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Can vitamin D prevent and/or alleviate hippocampal oxidative stress induced spatial memory impairment? An experimental study in male Long-Evans rats

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Abstract

Background: As spatial memory is essential for our every day lives, it's impairment should be alleviated or prevented. For this, drug treatments have extensive side effects and lengthy duration. **Objective:** To assess the effects of vitamin D on spatial memory performance and hippocampal oxidative stress markers in colchicine induced memory impaired male Long-Evans rats. **Methods:** 30 male Long-Evans rats (8±2 weeks; 225±75 gm) were grouped (6 rats/ group) into normal control, sham control, colchicine control, pre colchicine D and post colchicine D. Then, reference memory (RM) [Mean escape latency (EL) in acquisition phase, average EL of 5th and 6th acquisition days; number of target crossing and time spent in target in probe trial] and working memory (WM) [Mean EL and savings in training and test phase] were assessed in Morris water maze. After sacrifice, hippocampal malondialdehyde and glutathione peroxidase were estimated for oxidative stress assessment. Data were expressed as mean±SEM and statistical analysis was done by one-way ANOVA followed by Bonferroni's post hoc test. **Result:** Colchicine impaired both RM and WM as well as increased oxidative stress. Vitamin D prevented as well as alleviated WM impairment except savings and improved oxidative stress. However, after meticulous scrutiny of RM learning ability and consolidation, Pre D Exp rats had significantly better performance than those of Post D Exp rats. **Conclusion:** Vitamin D can prevent as well as alleviate colchicine induced spatial WM dysfunction along with oxidative stress but it was only found to be preventive, not alleviative in RM improvement.

Key words: Memory impairment, colchicine, oxidative stress, vitamin D, malondialdehyde, glutathione peroxidase, hippocampus, Morris water maze, reference memory, working memory

Introduction

Vitamin D, a fat-soluble vitamin, has physiological importance mainly on bone health and Ca^{2+} homeostasis.[1]Additionally, this vitamin has effective role on multiple chronic diseases, such as, malignancy,[2]cardiovascular disorder,[3] respiratory disorder[4,5]as well as enhancement of survival in COVID-19 patients.[6] Recently, the beneficial role of this vitamin on memory impairment both in animals[7,8] and human have been reported.[9] However, different mechanisms were postulated for alleviative effect of D on memory impairment, such as, by decreasing voltage gated Ca^{2+} channel (VGCC)[10] or clearing amyloid â plaques[7] or inhibiting expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).[11] In addition, this vitamin was also found to reduce oxidative stress and improve memory impairment by decrement of malondialdehyde (MDA)[12]along with increment of glutathione peroxidase (GPx) level in hippocampus.[7]

Spatial memory refers to the part of the memory system that stores and recalls of information within the brain that is needed both to plan a route to a desired location and to remember where an object is located or where an event occurred.[13] Both reference memory [form of long-term memory that stores information for solving tasks and reinforced by repeated training[14]] and working memory [form of short-term memory that provides information for brief periods of time while a person makes plans for action using it[14]] depend on the hippocampus, the most medial part of the temporal lobe. So, hippocampal neurodegeneration may cause memory impairment and oxidative stress is one of its most plausible causes.[15,16]

Spatial memory impairment refers to unusual forgetfulness which is distinctive from age-related forgetfulness. It is one of the important components of dementia whose prevalence is currently more than 55 million worldwid.[17] It is related to sedentary lifestyles, physical inactivity as well as stressful lifestyles, along with clinical disorders, including Alzheimer's disease, brain tumors, strokes, infections, and trauma to the brain.[18]

The term 'oxidative stress' describes alterations in cells brought about by reactive oxygen species (ROS), which are known as free radicals.[19]By various mechanisms, it can damage the cellular membrane such as, lipid peroxidation, protein oxidation and damage to DNA or RNA. Lipid peroxidation end product MDA is commonly measured as an indirect index of oxidative stress.[20] On the other hand, antioxidant is the scavenging molecule that limits or prevents the damaging effects of oxidants. Among them, super oxide dismutase (SOD), catalase(CAT) and glutathione peroxidase (GPx) act as first line defense against oxidative injury. Due to high metabolic activity, high density of oxidizable substances, and relatively low antioxidant defense, brain is very sensitive to oxidative stress.[21]

In the last decade, few animal studies explored the alleviative effects of vitamin D in the improvement of memory impairment.[7,11,12,22] The effective dose, duration and route of this D supplementation in memory impairment in different animal studies were found to be 42 IU/kg for 21 days[7] and 50 IU/kg for 6 weeks[23] in subcutaneous route as well as 10 µg/kg for 14 days[24] and 10,000 IU/kg for 4 weeks[16] in intraperitoneal route. However, to the best of our knowledge, the protective benefits of vitamin D against memory impairment and oxidative stress in the hippocampal region have received little attention in researches. Therefore, the goal of the current study was to assess the impact of vitamin D (10,000 IU for consecutive 28 days) on hippocampal memory function and oxidative stress markers in male Long-Evans rats, both before and after colchicine-induced spatial memory impairment.

Methods

Design and setting

This experimental study was conducted in the Department of Physiology of Bangabandhu

Sheikh Mujib Medical University (BSMMU), Dhaka, Bangladesh from March 2023 to February 2024 after obtaining ethical clearancefrom the Institutional Review Board (Registration no. 4471)of that University.

Drugs

Colchicine was obtained from Incepta Pharmaceuticals Ltd, Bangladesh. Vitamin D (Incepta Pharmaceuticals Ltd, Bangladesh), Normal saline (Popular Pharmaceuticals Ltd, Bangladesh), gentamicin (Incepta Pharmaceuticals Ltd, Bangladesh), thiopental sodium (Gonosasthaya Pharmaceuticals Ltd) and di-ethyl ether (MERCK, Germany) were purchased from local market.

Procurement and maintenance of animals

A total of 30 male Long-Evans rats (8±2 weeks; 225±75 gm) were collected from the central animal house of BSMMU. Then under a 12/12-hour light/dark cycle, all the rats were kept in specially built plastic cages with 6 rats per cage and room temperature was maintained at 27°C, as the thermoneutral zone for rodents is around 27 to 28°C. Rats were provided with standard laboratory food and cooled boiled water ad libitum, during acclimatization. All the experiments were conducted according to the Guidelines for the Animal Experimentation Ethics Committee (AEEC, 15/05/2023)'of the International Centre for Diarrheal Disease Research, Bangladesh along with advice of an expert veterinarian of Bangladesh Agricultural University, Mymensingh, Bangladesh. All the experiments were performed at daytime between 08:00 and 16:00 hours to avoid circadian influences.

Experimental design and dose schedule

On the basis of treatments, all rats were randomly divided into the following groups (6 rats/groups):

- Normal control (NC): No stereotaxic surgery (SS); no normal saline (NS); no vitamin D.
- Sham control (SC): SS and hippocampal infusion of 1 µl NS,[25]no vitamin D.
- Colchicine control (ColC): SS and hippocampal infusion of 15 µg of colchicinein 1 µl NS[25] no vitamin D.
- Pre colchicine D (Pre D Exp): Intraperitoneal (i.p.) 10,000IU vitamin D[16] for 28 days followed by SS and hippocampal infusion of 15 µg of colchicine in 1 µl NS.
- Post colchicine D (Post D Exp): SS and hippocampal infusion of 15 µg of colchicine in 1 µl NS followed by i.p.10,000IU vitamin D[16]for 28 days.

Hippocampal colchicine application by stereotaxic surgery

According to previous researches,[25,26] the colchicine was administered into the rat hippocampus by stereotaxic surgery (SS). Each rat was deeply anesthetized with thiopental sodium (45 mg/kg; i.p.) and was positioned in stereotaxic apparatus. The scalp was incised, retracted and two holes were drilled in the skullaccording to the coordinates of the hippocampus: -3.6 mm anterior-posterior, ± 2 mm lateral-medial, and -3.4 mm dorso-ventral relative to bregma. Then rat was infused through a Hamilton micro syringe with 15µg of colchicine in 1 µl normal saline in each hippocampusvery slowly over 1 minute and the micro syringe was kept place for the next minute (60 seconds) before being slowly withdrawn. The scalp was then closed with sutures and postoperative gentamycin (5 mg/kg, i.p.) was given to prevent sepsis.

Spatial memory assessment by Morris Water Maze (MWM) test

Test tools and circumstances

According to previous researchers,[27]the apparatus consisted of a large circular pool (150 cm in diameter X 50 cm high) filled with water. The whole inner wall of the pool and platform was painted withnon-toxic blackcolor to avoid visual clue in the pool. The pool was placed in a room containing extra maze cues, those could be used by rats for their orientation. Eight points,

north (N), south (S), east (E), west (W), north-east (NE), north-west (NW), south-east (SE) and south-west (SW) were used as starting positions and divided the pool arbitrarily into four quadrants. A black round platform (15 cm in diameter X 28cm high) was placed in the center of the NE quadrant with its top 2 cm below the water surface, so as to be invisible from within the pool. In Figure 1 and Figure 2, all five groups of rats' work plans were described.

Reference memory assessment

As shown in figure 1 and figure 2, ratswere swimming for 3 minutes without platform for consecutive 3 days for instrumental acclimatization as well as for habituation phase of reference memory version. Then in acquisition phase, the rats were given four trials per day over 6 consecutive days kept in a fixed position (NE quadrant). In each trial, the rats were released from a different starting point in a different sequence in each day and given 60 sec to find the platform and to climb onto it. A 50 sec (20 sec on platform+30 sec for self-drying) intertrial time was permitted. The latency to locate the platform [mean escape latency (EL); time from the moment of a rat's entrance into the water up to it's arrival at the platform] was measured by using a stop watch to assess rats' learning ability. Similar 4 trials were given for all rats for consecutive 6 days. On the first trial each day rats reached the platform by chance, that constituted the information stage and the subsequent trials required matching to the novel position for every day, as platform was fixed for 6 days. The average EL of 5^{th} and 6^{th} acquisition days was measured to assess memory consolidation. About 24 hours after last trial of day 6 (on day 7), the platform was removed from pool and final spatial probe trial was given to assess learning strength/ retrieval. In this probe trial, rats were allowed to swim *ad libitum* for 60 sec during which target crossing (TC, the quadrant of MWM from where the platform was removed) and time spent in target (TT, the time spent in the quadrant from where the platform was removed) were measured.[28]

Working memory assessment

As shown in figure 1 and figure 2, about 48 hours after probe trial, the working memory version of the test was administered using a testing paradigm adapted from Sarihi et al. (2000).[39] Here, the 6 days acquisition phase of reference memory test was regarded as pre training phase of working memory assessment. Then, a training and test phase was conducted over 4 consecutive days with 4 trials each day. Everyday, the platform position was changed but kept constant for daily 4 trials. However, every rat was released from 4 different starting points in the 4 daily trials all of which were distant from platform position. On the first trial of each day, rats reached the platform by chance, that constituted the information stage and the subsequent trials required matching to the novel position for that day as platform was changed every day. The mean EL in training and test phase was recorded as above to assess learning ability and savings (the difference in latency scores between trials 1 and 2, expressed as percentage of trial 1 was measured to assess leaning efficiency.[28]

Hippocampal oxidative stress marker assessment

Hippocampal tissue preparation and oxidative stress markers assessment

Rats were sacrificed by decapitation under di-ethyl ether (99%)anesthesia. The brains were then quickly removed and hippocampus was separated immediately followed by washing withice cold phosphate buffer solution [(PBS); 0.1 M, pH:7.40].Next, using a glass homogenizer, the tissues were weighed and homogenized in PBS using Weight (gm): Volume (ml) = 1:4 ratio. After that, the homogenate was centrifuged at 3500 rpm for 10 minutes in order to extract the supernatant. The supernatant was collected for analysis of MDA and GPx(Elab Science Biotechnology, USA)through ELISA. If there was any unintended delay then the supernatant was kept in -20^0C in laboratory.

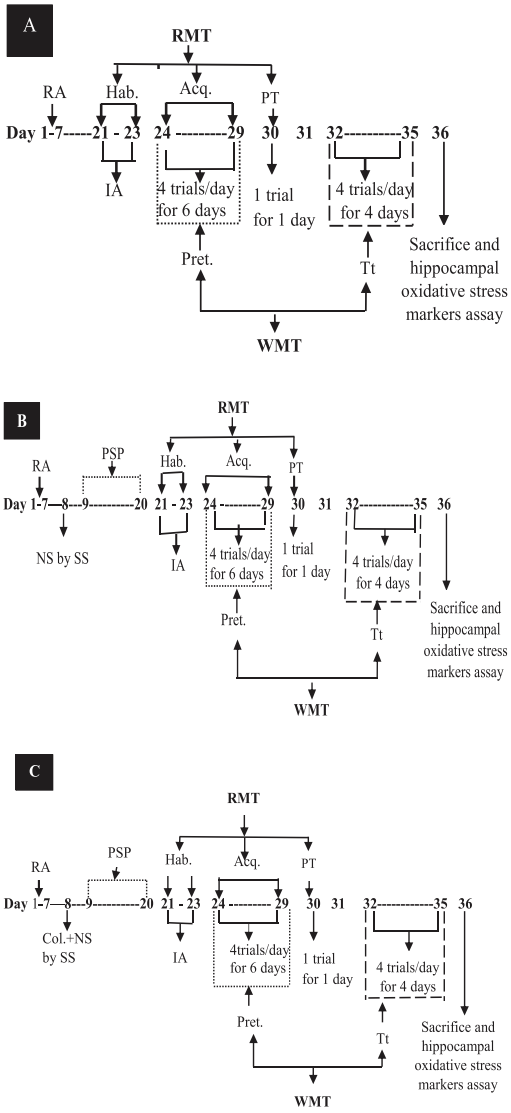

Figure 1: Working plan for control rats A) normal control; B) sham control; C) colchicine control RMT: Reference memory test; WMT: Working memory test; RA=Room acclimatization; PSP=Post-surgical period; Hab.=Habituation; Acq.=Acquisition; PT=Probe trial; IA=Instrumental acclimatization; Pret.= Pretraining; Tt=Training and test; NS=Normal saline;SS=Stereotaxic surgery;Col.=Colchicine

Statistical analysis

All data were expressed as mean±SEM of the study variables and analyzed using ANOVA (among groups) followed by Bonferroni post hoc test (between groups) in SPSS (version 25.0), where $p \leq 0.05$ was considered as statistically significant.

Results

Effect of vitamin D on spatial memory impairment induced by hippocampal colchicine

For reference memory performance

ColC rats showed significantly ($p \leq 0.001$) higher mean EL in comparison to those of SC rats. However, Dsupplementation improvedlearning ability performance, as evidenced by statistically significant ($p \leq 0.001$) differences in mean EL of our ColC and experimental (Pre D Exp and Post D Exp) rats. Strikingly, the differences of this variable between experimental (Pre D Exp and Post D Exp) and NC rats were found statistically non-significant in the last acquisition day. However, the differences of mean EL between Pre D Exp vs Post D Exp rats were found statistically non-significant in all acquisition days (Figure 3A). These data demonstrated that the learning ability of reference memory was impaired by colchicine and reversed almost to normal by systemic D.

In figure 3B, the mean value of average EL in 5th and 6th acquisition days was significantly ($p \leq 0.01$) higher in our ColC rats than those of SC rats. Moreover, D supplementation showed significantly lower average EL in both groups of our experimental [Pre D Exp ($p \leq 0.01$) and Post D Exp($p \leq 0.05$)] rats than that of ColC rats. On the contrary, the differences of this variable between our NC vs experimental rats along with Pre D Exp vs Post D Exp rats were found statistically non-significant. This result represented that colchicine induced consolidation disability in our ColC rats and our vitamin improved it similar to normal rats.

In our study, ColC rats showed retrieval impairment, as evidenced by significantly ($p \leq 0.001$) lower TC and TT than those of SC

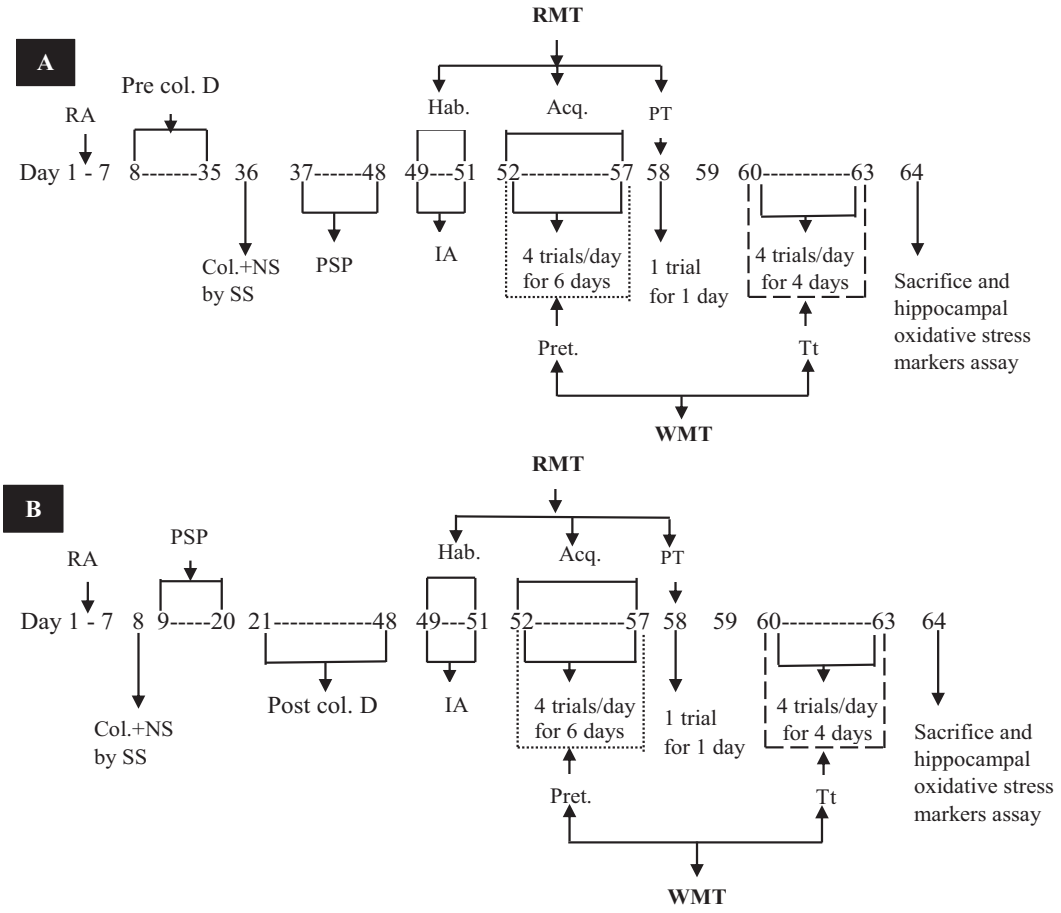

Figure 2: Working plan for experimental rats A) Pre colchicine D; B) Post colchicine DRMT: Reference memory test; WMT: Working memory test; RA=Room acclimatization; PSP=Post-surgical period; Hab.=Habituation; Acq.=Acquisition; PT=Probe trial; IA=Instrumental acclimatization; Pret.= Pretraining; Tt=Training and test; NS=Normal saline; SS=Stereotaxic surgery; Col.=Colchicine.

Table I: Study variables

	Spatial memory type	**Spatial memory perspective**	**Variables; Units**
For memory performance	Reference memory	Learning ability	Mean escape latency (in acquisition phase); seconds
		Consolidation	Average escape latency of 5th and 6th acquisition days (in acquisition phase); seconds
		Learning strength (Retrieval)	Target crossing; frequency/minute, Time spent in target; seconds/minute
	Working memory	Learning ability	Mean escape latency (in training and test phase); seconds
		Learning efficiency	Savings; seconds
For hippocampal oxidative stress		Oxidant	Malondialdehyde (MDA); ng/mg protein
		Antioxidant	Glutathione peroxidase (GPx); pg/mg protein

rats.This vitamin carried out significantly ($p \leq 0.001$) higher TC and TT in both of our experimental (Pre D Exp and Post D Exp) rats than that of ColC rats.And, statistical analysis revealed that the differences in these variables between our NC vs experimental rats and Pre D Exp vs Post D Exp rats were not significant (Figure3C, Figure 3D).These data indicated that learning strength (retrieval) was hampered by colchicine and prevented as well as alleviated almost to normal by vitamin D.

for working memory performance

Here, figure 4A showed that ColC rats had significantly ($p \leq 0.001$) higher mean EL in comparison to those of SC rats. However, vitamin D improved learning ability performance, as evidenced by statistically ($p \leq 0.01$ in trial 1, 2, 3; $p \leq 0.001$ in trial 4) significant differences in mean EL of our ColC and experimental (Pre D Exp and Post D Exp) rats.Strikingly, the differences of this variable between NC vs experimental ratsin trial 4 and Pre D Exp vs Post D Exp rats in all trials in

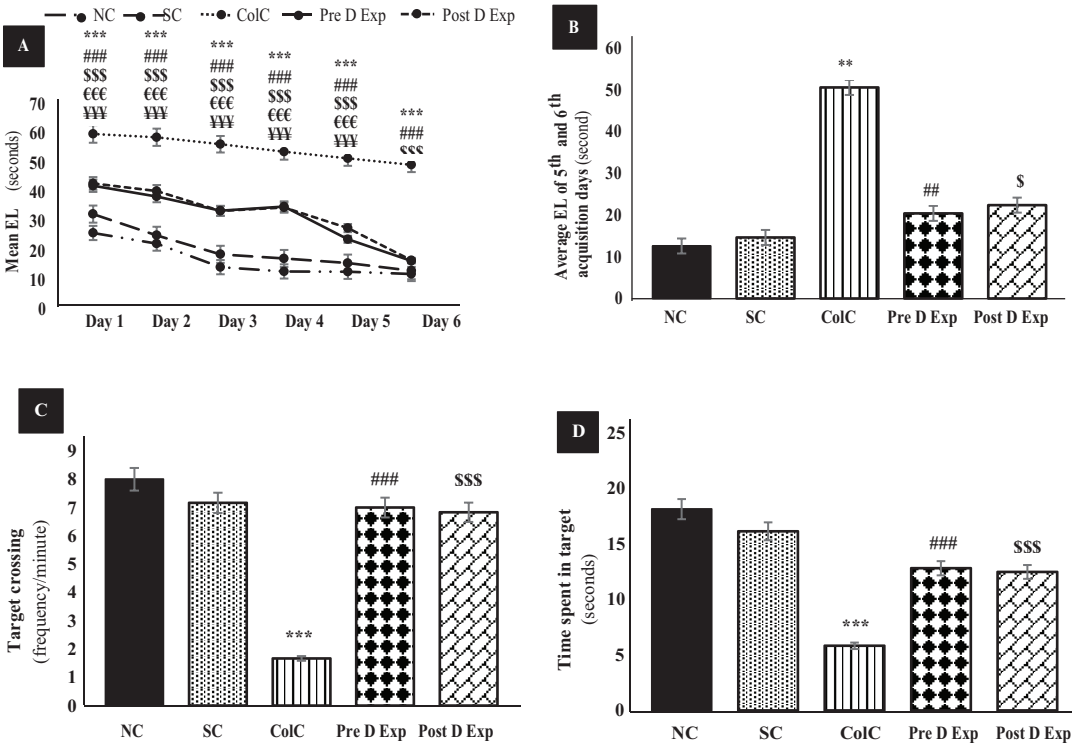

Figure 3: Reference memory performance in the Morris Water Maze test. Mean escape latency (EL) of 4 trials (1^{st} /2^{nd} /3^{rd} /4^{th}) in consecutive 6 days (A); average EL of 5^{th} and 6^{th} acquisition days (B);target crossing (C);and time spent in target (D).Data were in mean±SEM for 6 rats.Statistical analysis was done by ANOVA (among groups) followed by Bonferroni's post hoc test (between groups). * = SC vs ColC, # = ColC vs Pre D Exp, $ = ColC vs Post D Exp, • = NC vs Pre D Exp, ¥ = NC vs Post D Exp. In the interpretation of results, $p \leq 0.05$ was considered as significant. */#/$/•/¥ = $p \leq 0.05$; **/##/$$/••/¥¥ = $p \leq 0.01$; ***/###/$$$/•••/¥¥¥ = $p \leq 0.001$.NS=Normal saline; SS=Stereotaxic surgery. Normal control=No NS, no SS, no D, Sham control= NS with SS, no D; Colchicine control=Colchicine with SS, no D; Pre colchicine D=10,000IU D for 28 days, before colchicine with SS; Post colchicine D= 10,000IU D for 28 days, after colchicine with SS

all test days were found statistically non-significant.These data revealed that colchicine impaired the learning ability of working memory and this impairment was reversed near to normal by systemic D.

In our experiment, ColC rats showed significantly (p≤0.01)lower savings (i.e., learning inefficiency) in comparison to those of SC rats. But D supplementation could not improve savings in our experimentalrats, as evidenced by non-significant difference between our ColC and experimental rats. However, the differences of this variable were found statistically significant between NC vs Pre D Exp(p≤0.01) and NC vs Post D Exp(p≤0.05) but statistically non-significant between Pre D Exp vs Post D Exp rats (Figure 4B). Above data indicated that our D supplementation protocol could not prevent or alleviate colchicine induced learning inefficiency.

Effect of vitamin D on hippocampal oxidative status in duced by colchicine -

In figure 5, we observed that our ColC rats showed significantly (p≤0.001) higher hippocampal MD Aand lower GPx level in comparison to those of Sc rats. However, vitamin D caused a significantly lower MDA and higher GPx in Pre D Exp (p≤0.001) as well as in Post D Exp (p≤0.01; p≤0.001) rats in comparison to those of ColC rats. Strikingly, therewas non-significant differences between NC vs Pre D Exp and NC vs Post D Exp as well as Pre D Exp vs Post D Exp rats. Following data denoted that, colchicine caused hippocampal oxidative stress and our D could prevent and alleviate this stress near to normal.

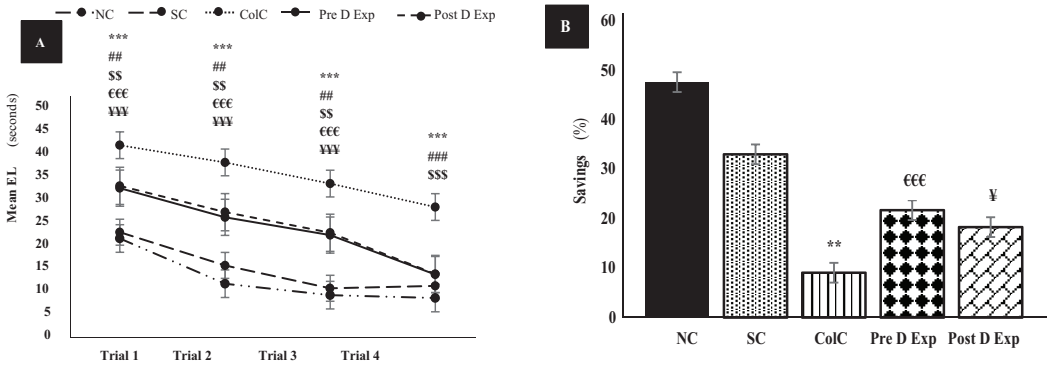

Figure 4: Working memory performance in the Morris Water Maze test. Mean escape latency (EL) of 4 trials (1s,2s,3s,4s) in consecutive 4 days; and savings (B). Data were in mean±SEM for 6 rats. Savings =Differences of trial 1 and trial 2, expressed as percentage of trial 1. Statistical analysis was doneby ANOVA (among groups) followed by Bonferroni's post hoc test (between groups). * = SC vs ColC, # = ColC vs Pre D Exp, $ = ColC vs Post D Exp, • = NC vs Pre D Exp, ¥ = NC vs Post D Exp. In the interpretation of results, p≤0.05 was considered as significant. */#/$/•/¥ = p≤0.05; **/##/$$/••/¥¥ = p≤0.01; ***/###/$$$/•••/¥¥¥ = p≤0.001. NS=Normal saline; SS=Stereotaxic surgery. Normal control=No NS, no SS, no D, Sham control= NS with SS, no D; Colchicine control=Colchicine with SS, no D; Pre colchicine D=10,000IU D for 28 days, before colchicine with SS; Post colchicine D= 10,000IU D for 28 days, after colchicine with SS

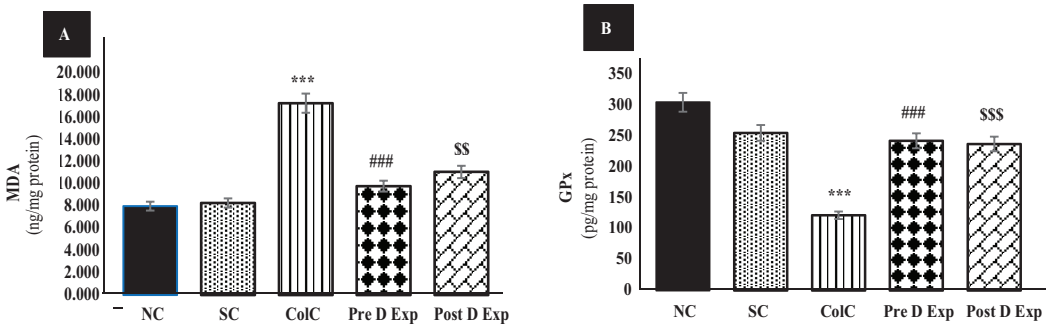

Figure 5: Hippocampal Malondialdehyde (MDA) (A) and Hippocampal Glutathione peroxidase (GPx) (B) level in different groups of rats. Data were in mean±SEM for 6 rats. Statistical analysis was done by ANOVA (among groups) followed by Bonferroni's post hoc test (between groups). * = SC vs ColC, # = ColC vs Pre D Exp, $ = ColC vs Post D Exp, • = NC vs Pre D Exp, ¥ = NC vs Post D Exp. In the interpretation of results, p≤0.05 was considered as significant. **/##/$$/••/¥¥ = p≤0.01; ***/###/$$$/•••/¥¥¥ = p≤0.001. NC=Normal control; NS=Normal saline; SS=Stereotaxic surgery; SC=Sham control; ColC=Colchicine control; Pre D Exp=Pre colchicine D; Post D Exp=Post colchicine D. Normal control=No NS, no SS, no D, Sham control= NS with SS, no D; Colchicine control=Colchicine with SS, no D; Pre colchicine D=10,000IU D for 28 days, before colchicine with SS; Post colchicine D= 10,000IU D for 28 days, after colchicine with SS.

Discussion

Memory, especially spatial memory, one of the most complex process of the brain, which is necessary for proper functioning of any living being. If it is impaired, one's intellectual ability would be hindered sufficiently enough, to interfere his/her personal behaviors and social communications.

Here, our spatial memory impaired rats showed both reference and working memory deficit along with oxidative stress as evidenced by lowered learning ability, consolidation, learning efficiency, learning strength (retrieval) as well as increment of malondialdehyde (MDA) and decrement of glutathione peroxidase (GPx) in comparison to those of normal and sham control rats. Similar findings were reported by Kumar et al.[29] and Pourkhhodadad et al.[30] in colchicine induced memory impaired rats.

In our spatial memory impaired rats, colchicine might increase the formation of Beta amyloid protein (BAP)[31] by increasing the action of β and γ secretase on amyloid precursor protein (APP)[14]. This BAP might accumulate extracellularly forming Aβplaques[14,32]resulted in neuronal death. These plaques might also initiate an inflammatory response,[14] along with direct action of colchicine on increment of cyclooxygenase-2 (COX-2) mRNA expression in hippocampus,[33] resulted in hippocampal neuroinflammation and neuronal death.

Moreover, colchicine also might induce direct nitric oxide (NO) production[34] by activation of inducible nitric oxide synthase (iNOS) enzyme.[32] This NO might react with superoxide anions ($\cdot O^-_2$) rapidly to form peroxynitrite ($ONOO\cdot$) resulted in oxidative stress and hippocampal neuronal death. In addition, this neurotoxic agent might elevate glutamate binding site in hippocampal neurons.[25]Excessive glutamate activity might cause α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA) receptor mediated Na^+ and Ca^{2+} influx and neuronal excitotoxicity. This might further enhance Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptor in neurons.

Both of these effects resulted in excess depolarization of hippocampal neurons causing increased ATP consumption by NA^+/K^+ ATPase. This might stimulate oxidative phosphorylation followed by $\cdot O^{-2-}$ anion formation.[51] In addition to increment of ATP consumption, this depolarization might open a large number of voltage gated Ca^{2+} channel (VGCC) and might increase intracellular Ca^{2+}, causing augmentation of NOS activity.[36] This process might be responsible oxidative stress[36] and neuronal death in hippocampus. Moreover, this markedly increased $\cdot O^{-2}$ and $ONOO\cdot$ might cause lipid peroxidation[19] and might elevate hippocampal MDA level in our memory impaired rats.[30]In addition, this increment of oxidants might cause increased consumption of GPx[19] for oxidative balance and decrease GPx concentration[30] in hippocampus.

In our study, intraperitoneal vitamin D might cause neuroprotection by clearing of Aβ plaques[7,16] through enhancing it's phagocytosis by macrophages[37]. This vitamin might also reduce neuroinflammation by inhibiting the expression of COX-2 mRNA along with cessation of NO synthesis by inhibiting the iNOS activity directly.[11] In addition, D supplementation might enhance choline acetyl transferase (ChAT) and decrement of acetylcholine esterase (AhCE)[38] in hippocampus and improve the cholinergic system driven spatial learning and memory impairment in our rats. Moreover, this multipotent vitamin might regulate Ca^{2+} homeostasis by down regulating the VGCC in hippocampal neurons.[10]

In addition, D reduced oxidative stress in our experimental (pre and post treated) rats as evidenced by decrement of MDA and increment of GPx when compared to spatial memory impaired rats. Similar MDA reducing effect of vitamin D was reported byMohamed et al. (2015)[7] in colchicine induced memory impaired rat model.

Here, in this aspect,D might protect cell membrane of our experimental rats through inhibition of lipid peroxidation resulted in decrement of MDA[12,16] and increment of GPx leve[12,16,39] via stimulating the antioxidant gene expression of nuclear factor erythroid 2- related factor 2 (Nrf2) system.[40]

As a whole, both of our experimental rats (Pre D Exp and Post D Exp) showed almost similar reference memory (on last acquisition day; Figure 3A) and working memory (in last trial; Figure 4A) performances as well as oxidative stress status when compared to those of our normal rats, except savings. From these findings we proposed that our 28 days vitamin D schedule could reverse almost all the aspects of spatial memory disability and oxidative stress near to normal. These results also revealed that, the improvement of learning disability in reference (Figure 3A) as well as working (Figure 4) memory of our experimental rats were somewhat slower than those of normal control rats. It indicated that, their speed of acquisition[41] for learning was low.

Furthermore, when we compared all of these spatial memory and oxidative stress variables between our two experimental groups, they were visualized to be statistically similar preliminarily (Figure 3, 4,5). However, to scrutinize the learning ability of reference memory between our 2 experimental groups (Pre D Exp vs Post D Exp) more precisely, we analyzed the reference memory escape latencies (EL) through a trial by trial basis(Figure 6, table I). Here, in the 1st trial of 1st acquisition day, the latencies to find the platform was significantly higher in our memory impaired rats in comparison to those of other rats and were not decreased any of the following acquisition days. It indicated that our colchicine induced memory impaired rats neither acquire nor retain any new information in whole of our reference memory version of MWM.

On the contrary, all the other rats quickly improved their performances, that is EL, with repeated trials (Trial 1 to 4), though our experimental (Pre D Exp and Post D Exp) rats required a relatively longer EL in 1st trial from 3rd

acquisition day onwards in comparison to controls.In addition, our normal control rats reached a steady performance (i.e., similar EL in 2nd, 3rd, 4th trials along with similar EL between last and first trial of two consecutive days)[41] almost on 3rd acquisition day. Whereas, only the Pre D Exp rats (not Post D Exp) could avail this steady state on the 5th and 6thacquisition day (Table I) i.e., non-significant differences between EL on 2nd, 3rd, 4th trials (Figure 6; statistical analysis not shown) and similar EL between day 5trial 4 and day 6trial 1 ($p = 0.331$, table II).

These findings exhibited that, our D supplementation before hippocampal damage could help to regain the RM as well as WM learning ability as well as consolidation power, though it was a little bit slower to develop in comparison to those of normal rats. On the contrary, unlike to normal rats, our Post D Exp rats were unable to consolidate the learned information from previous day and had to systematically relearn the platform position again everyday.

This proposal was further supported by comparatively better consolidation power [average EL of 5th and 6th acquisition days (20.39 vs 22.37); figure 3B] along with comparatively better oxidative status [hippocampal MDA (9.85 vs 11.13) and GPx(312.99 vs 298.58)level, figure 5] in Pre D supplemented rats in comparison to those of Post D supplemented group, though the differences between them were statistically non-significant.

In addition, in our study, we used 15 µg of colchicine in each hippocampus to cause spatial memory impairment by extensive hippocampal cells damage along with hippocampal atrophy, as proposed by Nakagawa et al. (1987).[25]As the spatial memory improvement in our experimental rats were prevented by the 28 days vitamin D supplementation schedule, so it is functionally verified that both of hippocampal neurogenesis as well as volume increment have been occurred here. This proposal is further supported by Barrett et al. (2019).[14] But it was our limitation that we could not demonstrate it morphologically.

Conclusion

Vitamin D can prevent as well as alleviate intrahippocampal colchicine induced spatial working memory dysfunction along with oxidative stress in male Long-Evans rats. However, in perspective of spatial reference memory improvement, it was only found to be preventive, not alleviative. In addition, D supplementation was sufficient enough to prevent these alarming consequences to almost normal, but somewhat slowly.

Conflict of interest

Authors declare no conflict of interest.

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