to food restriction (10 g/day/rat) to maintain their body weights at 85–90% of free-feeding values for the first 10 days. Food restriction continued throughout the training period with free access to water. Animals were trained to consume the food rewards (Kellogg's Chocos flakes, ~20 mg) from four arms randomly chosen as baited arms within a stipulated time of 10 min. The baited arms remained the same for a given rat, but varied between rats. RAM training took place between 10:00 and 16:00 h daily and was continued till all the animals learned to respond to the food rewards with minimal errors. The rats underwent test for their spatial memory skills on days 1 and 5 immediately after SD.

Entries into un-baited arms (reference memory errors), re-entries into baited arms (working memory errors) and percentage of correct choices were recorded on every trial [32].

## Biochemical estimations

Tissue homogenates (10% w/v) were prepared in ice-cold 0.1 M phosphate buffer (pH 7.4) in a motor homogenizer and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatants were used for the biochemical analysis. The level of lipid peroxidation was measured by quantifying malondialdehyde (MDA), a thiobarbituric acid (TBA) reacting substance (TBARS), according to the method of Buege and Aust [33]. CAT activity was assayed by measuring the decomposition of  $H_2O_2$  [34]. GSH-R activity was determined following the procedures of Smith et al. [35] by measuring the disappearance of NADPH. SOD activity was estimated by the method of Marklund and Marklund [36]. The degree of inhibition of autoxidation of pyrogallol at an alkaline pH by SOD was used as a measure of the enzyme activity. GSH-Px activity was determined according to the method of Rotruck et al. [37]. Protein content in the tissue was determined according to the method of Lowry et al. [38].

## Statistical analysis

Assuming a 5% significance level and a two-sided test, a sample size of seven per group was obtained with an *S/N* ratio of 1.6 and a power of 80%. In the RAM test, based

on their poor performance during training, four animals were excluded from the study. Shapiro–Wilk test was done to check the normality of the data. All values expressed as mean  $\pm$  SEM.  $p \leq 0.05$  were considered as statistically significant. All statistical analyses were done in SPSS (version 16.0). For EPM and OFT parameters, Kruskal–Wallis test was done to compare among the groups and Wilcoxon signed rank test was used for comparison with the baseline. For RAM test parameters, Mann–Whitney test was used to make comparison between the groups and Wilcoxon signed rank test was used for comparison with the baseline. Various parameters were analyzed using one-way ANOVA with Tukey's post hoc test.

## Results

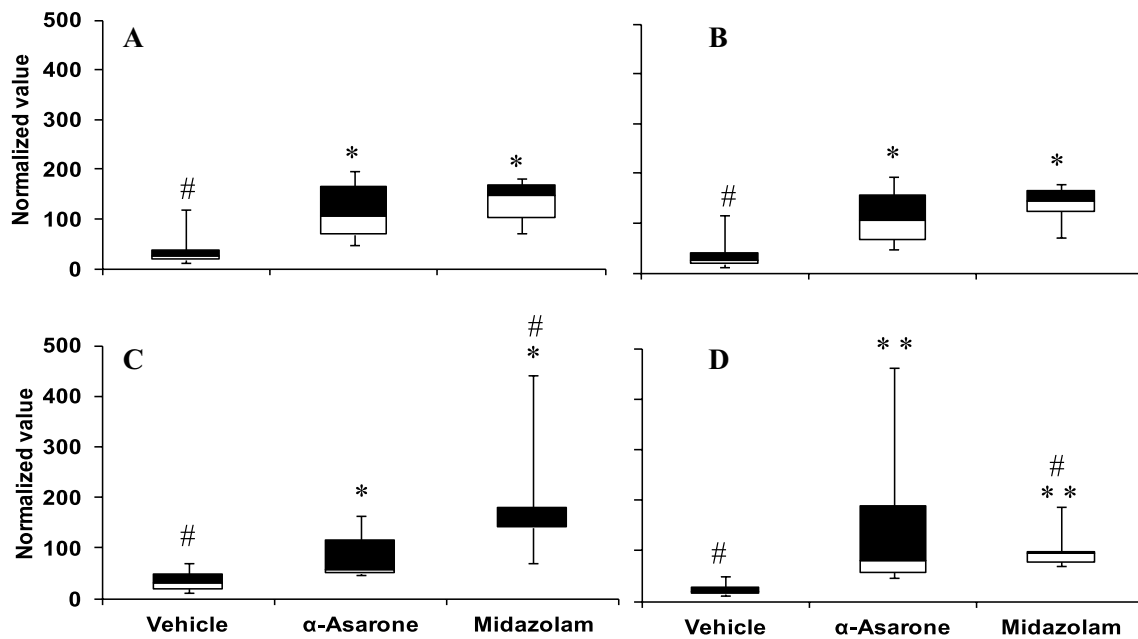
### Effects of administration of $\alpha$ -asarone on the anxiety levels in EPM test

The data from the vehicle-treated group showed that the time spent by the rats in the open arm was significantly reduced ( $p = 0.02$ ) after SD. The entries into the open arms were also markedly reduced ( $p = 0.01$ ) after 4 days of SD in those rats that received vehicle treatment. The total distance traveled and the average speed on the maze was also reduced ( $p = 0.03$ ) in these sleep-deprived rats (Fig. 2; Table 1).

On the other hand, the time spent in the open arm after SD was increased by day 4 ( $p = 0.008$ ) in the  $\alpha$ -asarone-treated rats (Fig. 2; Table 1). Entry in to the open arm was also improved ( $p = 0.04$ ) by day 4 (Fig. 2; Table 1). Furthermore, the distance traveled on EPM and the average speed of mobility was also improved ( $p = 0.01$ ) in  $\alpha$ -asarone group in comparison to the vehicle group (Fig. 2; Table 1). Though there was difference in the magnitude of change, effects of  $\alpha$ -asarone on most of the parameters were similar to that of midazolam in the sleep-deprived rats (Fig. 2; Table 1).

### Effect of $\alpha$ -asarone on the anxiety levels in OFT

After 5 days of SD, vehicle-treated rats made significantly decreased entries ( $p = 0.04$ ) and time spent ( $p = 0.03$ ) into the inner zone (Fig. 3; Table 2). Along with decreased time in the inner zone, the vehicle-treated rats had spent increased ( $p = 0.03$ ) time in the outer zone after 5 days of SD. But the  $\alpha$ -asarone-treated rats entered the open arm more frequently ( $p = 0.04$ ) even after 5 days of SD (Fig. 3). After 5 days of SD, there was reduction in mobility on maze in both vehicle- and  $\alpha$ -asarone-treated rats (Table 2). Even though not statistically significant,  $\alpha$ -asarone group spent more time in the inner zone than the midazolam group (Table 2).



**Fig. 2** Changes in EPM parameters after administration of vehicle,  $\alpha$ -asarone and midazolam in sleep-deprived rats. Changes in the parameters in EPM test show decreased levels of anxiety in those rats that received vehicle,  $\alpha$ -asarone and midazolam before SD. The box plot shows the **a** total distance traveled, **b** average speed, **c** the entries into the open arms and **d** the time spent in the open arms on day 4 of

SD as compared to the baseline values (taken as 100%) taken before SD. \*Indicates significant change from vehicle group and # indicates significance from the baseline. Levels of significance \*, #  $p \leq 0.05$  and \*\*, #  $p \leq 0.01$ .  $N=7$  for vehicle group,  $N=8$  for  $\alpha$ -asarone group and  $N=5$  for midazolam group

### Effects of administration of $\alpha$ -asarone on spatial memory in RAM test

As indicated in Fig. 4 and Table 3, the percentage of correct choices was significantly higher and working memory and reference memory errors were significantly lower in the  $\alpha$ -asarone-treated rats when compared to the vehicle-treated group. The correct choices made by vehicle group were reduced ( $p=0.02$ ) after 5 days of SD (Fig. 4a; Table 3). Number of entries into the un-baited arm (reference memory errors) was significantly higher ( $p=0.03$ ) in the vehicle group when compared to the  $\alpha$ -asarone group by day 5 of SD (Fig. 4b; Table 3). The number of re-entries into the baited arm, indicating working memory error, was also significantly higher in the vehicle-treated group, as compared to the drug-treated group, by day 5 of SD (Fig. 4b; Table 3).

### Effects of administration of $\alpha$ -asarone on the antioxidant levels in cortex, subcortex and brainstem

Table 4 summarizes the changes in antioxidant levels in vehicle- and  $\alpha$ -asarone-treated SD rats. The MDA levels in the brainstem and subcortex lowered after 5 days of SD (Table 4). CAT activity was also significantly decreased in the cortex and subcortex in vehicle group after 5 days of

SD in comparison to the control group (Table 4). Increase in the activities of SOD, GSH-Px and GSH-R was also observed after 5 days of SD (Table 4).

In comparison to the vehicle group,  $\alpha$ -asarone reduced ( $p=0.02$ ) the MDA levels in the subcortex and brainstem after 5 days of SD (Table 4). CAT activity in the cortex and subcortex increased ( $p=0.03$ ) on day 5 in SD rats when treated with  $\alpha$ -asarone (Table 4). Subcortical region also showed increased GSH-R activity ( $p=0.05$ ) in the  $\alpha$ -asarone-treated rats after 5 days of SD.

## Discussion

Increased anxiety levels and errors in reference and working memory in sleep-deprived rats were alleviated by  $\alpha$ -asarone administration. This was associated with decreased MDA level and increased activities of CAT and GSH-R.

### Changes in anxiety and cognition produced by SD

Increased anxiety after SD observed in the present study is supported by an earlier report and contradicted by few other reports [39–41]. Cohen et al. [39] reported that SD by gentle handling for 6 h induced anxiety in rats. On the contrary, the same method was found to reduce anxiety and

**Table 1** Values of all parameters of EPM after administration of  $\alpha$ -asarone, midazolam and vehicle in SD rats

Parameters	Vehicle			$\alpha$ -Asarone			Midazolam		
	Baseline (no drug)	SD day 1	SD day 4	Baseline (no drug)	SD day 1	SD day 4	Baseline (no drug)	SD day 1	SD day 4
Total distance traveled (m)	7.3 $\pm$ 1.7	5.0 $\pm$ 1.3	2.1 $\pm$ 1.1 <sup>#</sup>	9.0 $\pm$ 1.1	5.2 $\pm$ 0.7 <sup>#</sup>	7.1 $\pm$ 1.3*	7.2 $\pm$ 1.3	5.7 $\pm$ 0.8	6.7 $\pm$ 1.0*
Average speed (cm/s)	2.4 $\pm$ 0.6	1.3 $\pm$ 0.4	0.7 $\pm$ 0.4 <sup>#</sup>	3.1 $\pm$ 0.3	1.7 $\pm$ 0.2 <sup>#</sup>	2.2 $\pm$ 0.4*	1.8 $\pm$ 0.2	1.9 $\pm$ 0.3	0.02 $\pm$ 0.0*
Time mobile (s)	93.3 $\pm$ 20.1	82.1 $\pm$ 26.9	40.8 $\pm$ 17.5	113.1 $\pm$ 26.0	51.1 $\pm$ 7.4	52.0 $\pm$ 8.8 <sup>#</sup>	72.9 $\pm$ 18.2	53.5 $\pm$ 5.7	49.9 $\pm$ 8.5
Time spent in open arms (s)	38.0 $\pm$ 11.9	5.0 $\pm$ 2.1 <sup>#</sup>	5.1 $\pm$ 3.9 <sup>#</sup>	31.5 $\pm$ 12.7	15.2 $\pm$ 4.4 <sup>♦</sup>	23.9 $\pm$ 4.2**	22.6 $\pm$ 4.43	30.2 $\pm$ 3.9	29.8 $\pm$ 4.8** <sup>#</sup>
Time spent in closed arms (s)	165.7 $\pm$ 36.6	213.4 $\pm$ 35.4	144.1 $\pm$ 48.9	233.7 $\pm$ 16.2	250.1 $\pm$ 9.1	231.2 $\pm$ 21.3	247.4 $\pm$ 4.7	242.8 $\pm$ 4.3	248.9 $\pm$ 5.1
Time spent in center zone (s)	96.3 $\pm$ 34.3	81.6 $\pm$ 35.7	150.8 $\pm$ 50.6	34.8 $\pm$ 7.7	34.7 $\pm$ 5.9	45.0 $\pm$ 19.5	36.1 $\pm$ 7.2	33.0 $\pm$ 7.1	21.2 $\pm$ 3.3
No. of entries in open arms	7.0 $\pm$ 1.5	3.1 $\pm$ 1.3	1.4 $\pm$ 0.5 <sup>#</sup>	7.8 $\pm$ 1.9	4.3 $\pm$ 0.9	04.6 $\pm$ 1.0*	4.2 $\pm$ 1.6	5.8 $\pm$ 1.9 <sup>#</sup>	8.6 $\pm$ 1.9* <sup>#</sup>
No. of entries in closed arms	8.6 $\pm$ 1.8	12.0 $\pm$ 2.6	6.3 $\pm$ 1.8	12.6 $\pm$ 1.5	9.4 $\pm$ 1.6	9.1 $\pm$ 1.4 <sup>#</sup>	13.8 $\pm$ 3.6	15.2 $\pm$ 3.2	14.8 $\pm$ 3.4

The data are represented as mean  $\pm$  SEM

<sup>#</sup>Significance from the baseline

\*Significance between vehicle and  $\alpha$ -asarone group on days 1 and 4 of SD and  $N=7$  for vehicle group,  $N=8$  for  $\alpha$ -asarone group and  $N=5$  for midazolam group

<sup>♦</sup>Significance between  $\alpha$ -asarone and midazolam group on days 1 and 4 of SD. Levels of significance \*<sup>#</sup>, <sup>♦</sup> $p < 0.05$  and \*\* $p < 0.01$

increase risk-taking behavior in EPM test [40] and produced no anxiety in the OFT [41]. It is noted that this is the first report on the effect of repeated SD by gentle handling on anxiety. Repeated SD by other methods, however, has shown to increase anxiety [7, 13].

Cognitive decline, observed in the present study, as a result of SD is supported by earlier reports [42, 43]. Impairment in the spatial reference memory was observed in rats subjected to 6 h of SD by gentle handling [42]. Furthermore, mice subjected to 3 h of SD for 30 days by gentle handling, showed significantly impaired spatial learning and spatial memory (reference and working) retention [43].

### Anxiolytic and cognitive enhancing effect of $\alpha$ -asarone

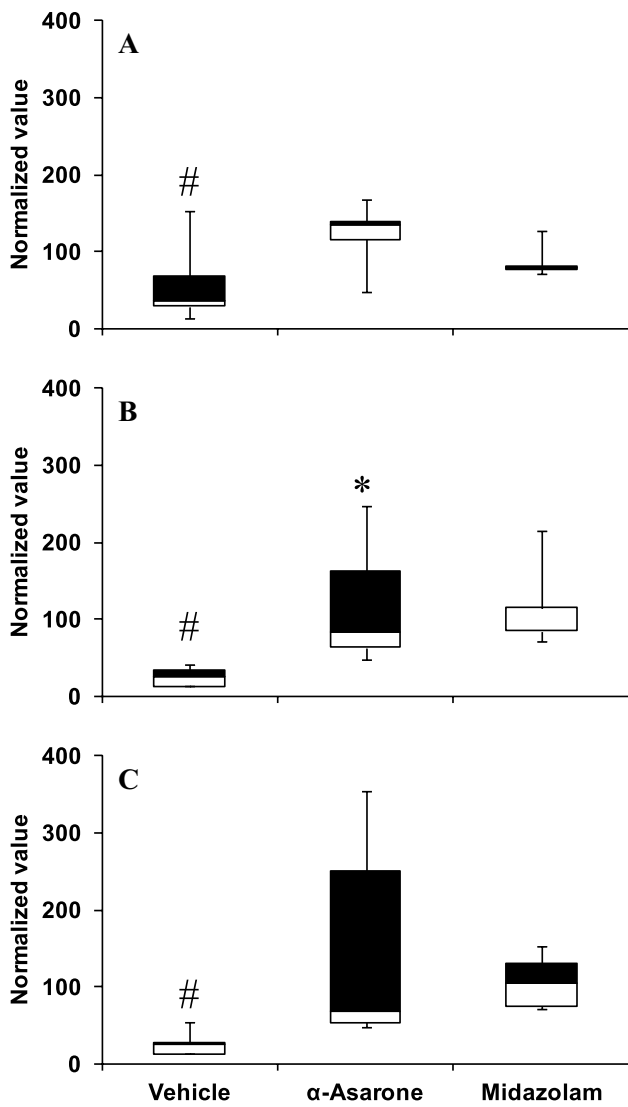
The animals treated with 10 mg/kg of  $\alpha$ -asarone were less anxious when compared to their vehicle counterpart after

being subjected to SD. Moreover, the anxiolysis of  $\alpha$ -asarone was on par with the commonly used anxiolytic midazolam [44, 45]. This indicates that  $\alpha$ -asarone at a dose of 10 mg/kg is not just an effective hypnotic [22], but also a potent anxiolytic to alleviate anxiety associated with SD.

In the present study,  $\alpha$ -asarone improved reference as well as working memory and enhanced the performance of rats on RAM. This signifies the efficacy of  $\alpha$ -asarone in alleviating the cognitive decline associated with SD and thus, may be considered as a better alternative for the management of insomnia since the commonly used hypnotics produces cognitive deficits [17, 18].

### Effects of administration of $\alpha$ -asarone on the antioxidant levels

In the present study, oxidative stress level in cortex, sub-cortex and brainstem was altered in the rats subjected to SD. Even though SD for 5 h produced moderate oxidative



**Fig. 3** Changes in OFT parameters after administration of vehicle,  $\alpha$ -asarone and midazolam in sleep-deprived rats. Changes in the parameters in OFT shows decreased levels of anxiety in those rats that received vehicle,  $\alpha$ -asarone and midazolam before SD. The box plot shows **a** the time spent in the outer zone, **b** entries in to inner zone and **c** the time spent in the inner zone on day 5 of SD. Effect of 5 days SD is compared to the baseline values (taken as 100%) before SD. \*Indicates significant change from vehicle group and #indicates significance from the baseline. Levels of significance \* $p \leq 0.05$ .  $N=7$  for vehicle group,  $N=8$  for  $\alpha$ -asarone group and  $N=5$  for midazolam group

stress, by day 5 of SD, the lipid peroxidation was considerably lowered along with an increase in the GSH-R and GSH-Px activities. This might be a cellular adaptive response to recoup from the acute stress associated with SD as previously reported [46, 47]. It is noted that the brain is capable of responding to the stress associated with acute sleep loss and thereby preventing oxidative stress [48].

**Table 2** Values of all parameters of OFT after administration of  $\alpha$ -Asarone, midazolam and vehicle in SD rats

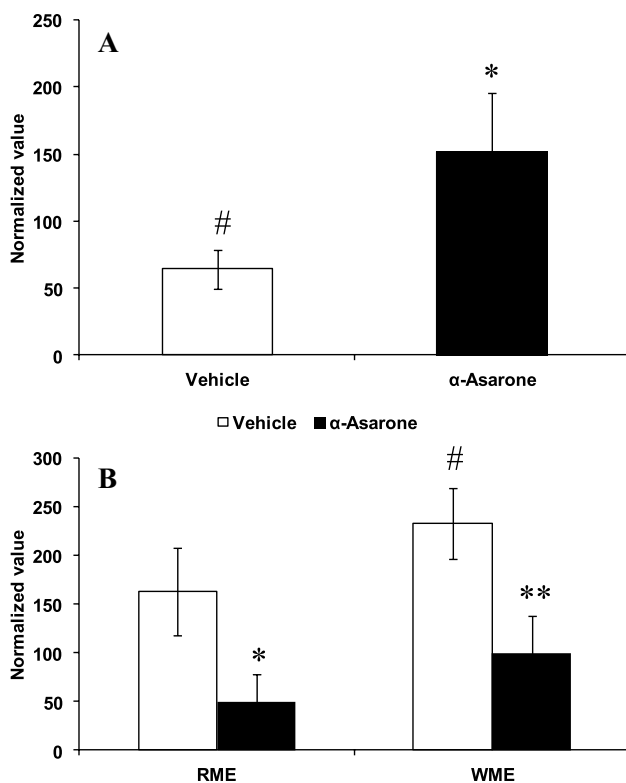
Parameters	Vehicle		$\alpha$ -Asarone		Midazolam	
	Baseline (no drug)	SD day 5	Baseline (no drug)	SD day 2	SD day 5	SD day 2
Total distance traveled (m)	28.8 $\pm$ 3.6	17.5 $\pm$ 4.5 <sup>#</sup>	25.6 $\pm$ 3.7	21.7 $\pm$ 4.0	13.3 $\pm$ 3.2 <sup>#</sup>	26.3 $\pm$ 4.1
Average speed (cm/s)	9.6 $\pm$ 1.2	5.8 $\pm$ 1.2 <sup>#</sup>	9.0 $\pm$ 1.1	7.0 $\pm$ 1.2	4.6 $\pm$ 0.9 <sup>#</sup>	8.8 $\pm$ 1.4
Time mobile (s)	208.8 $\pm$ 18.2	114.3 $\pm$ 19.7 <sup>#</sup>	186.4 $\pm$ 21.9	146.4 $\pm$ 20.3	103.2 $\pm$ 18.9 <sup>#</sup>	172.3 $\pm$ 25.9
Time spent in outer zone (s)	222.5 $\pm$ 20.7	276.3 $\pm$ 4.0 <sup>#</sup>	254.6 $\pm$ 10.5	267.1 $\pm$ 7.4	245.9 $\pm$ 29.3	277.9 $\pm$ 8.9
Time spent in inner zone (s)	28.6 $\pm$ 14.8	7.6 $\pm$ 1.8	17.7 $\pm$ 6.7	11.4 $\pm$ 3.2	16.4 $\pm$ 9.7	3.8 $\pm$ 2.6
No. of entries in outer zone	18.0 $\pm$ 3.2	10.3 $\pm$ 2.6	14.3 $\pm$ 1.6	11.0 $\pm$ 2.7	8.5 $\pm$ 1.5 <sup>#</sup>	12.2 $\pm$ 2.4
No. of entries in inner zone	11.1 $\pm$ 2.5	5.3 $\pm$ 1.2	8.4 $\pm$ 1.7	6.4 $\pm$ 1.8	4.6 $\pm$ 1.9 <sup>*</sup>	3.4 $\pm$ 1.4

The data are represented as mean  $\pm$  SEM

Levels of significance \* $p < 0.05$  and <sup>#</sup> $p < 0.01$ .  $N=7$  for vehicle group,  $N=8$  for  $\alpha$ -asarone group and  $N=5$  for midazolam group

<sup>#</sup>Significance from the baseline

\*Significance between vehicle and  $\alpha$ -asarone group on days 2 and 5 of SD



**Fig. 4** Changes in RAM parameters after administration of  $\alpha$ -asarone and vehicle in sleep-deprived rats. Changes in the parameters in RAM test show increased spatial memory in those rats that received  $\alpha$ -asarone before SD. The graph shows **a** the percentage of correct choices made and **b** reference memory (RME) and working memory (WME) errors on day 5 of SD compared to the baseline values (taken as 100%) taken before SD. \*Indicates significance of the differences between vehicle and  $\alpha$ -asarone group and #indicates significance from the baseline. Levels of significance \* $p < 0.05$  and \*\* $p < 0.01$ .  $N = 5$  for each group

On the other hand, the cellular adaptive response after 5 days of SD was not evident in the behavior of the animals. They showed an increased anxiety and reduced spatial memory after 5 days of SD. This might be due to the reduction in the activity of CAT especially in the subcortical region along with increase in the SOD activity. Catalase over-expression

is reported to be sufficient to enhance cognition and reduce anxiety even in the absence of alteration in levels of oxidative stress [49]. Furthermore, in the present study, both subcortex and cortex were affected the most by SD associated oxidative stress which in turn would have led to cognitive decline and anxiety. Previously, a systematic review reported that the brain areas, namely, cortex, hypothalamus, hippocampus, thalamus and amygdala are found to be more vulnerable to oxidative stress [50].

The anxiolytic and cognitive enhancing effect of  $\alpha$ -asarone in the present study may be partly due to its antioxidant property. The present study indicated that the oxidative cellular damage induced by sleep loss was alleviated moderately by  $\alpha$ -asarone treatment which reduced the MDA levels and increased the activity of the antioxidants CAT and GSH-R in the subcortex and the brainstem regions. It is further noted that the effect of  $\alpha$ -asarone in the present study was more drastic in the subcortical region which probably reflected in the anxiety and the cognitive testing in the rats subjected to SD. In various other models, improvement in the cognitive functions after  $\alpha$ -asarone administration was associated with an increased antioxidant status, mainly in the subcortical regions like hippocampus and striatum, and to a lesser extent in the cortex [24, 26, 29, 51]. It is also emphasized that the effect of  $\alpha$ -asarone on the antioxidant status of brain may be dose dependent and other doses might probably showcase an entirely different pattern of antioxidant activity.

Improvement in sleep quality by  $\alpha$ -asarone treatment is also associated with lowering of hyperthermia caused by 5 h SD for 5 days [22]. Hyperthermia is a leading cause to anxiety and cognitive impairment [52, 53] and is linked to increased oxidative stress [54]. So the antioxidant effect observed in the present study, along with mild temperature lowering effect of  $\alpha$ -asarone might have facilitated sleep, anxiolysis and enhancement of cognition.

It is not possible to have an ideal animal model for insomnia, but the SD procedure done in this study can be considered acceptable for studying insomnia [10, 11]. All the SD-induced changes in sleep like increased latency to sleep (difficulty in falling asleep), sleep fragmentation/ increased

**Table 3** Values of all parameters of RAM after administration of  $\alpha$ -asarone and vehicle in SD rats

Groups	Vehicle			$\alpha$ -Asarone		
	Baseline (no drug)	SD day 1	SD day 5	Baseline (no drug)	SD day 1	SD day 5
Correct choices made (%)	43.7 $\pm$ 5.9	53.7 $\pm$ 4.4	29.1 $\pm$ 6.3 <sup>#</sup>	49.1 $\pm$ 3.8	62.1 $\pm$ 8.6	70.0 $\pm$ 13.3*
Reference memory error	5.0 $\pm$ 1.3	2.6 $\pm$ 0.4	7.4 $\pm$ 2.0	3.8 $\pm$ 0.6	2.4 $\pm$ 0.7	1.6 $\pm$ 0.8*
Working memory error	1.2 $\pm$ 0.7	0.6 $\pm$ 0.2	2.8 $\pm$ 0.6 <sup>#</sup>	0.6 $\pm$ 0.3	0.6 $\pm$ 0.2	0.6 $\pm$ 0.2**

The data are represented as mean  $\pm$  SEM

Levels of significance \* $p \leq 0.05$  and \*\* $p \leq 0.01$ .  $N = 5$  for both groups

<sup>#</sup>Significance from the baseline

\*Significance of the differences between vehicle and  $\alpha$ -asarone group



**Table 4** Effect of acute SD by gentle handling on antioxidant levels after administration of vehicle and  $\alpha$ -Asarone

	Cortex						Subcortex						Brainstem					
	SD day 1		SD day 5		C		SD day 1		SD day 5		C		SD day 1		SD day 5		C	
	Veh	$\alpha$ -As	Veh	$\alpha$ -As	Veh	$\alpha$ -As	Veh	$\alpha$ -As	Veh	$\alpha$ -As	Veh	$\alpha$ -As	Veh	$\alpha$ -As	Veh	$\alpha$ -As	Veh	$\alpha$ -As
MDA (nmol/mg protein)	6.0±0.4	5.0±0.4	5.2±0.3	5.3±0.5	4.9±1.3	3.8±0.2	4.1±0.1	3.8±0.1	0.9±0.1 <sup>###</sup>	0.6±0.0 <sup>*###</sup>	3.1±0.9	5.3±0.6 <sup>#</sup>	4.2±0.6	1.6±0.6 <sup>###</sup>	0.8±0.1 <sup>*,##</sup>			
CAT (U/mg protein)	1.7±0.1	1.3±0.2	1.3±0.1	1.1±0.1 <sup>#</sup>	1.5±0.1 <sup>*</sup>	1.4±0.1	1.1±0.0 <sup>#</sup>	1.3±0.1 <sup>*</sup>	1.0±0.1 <sup>#</sup>	2.4±0.3 <sup>*##</sup>	3.1±0.3	2.0±0.2 <sup>#</sup>	3.1±0.5 <sup>*</sup>	3.1±0.2	2.7±0.1			
GSH-R (U/mg protein)	2.6±0.4	0.8±0.3 <sup>##</sup>	1.3±0.3 <sup>##</sup>	4.6±0.2 <sup>##</sup>	4.5±0.5 <sup>#</sup>	1.9±0.4	1.3±0.4	1.4±0.2	2.8±0.4	3.6±0.3 <sup>*##</sup>	1.6±0.2	1.6±0.2	1.4±0.2	2.3±0.3	2.2±0.1			
SOD (U/mg protein)	11.6±1.9	11.2±0.8	10.1±1.5	15.8±2.3 <sup>#</sup>	13.7±0.6	8.7±0.3	15.0±1.4 <sup>#</sup>	12.8±0.9	11.2±1.4 <sup>#</sup>	6.5±0.5	10.1±1.9	13.2±1.5	11.8±1.4	8.4±0.6	7.9±0.5			
GSH-Px (mU/mg protein)	34.4±6.5	9.1±3.2 <sup>##</sup>	16.9±3.2 <sup>#</sup>	65.6±13.2 <sup>##</sup>	59.2±9.7 <sup>##</sup>	19.1±2.0	12.7±1.1 <sup>#</sup>	11.8±1.8 <sup>#</sup>	42.8±11.6 <sup>#</sup>	30.8±4.8 <sup>##</sup>	15.7±2.3	20.6±1.1	14.8±1.7	56.8±20.2	24.1±3.6			

Levels of significance \*<sup>#</sup> $p < 0.05$ , <sup>##</sup> $p < 0.01$  and <sup>###</sup> $p < 0.001$ .  $N = 7$  for Control (C),  $N = 6$  for Vehicle SD day 1,  $N = 7$  for  $\alpha$ -asarone SD day 1,  $N = 5$  for vehicle SD day 5 and  $N = 5$  for  $\alpha$ -asarone SD day 5

<sup>#</sup>Significance from untreated control

\*Significance between vehicle (Veh) and  $\alpha$ -asarone ( $\alpha$ -As) group

arousal index (frequent awakenings) and reduced sleep quality [22], satisfy the clinical guidelines laid down as criteria for chronic insomnia [10]. Moreover, the SD procedure used in the present study (gentle handling) is reported to be a valid animal model of insomnia with many overlapping pathologies [11]. So it is reasonable to expect similar results in patients with insomnia.

## Conclusion

$\alpha$ -Asarone 10 mg/kg reduced the SD-induced anxiety and memory deficit with reduced oxidative stress in the subcortex. This study provided evidences to indicate that the hypnotic dose of  $\alpha$ -asarone (10 mg/kg) can effectively manage anxiety associated with sleep loss and could also assist in counteracting the associative cognitive deficits under such conditions. Improved antioxidant level especially CAT activity might have facilitated the improvement in the cognitive functioning. This is the first report to show that the hypnotic  $\alpha$ -asarone may be a potential therapeutic agent in the management of insomnia-associated changes in anxiety and memory.

**Acknowledgements** The work was supported by the research grant from the Council of Scientific and Industrial Research, New Delhi, India (CSIR Sanction No: 37(1543)/12-EMR II). AR was supported by CSIR Junior Research Fellowship. We acknowledge Dr. Lakshmi R for her assistance in the RAM test.

## Compliance with ethical standards

**Conflict of interest** None of the authors has any financial interest or conflicts of interest related to this work.

**Ethical approval** All the procedures employed in this study were approved by the Institutional Animal Ethics Committee of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala.

**Animals** Wistar rats were obtained from the Division of Laboratory Animal Sciences, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala.

## References

- Alkadhi K, Zagaar M, Alhaider I, Salim S, Aleisa A. Neurobiological consequences of sleep deprivation. *Curr Neuropharmacol*. 2013;11:231.
- Goel N, Rao H, Durmer JS, Dinges DF. Neurocognitive consequences of sleep deprivation. *Semin Neurol*. 2009;29:320 (NIH Public Access).
- American Psychiatric Association. Committee on Nomenclature and Statistics. Diagnostic and Statistical Manual of Mental Disorders (DSM). Revised fourth edition. Washington DC; 1994. pp. 143–7.
- Ramsawh HJ, Stein MB, Belik SL, Jacobi F, Sareen J. Relationship of anxiety disorders, sleep quality, and functional impairment in a community sample. *J Psychiatr Res*. 2009;43:926–33.
- Bourdet C, Goldenberg F. Insomnia in anxiety: sleep EEG changes. *J Psychosom Res*. 1994;38:93–104.
- Silva RH, Kameda SR, Carvalho RC, Takatsu-Coleman AL, Niigaki ST, Abílio VC, Tufik S, Frussa-Filho R. Anxiogenic effect of SD in the elevated plus-maze test in mice. *Psychopharmacology*. 2004;176:115–22.
- Vollert C, Zagaar M, Hovatta I, Taneja M, Vu A, Dao A, Levine A, Alkadhi K, Salim S. Exercise prevents sleep deprivation-associated anxiety-like behavior in rats: potential role of oxidative stress mechanisms. *Behav Brain Res*. 2011;224:233–40.
- Ferrara M, Iaria G, Tempesta D, Curcio G, Moroni F, Marzano C, De Gennaro L, Pacitti C. Sleep to find your way: the role of sleep in the consolidation of memory for navigation in humans. *Hippocampus*. 2008;18:844–51.
- Yoo SS, Hu PT, Gujar N, Jolesz FA, Walker MP. A deficit in the ability to form new human memories without sleep. *Nat Neurosci*. 2007;10:385–92.
- Schutte-Rodin S, Broch L, Buysse D, Dorsey C, Sateia M. Clinical guideline for the evaluation and management of chronic insomnia in adults. *JCSM*. 2008;4:487.
- Revel FG, Gottowik J, Gatti S, Wettstein JG, Moreau J-L. Rodent models of insomnia: a review of experimental procedures that induce sleep disturbances. *Neurosci Biobehav Rev*. 2009;33:874–99.
- Bouayed J, Rammal H, Soulimani R. Oxidative stress and anxiety: relationship and cellular pathways. *Oxid Med Cell Longev*. 2009;2:63–7.
- Hassan W, Silva CEB, Mohammadzai IU, da Rocha JBT, Landeira-Fernandez J. Association of oxidative stress to the genesis of anxiety: implications for possible therapeutic interventions. *Curr Neuropharmacol*. 2014;12:120–39.
- Reimund E. The free radical flux theory of sleep. *Med Hypotheses*. 1994;4:231–3.
- Parrino L, Terzano MG. Polysomnographic effects of hypnotic drugs. *Psychopharmacology*. 1996;126:1–16.
- Nishino T, Takeuchi T, Takechi K, Kamei C. Evaluation of anxiolytic-like effects of some short-acting benzodiazepine hypnotics in mice. *J Pharmacol Sci*. 2008;107:349–54.
- Lister RG. The amnesic action of benzodiazepines in man. *Neurosci Biobehav Rev*. 1985;9:87–94.
- de Gage SB, Moride Y, Ducruet T, Kurth T, Verdoux H, Tournier M, Pariente A, Begaud B. Benzodiazepine use and risk of Alzheimer's disease: case-control study. *BMJ*. 2014;349:g5205.
- Ashton H. Guidelines for the rational use of benzodiazepines. *Drugs*. 1994;48:25–40.
- Toner LC, Tsambiras BM, Catalano G, Catalano MC, Cooper DS. Central nervous system side effects associated with Zolpidem treatment. *Clin Neuropharmacol*. 2000;23:54–8.
- Kumar VM, Gulia KK. Sleep medicine in *Ayurveda*. *Sleep Med Rev*. 2016;25:131.
- Radhakrishnan A, Jayakumari N, Kumar VM, Gulia KK. Sleep promoting potential of low dose  $\alpha$ -asarone in rat model. *Neuropharmacology*. 2017;125:13–29.
- Dandiya P, Menon M. Actions of asarone on behavior, stress, and hyperpyrexia, and its interaction with central stimulants. *J Pharmacol Exp Ther*. 1964;145:42–6.
- Kumar H, Kim BW, Song SY, Kim JS, Kim IS, Kwon YS, Koppula S, Choi DK. Cognitive enhancing effects of alpha asarone in amnesic mice by influencing cholinergic and antioxidant defense mechanisms. *Biosci Biotechnol Biochem*. 2012;76:1518–22.
- Shin JW, Cheong YJ, Koo YM, Kim S, Noh CK, Son YH, Kang C, Sohn NW.  $\alpha$ -Asarone ameliorates memory deficit in

- lipopolysaccharide-treated mice via suppression of pro-inflammatory cytokines and microglial activation. *Biomol Ther.* 2014;22:17.
26. Limon ID, Mendieta L, Diaz A, Chamorro G, Espinosa B, Zenteno E, Guevara J. Neuroprotective effect of alpha-asarone on spatial memory and nitric oxide levels in rats injected with amyloid- $\beta$  (25–35). *Neurosci Lett.* 2009;453:98–103.
  27. Liu S, Chen SW, Xu N, Liu XH, Zhang H, Wang YZ, Xu XD. Anxiolytic-like effect of  $\alpha$ -asarone in mice. *Phytother Res.* 2012;26:1476–81.
  28. Lee B, Sur B, Yeom M, Shim I, Lee H, Hahm DH. Alpha-asarone, a major component of *Acorus gramineus*, attenuates corticosterone-induced anxiety-like behaviours via modulating TrkB signaling process. *Korean J Physiol Pharmacol.* 2014;18:191–200.
  29. Manikandan S, Devi RS. Antioxidant property of  $\alpha$ -asarone against noise-stress-induced changes in different regions of rat brain. *Pharmacol Res.* 2005;52:467–74.
  30. Pace-Schott EF, Hobson JA. The neurobiology of sleep: genetics, cellular physiology and subcortical networks. *Nat Rev Neurosci.* 2002;3:591–605.
  31. Gulia KK, Patel N, Kumar VM. Increased ultrasonic vocalizations and risk-taking in rat pups of sleep-deprived dams. *Physiol Behav.* 2015;139:59–66.
  32. Wenk GL. Assessment of spatial memory using the radial arm maze and Morris water maze. In: *Curr Protoc Neurosci/editorial board*, Crawley JN, et al., Chap. 8, Unit 8, 5A. 2004.
  33. Buege JA, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol.* 1978;52:302–10.
  34. Abei H. Catalase in vitro. *Methods Enzymol.* 1984;105:121–6.
  35. Smith IK, Vierheller TL, Thorne CA. Assay of glutathione reductase in crude tissue homogenates using 5, 5'-dithiobis (2-nitrobenzoic acid). *Anal Biochem.* 1988;175:408–13.
  36. Marklund S, Marklund G. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem.* 1974;47:469–74.
  37. Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG, Hoekstra WG. Selenium: biochemical role as a component of glutathione peroxidase. *Science.* 1973;179:588–90.
  38. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem.* 1951;193:265–75.
  39. Berro LF, Hollais AW, Patti CL, Fukushiro DF, Mari-Kawamoto E, Talhati F, Costa JM, Zanin KA, Lopes-Silva LB, Ceccon LM, Santos R. Sleep deprivation impairs the extinction of cocaine-induced environmental conditioning in mice. *Pharmacol Biochem Behav.* 2014;124:13–8.
  40. Cohen S, Kozlovsky N, Matar MA, Kaplan Z, Zohar J, Cohen H. Post-exposure sleep deprivation facilitates correctly timed interactions between glucocorticoid and adrenergic systems, which attenuate traumatic stress responses. *Neuropsychopharmacology.* 2012;37:2388–404.
  41. Cortese BM, Mitchell TR, Galloway MP, Prevost KE, Fang J, Moore GJ, Uhde TW. Region-specific alteration in brain glutamate: possible relationship to risk-taking behavior. *Physiol Behav.* 2010;99:445–50.
  42. Guan Z, Peng X, Fang J. Sleep deprivation impairs spatial memory and decreases extracellular signal-regulated kinase phosphorylation in the hippocampus. *Brain Res.* 2004;1018:38–47.
  43. Xu ZQ, Gao CY, Fang CQ, Zhou HD, Jiang XJ. The mechanism and characterization of learning and memory impairment in sleep-deprived mice. *Cell Biochem Biophys.* 2010;58:137–40.
  44. Anseloni VZ, Brandao ML. Ethopharmacological analysis of behaviour of rats using variations of the elevated plus-maze. *Behav pharmacol.* 1997;8:533–40.
  45. Zangrossi H, Viana MB, Graeff FG. Anxiolytic effect of intramygdala injection of midazolam and 8-hydroxy-2-(di-n-propylamino) tetralin in the elevated T-maze. *Eur J Pharmacol.* 1999;369:267–70.
  46. Ramanathan L, Gulyani S, Nienhuis R, Siegel JM. Sleep deprivation decreases superoxide dismutase activity in rat hippocampus and brainstem. *Neuroreport.* 2002;13:1387–90.
  47. Ramanathan L, Hu S, Frautschy SA, Siegel JM. Short-term total sleep deprivation in the rat increases antioxidant responses in multiple brain regions without impairing spontaneous alternation behavior. *Behav Brain Res.* 2010;207:305–9.
  48. Cirelli C. Cellular consequences of sleep deprivation in the brain. *Sleep Med Rev.* 2006;10:307–21.
  49. Olsen RHJ, Johnson LA, Zuloaga DG, Limoli CL, Raber J. Enhanced hippocampus-dependent memory and reduced anxiety in mice over-expressing human catalase in mitochondria. *J Neurochem.* 2013;125:303–13.
  50. Villafuerte G, Miguel-Puga A, Rodríguez EM, Machado S, Manjarrez E, Arias-Carrión O. Sleep deprivation and oxidative stress in animal models: a systematic review. *Oxid Med Cell Longev.* 2015;2015:234952. <https://doi.org/10.1155/2015/234952>
  51. Pages N, Maurois P, Delplanque B, Bac P, Stables JP, Tamariz J, Chamorro G, Vamecq J. Activities of  $\alpha$ -asarone in various animal seizure models and in biochemical assays might be essentially accounted for by antioxidant properties. *Neurosci Res.* 2010;68:337–44.
  52. Groenink L, Vinkers C, Oorschot R, Olivier B. Models of anxiety: Stress-induced hyperthermia (SIH) in singly housed mice. *Curr Protoc Pharmacol.* 2009;5:16.
  53. Shibasaki M, Namba M, Oshiro M, Kakigi R, Nakata H. Suppression of cognitive function in hyperthermia; from the viewpoint of executive and inhibitive cognitive processing. *Sci Rep.* 2008;7:43528.
  54. Flanagan SW, Moseley PL, Buettner GR. Increased flux of free radicals in cells subjected to hyperthermia: detection by electron paramagnetic resonance spin trapping. *FEBS Lett.* 1998;431:285–6.