

Effect of *Ganoderma lucidum*, *Spirulina* and Vitamin D on Diazepam-Induced Anterograde Amnesia in Male Wistar Rats: An Experimental Study

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ABSTRACT

Objective: Current study was to check the effect of *Ganoderma lucidum*, *Spirulina*, and vitamin D on Diazepam-induced anterograde amnesia in male Wistar rats. **Materials and Methods:** Six groups (I–VI; n = 10) of healthy male Wistar rats (150–180 g) were used for the study. The induction of anterograde amnesia was done by diazepam. The Morris water maze was used to study the amnesia where the rats must swim to hidden escape platform. The rats were trained to locate a hidden escape platform by releasing them into water for four times a day for four consecutive days. The acquisition of this task was measured by noting the time taken to escape the platform. On day 5 of the study, retrieval of this learnt task was measured by noting the time taken to search for the missing hidden escape platform. These two test times were then compared with the test groups and drug-induced amnesia groups. The time was expressed as mean ± standard error of mean. **Results:** *Ganoderma lucidum*, and *Spirulina* completely ameliorated the induction of anterograde amnesia by diazepam. The escape latency time of vitamin D-administered rats was less when that of diazepam-administered rats but was not statistically significant.

Conclusion: *Ganoderma lucidum*, *Spirulina*, and vitamin D ameliorated the oxidative stress-induced anterograde amnesia pointing toward a possible antioxidant role of them. These, at the commonly prescribed clinical doses, can therefore potentially inhibit oxidative stress-induced neurodegeneration, especially in the ageing population. Further studies are needed to substantiate these findings.

Key words: Anterograde amnesia, *Ganoderma lucidum*, *Spirulina*, Vitamin D, Morris water maze.

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INTRODUCTION

Memory is one of the fascinating functions of the brain, which projected man to dominate all forms of life. It complicates our understanding due to its many diverse functions, numerous circuits, and interconnections. It has been proposed that memory is stored in the brain by ongoing electrical activity in circuits or networks of nerve cells.¹ The protein synthesis is the underlying stage of memory formation and the general memory functions including encoding, storage, and retrieval of knowledge.¹⁻² Amnesia is a pathological state of memory loss, which occurs either by physical damage to the brain, especially in hippocampus, fornix or mammillary bodies, or by substance abuse such as alcohol. Amnesia is characterized by the inability to encode new and recalling posttraumatic memories is called anterograde amnesia, whereas failure in retrieval of pretraumatic memories is called retrograde amnesia. Anterograde amnesia is more severe than retrograde amnesia. Stroke, infections, dementia, Alzheimer's disease, improper oxygen supply to brain, alcohol abuse, lack of acetylcholine, and certain medications are also the causative agents of amnesia.³⁻⁴ In 2001, 24.3 million people had dementia and 4.6 million new cases are diagnosed every year. It has been predicted that the number may double in next 20 years.⁵ In future, India will have one of the largest numbers of elderly with dementia.⁶ Cognitive functions rely on neurotransmission, especially cholinergic neurotransmission and the neurotransmitters involved in cholinergic neurotransmission were predominantly involved in learning and memory functions and their modification plays a significant role in management of amnesia.⁷

Ganoderma lucidum is a white rot fungus, which is used as a traditional medicine in various countries such as China, Korea, and Japan. It consists of various physiological substances such as nucleosides, steroids, alkaloids, proteins, and amino acids,⁴ and it has shown to improve memory by its neuroprotective property.⁸ *Spirulina* is a blue-green algae, which contains

high-quality protein and essential macro- and micronutrients having anti-oxidative and anti-inflammatory activities.⁹ It is also effective in treating cancer, hyperlipidemia, and preventing memory dysfunction.⁹⁻¹⁰ Vitamin D is a multifunctional steroid, which can regulate neuron health¹¹ and its deficiency has been implicated in psychiatric and neurologic disorders. Though conflicting, a number of studies indicated that vitamin D has a role in cognitive, mood, and physical performance in older adults.¹²⁻¹³

Ganoderma lucidum, *Spirulina*, and vitamin D may be effective in treatment of amnesia; however, there is paucity of literature regarding their effects on amnesia in animals. Also, reports are conflicting regarding the role of vitamin D in amnesia. In this view, present study planned to explore the antiamnesic effects of *Ganoderma lucidum*, *Spirulina*, and vitamin D on experimentally induced anterograde amnesia in male Wistar rats.

MATERIALS AND METHODS

Animals

Adult healthy male Wistar rats weighing 150–180 g was obtained from the Central Animal House, J.N. Medical College, Belagavi and were accustomed to 12:12 h light: dark cycle for 10 days prior to the commencement of experiment. Animals were maintained on standard rat chow pellet and water *ad libitum*. The study was approved by the Institutional Animal Ethical Committee (IAEC) maintained as per the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi.

Rats were selected based on their swimming ability showing minimal floating tendencies and normal behavior. Rats that are unable to swim,

failing to locate the platform on all occasions for two consecutive days, and showing predominant tendency to float were not included.

Selected rats were randomly divided into six groups comprising 10 rats in each group ($n = 6$).

Following selected treatments were given to the respective groups. In each group a suspension was prepared using gum acacia and sterile water with allotted drug except in group I.

- Group I: Control (0.5 mL of 1% gum acacia orally) was given 30 min before trial.
- Group II: Diazepam (1 mg/kg intraperitoneally) given 30 min before trial.
- Group III: Diazepam (1 mg/kg intraperitoneally) plus a fruit body product of *Ganoderma lucidum* (292 mg/kg) given orally 60 min before commencing acquisition trial.
- Group IV: Diazepam (1 mg/kg intraperitoneally) plus mycelium product of *Ganoderma lucidum* (486 mg/kg oral) 60 min from trial.
- Group V: Diazepam (1 mg/kg intraperitoneally) plus *Spirulina* (324 mg/kg oral) 60 min before commencing trial.
- Group VI: Diazepam (1 mg/kg intraperitoneally) plus vitamin D (1 mcg/kg intraperitoneally) before trial.

Morris water maze

Morris water maze is a circular water tank having a diameter of 150 cm and height of 50 cm with nonreflecting interior surface. The inner surface was opaque and filled with opaque water at $25 \pm 5^\circ\text{C}$ up to 30 cm. water was made opaque by dissolving 3 L of fresh milk. Tank was divided into four quadrants using wires. A square platform having a height of 29 cm was placed in the middle of the one quadrant (goal quadrant—Qg) and its position was kept unaltered throughout the training and acquisition trials.

Acquisition trial

On day 1 of the experiment, all rats were familiarized with the task and trials were not counted and further this familiarization was confirmed when animals learnt about escape route from this aversive condition.

From day 2–5 of the experiment acquisition trial was performed. All control and drug-treated rats were released into the water facing towards the wall in one of the quadrants. They were subjected to four trials per day for 4 days. During successive trials and successive days, starting points were changed every time. Several distal cues were provided to the rats with strict adherence to their same fixity for all the days of the trial and the position of the examiner was fixed with respect to the distal cues. Subsequently, they were allowed to escape the platform and stay there for 20 s to generate a spatial memory of the hidden platform with the help of distal cues. The time required to escape the platform—escape latency time (ELT) was noted and compared amongst different days and different groups. Rats unable to locate the platform within 120 s were guided to the platform by hand and again kept there for 20 s. Rats failing the task on consecutive trials for two successive days were excluded from the study.

Retrieval trial

On day 6 of the experiment, platform from the Qg was removed and all groups were administered with vehicle (0.5 mL of 1% gum acacia orally 30 min before the trial) and evaluated for the time spent in previously Qg (index of retrieval). This was done only once and the farthest quadrant from the goal quadrant was chosen to release the rats. This quadrant was kept the same for all groups. The time spent in the previously Qg was compared among control, drug-induced amnesia, and drug-treated groups.

Statistical analysis

The data for all the groups were expressed as mean \pm standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by post hoc Dunnett's test using Graph pad prism 4 software. A difference in groups with $p \leq 0.05$ was considered statistically significant.

RESULTS

A total of 10 rats were selected per group based on their ability to swim. Rats that preferentially floated were excluded from the study and no mortality was encountered during the study period. (Table 1)

Control group rats learnt and acquired the task of locating the submerged, invisible platform quickly. The day 4 ELT was significantly reduced compared to the day 1 ELT. While acquiring the task, their trajectory toward the Qg changed from circumferential on day 1 to goal directed on day 4. On removing the platform, they could retrieve the previous location by spending more time in the Qg.

Group II diazepam-treated rats failed to acquire the task. On comparison of the mean ELT among all the 4 days, no significant difference was observed from day 1. Even on the day 4, they were swimming circumferentially with minimal goal directed behavior. As there was no acquisition of the trial, when released into quadrant on the day 6, the time spent in the previous Qg was similar to time spent in all other quadrants.

In group III, *Ganoderma lucidum* (fruit product)-treated rats, the acquisition of the task was satisfactory as significant decrease in the time required to escape to the platform was seen with change in swimming trajectory.

In group IV, *Ganoderma lucidum* (mycelium product)-treated rats, significant difference in the mean ELT was observed at day 4 compared to the day 1. In addition, their ways were increasingly goal directed. On the day 6, the rats spent majority of their swimming time locating for the missing platform in the Qg.

Spirulina-administered animals also showed a significant decrease in ELT.

In vitamin D-administered animals, although a difference was observed in the ELT at day 4 when compared to the ELT at day 1, the rats did search for the hidden platform, but difference was not significant. Additionally, as evident on the day 6, rats showed retrieval of learnt task by spending maximum time in the previously Qg.

DISCUSSION

Diazepam is a benzodiazepine, which affects both learning and retrieval of memory.¹⁴ It enhances the inhibitory action of neurotransmitter such as GABA by facilitating its binding with the receptor subunit—GABA_A and brings about prospective and retrospective memory impairment.^{15–16}

Table 1: Comparison of mean escape latency time of rats.

Groups (n = 10)	Day 1	Day 2	Day 3	Day 4	Index of retrieval (s)
I	30.05 \pm 5.28	18.48 \pm 4.12	13.83 \pm 1.25	8.45 \pm 0.86	65.60 \pm 2.90
II	33.03 \pm 3.28	16.98 \pm 2.87	15.18 \pm 1.19	13.2 \pm 1.43	44.90 \pm 2.11
III	24.98 \pm 3.95	16.70 \pm 3.10	13.38 \pm 2.22	8.02 \pm 1.70*	79.08 \pm 3.51*
IV	24.10 \pm 3.99	11.28 \pm 2.08	9.68 \pm 1.56	6.33 \pm 0.72*	76.63 \pm 1.82*
V	9.77 \pm 3.09	8.075 \pm 1.23	6.12 \pm 0.77	4.69 \pm 0.29*	81.05 \pm 3.03*
VI	23.66 \pm 5.04	15.93 \pm 2.71	13.76 \pm 3.04	10.6 \pm 3.03	63.40 \pm 1.86

*Mean with p-value lesser than 0.05 is considered as statistically significant (ANOVA followed by Post hoc-Dunnett's test)

GABA_A receptor is a recognition site for certain agonists such as barbiturates, GABA, and benzodiazepines. Activation of GABA_A receptor by these agonists in medial septal region leads to inhibition of neuron firing. In rat brain, this activation linked with cholinergic nerve terminal leading to inhibition of the neurotransmitter acetylcholine, which is released from the hippocampal synaptosomes of mouse.¹⁶ Acetylcholine esterase hydrolyses the remaining acetylcholine, resulting in abnormal neurotransmission, and memory impairment. It has reported that diazepam also produces reactive oxygen species-mediated changes.¹⁷ This drug is extensively used in anxiety, epilepsy, and other neural disorder studies.¹⁸ Hence, current study employed diazepam to induce amnesia in rats.

Morris water maze is a frequently used laboratory tool to study spatial learning and memory neuroscience in rats. No direction-specific behavior needs to be prompted, and it gained the core position in neuroscience research.¹⁹⁻²⁰

Alteration of neurotransmitter plays a significant role in treating amnesia, especially in the hippocampus and cerebellum regions of the brain.⁷ Maintaining the levels of acetylcholine by inhibiting the acetylcholine esterase also plays a significant role in treating neurodegenerative diseases²¹ as patients with dementia produce less amount of acetylcholine, and it gets broken down by this enzyme.⁴ Oxidative stress also implicates to the neurodegenerative diseases such as Alzheimer's disease.²¹

Ganoderma lucidum protects the hippocampal neurons due to its anti-oxidant and anti-inflammatory activities, and it can pass through the blood-brain barrier.²² Acetylcholine esterase inhibition activity was correlated with total phenolic and flavonoid content.²³ It has been reported that *Ganoderma lucidum* possess steroids, phenols, and terpenoids and its fruiting bodies possess lanostane triterpenes, which is the best inhibitor of acetylcholine esterase.²⁴⁻²⁵ A study by Yu jin choi⁴ showed that *Ganoderma lucidum* water extract fermented by lactic acid bacteria is effective in inhibiting acetylcholine esterase enzyme. Also, Shevelev *et al.*²⁶ showed that *Ganoderma lucidum* reduces the frequency of the inhibitory neurotransmitters in the brain. Resembling results are obtained in our study where both fruit body and mycelium of *Ganoderma lucidum*-treated rats showed significant decrease in time required to escape to the platform when compared to day 1; however, and on day 6, most of the time spent on the target quadrant suggesting storing and retrieval of memory got restored to normal. Study by Yu Jin Choi⁴ also showed a similar result where *Ganoderma lucidum* water extract-treated group showed increased swimming time in Qg finding missing platform when compared to scopolamine-treated group. These findings suggest that *Ganoderma lucidum* restores the activity of neurotransmitters and enhances the cognitive performance and rectifies learning and memory deficits.

Oxidative damage and accumulation of iron in the brain are also thought as major pathological features of neurodegenerative diseases such as Alzheimer's disease. *Spirulina* is believed as a radical scavenger and possess iron-chelating as well as antioxidant property.²⁷ A study by Wang *et al.*²⁸ reported that antioxidants play a significant role in neurodegenerative disorders and *Spirulina* showed increased post-stroke locomotor activity. In our study, *Spirulina*-treated group showed a decreased ELT and on day 6, rats spent majority of swimming time in Qg, which suggests that the *spirulina*, in addition to its antioxidant capabilities, is a potent neuro-protective organism.

Study by Wilkins *et al.*¹³ confirmed that vitamin D deficiency was associated with low mood and impairment in cognitive performance, and low concentration leads to neurodegenerative disease such as Alzheimer's disease. Vitamin D has specific actions on organs such as the central nervous system; its receptor are present in the neurons and has a various physiological processes in the hippocampus and on certain regions

connected to cognition.²⁹ In our study vitamin D showed a difference in ELT on day 4 when compared to day 1 and rats did search for the hidden platform but was not significant. However, a study by Taghizadeh *et al.*¹² showed that vitamin D negatively affected the maze learning with significantly lower performance compared to control group; however, in another study by Latimer *et al.* three therapeutic levels of calcium administration to groups concluded that vitamin D has a positive effect on spatial learning and memory.³⁰ This may also explain the partial response seen after administration of vitamin D in this study.

Further studies are required to determine the long-term effects and mechanism behind the neurotransmitter misbalancing, which impairs the spatial learning, consolidation, and retrieval of memory in animals.

CONCLUSION

Study finding suggests that the *Ganoderma lucidum* and *Spirulina* has a definite role in ameliorating anterograde amnesia induced by diazepam, and vitamin D also has a partial response after administration.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

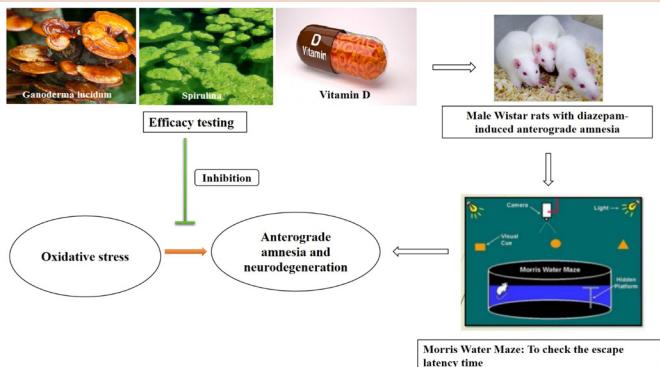
IAEC: Institutional Animal Ethical Committee; **CPCSEA:** Committee for the Purpose of Control and Supervision of Experiments on Animals
Qg: goal quadrant; **ELT:** Escape latency time; **SEM:** standard error of the mean; **ANOVA:** Analysis of variance; **mL:** millilitre; **mg:** milligram; **kg:** kilogram; **cm:** centimetre.

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PICTORIAL ABSTRACT



SUMMARY

- *Ganoderma lucidum*, Spirulina, and vitamin D have possible antioxidant effects.
- These can ameliorate the oxidative stress-induced anterograde amnesia.
- At commonly prescribed doses, these can potentially inhibit oxidative stress-induced neurodegeneration, especially in aging population.

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