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Vitamin D3 attenuates oxidative stress and cognitive deficits in a model of toxic demyelination

Sepideh Tarbali [1], Shiva Khezri [1*]

[1] Department of Biology, Faculty of Science, Urmia University, Urmia, Iran

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ABSTRACT

Objective(s): Multiple sclerosis (MS) is a demyelinating disease. The prevalence of MS is highest where environmental supplies of vitamin D are low. Cognitive deficits have been observed in patients with MS. Oxidative damage may contribute to the formation of MS lesions. Considering the involvement of hippocampus in MS, an attempt is made in this study to investigate the effects of vitamin D3 on behavioral process and the oxidative status in the dorsal hippocampus (CA1 area) following the induction of experimental demyelination in rats.

Materials and Methods: Animals were divided into six groups. Control group: animals received no surgery and treatment; saline group: animals received normal saline; sham group: animals received 150 µl sesame oil IP; vitamin D3 group: animals received 5 µg/kg vitamin D3 IP; lysophosphatidyl choline (LPC) group (toxic demyelination's model): animals received LPC by stereotaxic intra-hippocampal injection of 2 µl LPC in CA1 area; Vitamin D3- treated group: animals were treated with vitamin D3 at doses of 5 µg/kg IP for 7 and 21 days post lesion. The spatial memory, biochemical parameters including catalase (CAT) activities and lipid peroxidation levels were investigated.

Results: Animals in LPC group had more deficits in spatial memory than the control group in radial arm maze. Vitamin D3 significantly improved spatial memory compared to LPC group. Also, results indicated that vitamin D3 caused a decrease in lipid peroxidation levels and an increase in CAT activities.

Conclusion: Current findings suggest that vitamin D3 may have a protective effect on cognitive deficits and oxidative stress in toxic demyelination's model.

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Introduction

Demyelination, the process or state resulting from the loss or destruction of myelin, is a distinguishing feature of diseases such as multiple sclerosis (MS) (1). Several studies on animal models have been conducted to study MS; among these, local models of demyelination are particularly useful for assessing the mechanisms involved in the processes of demyelination and remyelination. Lysophosphatidyl choline (LPC) is a specific toxin for myelination of the target cells with a less effect on other neural cells. Following injection into the white or gray matters of the brain or spinal cord, this gliotoxin can induce specific demyelination (2).

The adult hippocampus, an important center for learning and memory, is extremely vulnerable to various insults and neurological diseases (3). Cognitive deficits have been observed in MS patients suffering from hippocampus insults (4). Among these reported cognitive deficits, memory dysfunction is relatively common (5). The cause of memory dysfunction in MS is currently unknown, but neuroimaging studies suggest that hippocampal pathology is involved (6), particularly, atrophy of the CA1 region (7). Considering this, an attempt is made in this study to investigate cognitive deficits in an experimental model of MS.

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to detoxify the reactive intermediates or easily repair the resulting damage (8). It has been also proven that oxidative stress has an essential role in the inflammatory processes and in the pathogenesis of MS (9). The brain is particularly vulnerable to oxidative damage due to: i) elevated use of oxygen; ii) low antioxidant levels; and iii) high phospholipid levels (10). Furthermore, decreased cellular antioxidant defense systems in the central nervous system (CNS) can increase the injury observed in MS (11). Also, the hippocampus is a site for functionally significant

*Corresponding author: Shiva Khezri. Department of Biology, Faculty of Science, Urmia University, Urmia, Iran. Tel: +98-44-31942122; email: sh.khezri@urmia.ac.ir

oxidative damage (12). Therefore, in this study we chose dorsal hippocampus (CA1 area) to assess markers of oxidative stress in rats.

Mounting evidence indicates that vitamin D3 supplementation is correlated with a lower risk of developing MS (13, 14). Also, antioxidant such as vitamin D3 (15) has a protective role in neurons (16) and has been proposed to use by MS patients to attenuate the disease progression (17). In the present study, we used a toxin-induced model of demyelination in the CA1 area of adult rat to assess the effect of vitamin D3 on behavioral process. Also, the catalase (CAT) activities and lipid peroxidation levels were assessed as indices of oxidative status into the lesion site.

Materials and Methods

Animals

All experiments were carried out on adult male wistar rats with weight ranging from 200 to 250 g (Pastor Institute, Karaj, Iran). Animals were housed seven per cage under a 12-hr light/dark cycle in a room with controlled temperature (23± 2 ˚C). Food and water were available *ad libitum*. All the experiments were carried out according to the protocol approved by the Animal Ethics Committee of Urmia University, Urmia, Iran. Animals were divided into six groups; each group contained seven animals. Control group: animals received no surgery and treatment; saline group: animals received normal saline in CA1 area; sham group: animals received 150 µl sesame oil IP; vitamin D3 group: animals received 5 µg/kg vitamin D3 IP; LPC group (toxic demyelination's model): animals received LPC by stereotaxic intra-hippocampal injection of 2 µl LPC in CA1 area; Vitamin D3- treated group: animals were treated with vitamin D3 at doses of 5 µg/kg IP (from DSM Nutritional Products, Village-Neuf, France) (18, 19) for 7 and 21 days post lesion.

Stereotaxic surgery

After 1 week of acclimatization, rats were anesthetized with a mixture of ketamine hydrochloride (Sigma, Germany) and xylazine (10 and 2 mg/kg IP, respectively) (20) and mounted in a stereotaxic frame (Stoelting, USA) in the skull-flat position. Dura was exposed by using an electric high-speed drill at the appropriate previously labeled site on the skull. Demyelination was induced bilaterally by direct single injection of 2 µl of LPC 1% (Sigma, St. Louis, USA) in sterile saline 0.9% (21) into the CA1 area of hippocampal formation, using appropriate stereotaxic coordinates -3.8 mm posterior to bregma, ±2.2 mm lateral to the midline and +3.2 mm ventral of the dorsal surface of the skull (AP= -3.8; ML= ±2.2; DV= +3.2) (22) by using a 5 µl Hamilton syringe. Saline group (animals received saline in CA1 area) were injected with an equal volume of sterile saline. LPC or saline was injected at a rate of 1 µl/min using micro injection pump (Stoelting, USA), and the needle was kept in place for 5 min to allow the injected solutions and tissue to equilibrate and avoid the possible reflux through the needle track. Incisions were ligated with silk thread.

Radial arm maze test

Working and reference memory can be separately and simultaneously assessed in a version of the working and reference memory tasks in a radial arm maze (23). The maze consisted of eight arms (Nos. 1-8; 48×12 cm) extending radially from a central area. At the end of each arm, there was a food cup that held a single 50-mg food pellet. The apparatus was surrounded by various extra-maze cues such as a laboratory bench, posters and a clock. The extra-maze cues were placed in the same positions during the study (24). Prior to the performance of the maze task, the animals were kept on a restricted diet, and body weight was maintained at 85% of their free-feeding weights. Each animal was placed individually in the center of the maze where the same four arms (Nos. 1, 2, 4 and 7) were baited for each daily training trial. The other four arms (Nos. 3, 5, 6 and 8) were never baited (24). On the first day, for habituation of the rats to the new environment of the maze, they were individually placed inside the center of the maze and were given 5 min to explore the maze. During the habituation day, rats did not receive food. The second and third days of training sessions consisted of two sessions per day for 5 min in the morning and evening. An arm entry was counted when all four limbs of the rat were within an arm. The test sessions, which were performed after training sessions, included one session per day on days 4-7 and 18-21. Measurements were made on the number of spatial reference memory errors (entering an arm that was not baited), spatial working memory errors (entering an arm containing food but previously entered) and the time taken to find food containing arms as a criterion for learning assessment (25, 26). The radial arm maze was cleaned with 70% ethanol and dried before each trial.

Measurements of oxidative stress markers in the hippocampus

For the biochemical assays, the animals were deeply anesthetized and the hippocampus removed. The hippocampus was washed with ice-cold saline solution 0.9%, weighed and kept at -80 ˚C until used for preparation of homogenates.

Lipid peroxidation assay

Lipid peroxidation was assayed by measuring the level of malondialdehyde (MDA) in the hippocampus. MDA results from degradation of polyunsaturated

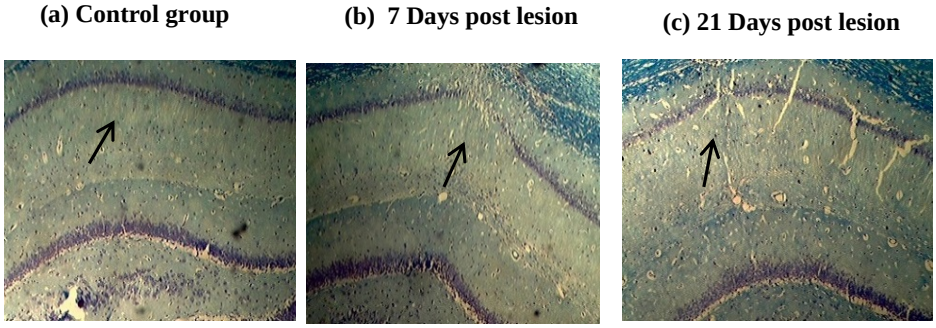


Figure 1. The approximate point of the lysophosphatidyl choline (LPC) injection in the dorsal hippocampus (CA1 area) after myelin specific staining using luxol fast blue and cresyl fast violet: Light micrographs for (a) control group (i.e., animals received no surgery and treatment, saline, sesame oil and vitamin D3 treated rats); (b) LPC-treated group on 7th days post lesion; and (c) LPC-treated group on 21st days post lesion are shown as representative. Arrows show demyelinated sites (× 100)

fatty acids. The production of this substance is used as a biomarker to measure the level of lipid peroxidation. MDA levels were determined according to Esterbauer and Cheeseman method (27). MDA reacts with thiobarbituricacid to produce a colored complex having peak absorbance at 535 nm. The resulting complex was quantified using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol of MDA per g tissue.

Catalase activity assay

CAT activity was assayed by measuring the absorbance decrease at 240 nm in a reaction medium containing 100 µl H_2O_2, 2.8 ml phosphate buffer pH 7.0 and 100 µl of the sample, according to Aebi method (28). One unit of enzyme is defined as 1 µmol of H_2O_2 consumed per min and the specific activity is reported as unit per g tissue.

Tissue processing and histology

Animals were re-anesthetized on days 7 and 21 post lesion and were perfused intracardially with 0.1 M phosphate buffered saline (PBS) and then with a solution of 4% paraformaldehyde in 0.1 M PBS (pH=7.4). The hemispheres were taken out and post fixed overnight in the same fixative at 4 °C. For paraffin embedding, tissues were first dehydrated in alcohol, cleared by incubations in xylene, and finally embedded in paraffin for 3 hr, and then blocked. Coronal serial sections (5 µm thickness) were obtained from the hemispheres using a rotary microtome and then were stained with 0.1 % luxol fast blue (British Drug House, UK) solution at 60 °C for 3 hr. Adequate contrast was made by transient immersion of preparations in 0.05 % lithium carbonate and 70 % alcohol. After washing with distilled water, the sections were counterstained with 0.1 % cresyl fast violet (Merck, Germany) for 4 min. Sections were washed in distilled water again and dehydrated in a graded series of alcohols, then cleared in xylene, cover slipped (29), and the sites of demyelination were verified. Only animals whose demyelination sites were exactly placed in the CA1 area were used for data analysis (Figure 1).

Statistical analysis

The results are expressed as mean ± SEM. Data from biochemical assessments were analyzed using one-way analysis of variance (ANOVA) followed by Tukey *post hoc*. The averages obtained from behavioral assessments were compared by using analysis of variance for repeated measures. The *P*-value less than 0.05 was considered to be statistically significant.

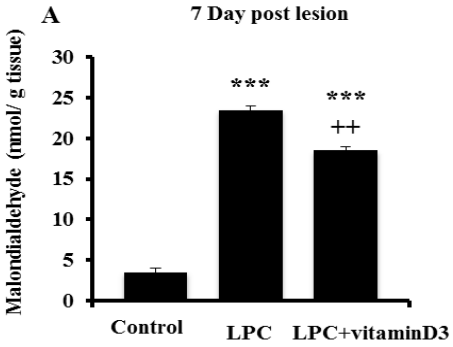
Results

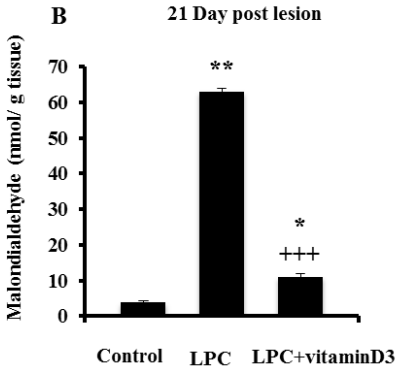
LPC-induced demyelination

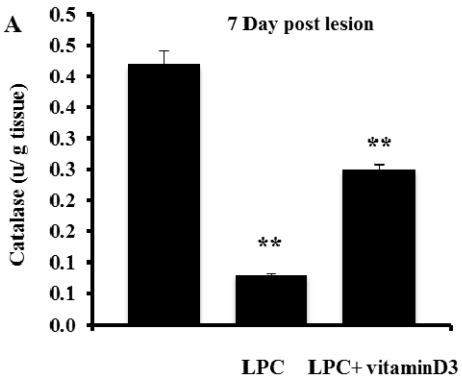
We profited from a demyelination model by LPC injection that was able to induce demyelination in CA1 area. Representative histological micrographs for control group, 7 and 21 days post lesion are shown in Figure 1. Since there was no difference between the control, saline, sham and vitamin D3 groups, the data for these four groups were merged and mentioned as control data.

Effect of vitamin D3 on lipid peroxidation

Figure 2 illustrates the MDA levels on day 7 and 21 post lesion for all the animals considered in the experiment. As is shown in Figure 2A, seven days post lesion, MDA levels in LPC- treated animals and vitamin D3- treated animals was significantly higher than that in control animals (both $P<0.001$). However, administration of vitamin D3 during this 7 days reversed this effect ($P<0.01$, Figure. 2A). Assessment of MDA levels on 21 days post lesion indicated a significant increase in MDA levels in LPC-treated animals and vitamin D3- treated animals compared to control animals ($P<0.01$ and $P<0.05$, respectively), while vitamin D3 administration for 21 days significantly ($P<0.001$) ameliorated this index compared to LPC-treated animals (Figure 2B).







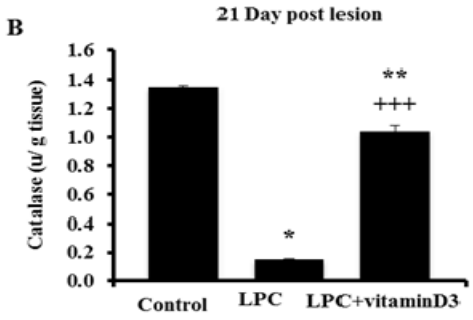


Figure 2. Effect of vitamin D3 on the malondialdehyde levels (MDA: nmol/g tissue) in hippocampus on (a) 7th and (b) 21st days after local injection of lysophosphatidyl choline (LPC) (Mean± SEM). Groups indicated with*, ** and*** have $P < 0.05$, $P<0.01$ and $P<0.001$ compared to the control groups (i.e., animals received no surgery and treatment, saline, sesame oil and vitamin D3 treated rats), respectively. Group indicated with ++ and +++ have $P< 0.05$ and $P<0.001$ compared to the LPC- treated rats, respectively

Figure 3. Effect of vitamin D3 on the catalase activity in hippocampus (CAT: u/g tissue; u= 1 µmol H_2O_2 min^{-1}) on (a) 7th and (b) 21st days after local injection of lysophosphatidyl choline (LPC) (Mean± SEM). Groups indicated with* and ** have $P<0.05$ and $P< 0.01$ compared to the control groups (i.e., animals received no surgery and treatment, saline, sesame oil and vitamin D3 treated rats), respectively. Group indicated with +++ have $P<0.001$ compared to the LPC- treated rats

Effect of vitamin D3 on catalase activity

On 7 days post lesion, the CAT activities in LPC-treated animals and vitamin D3- treated animals was significantly less than that in the control animals (both $P<0.01$, Figure 3A). However, administration of vitamin D3 during these 7 days did not show any significant effect on the CAT activities (Figure 3A). Also, on the day 21 the CAT activities in LPC- treated group was less than that in control group ($P<0.05$, Figure 3B). Vitamin D3 increased the CAT activities to an upper level than control group ($P<0.01$). Also animals that received vitamin D3 daily for 21 days showed a significant increase in the CAT activities compared to LPC- treated group ($P<0.001$, Figure 3B).

Effect of vitamin D3 on behavioral characteristics

To compare the performance of rats in the radial arm maze, the trial time, working memory and reference memory processes examined simultaneously. Our results showed that on days 4-7, trial time in LPC- treated animals (on the 6th and 7th days) ($P<0.05$ and $P<0.01$, respectively), and in vitamin D3- treated animals (on the 7th day) ($P<0.05$) was significantly higher than that in control animals. Compared to the LPC- treated group, administration of vitamin D3 for 7 days significantly decreased the trial time (on the 7th day) ($P<0.01$, Figure 4A). On days 18-21, trial time in LPC- treated animals on the 19th, 20th and 21st days ($P<0.05$, $P<0.01$ and $P<0.05$, respectively) and also in the vitamin D3- treated animals on the 19th, 20th and 21st days was significantly higher than that in control animals ($P<0.01$, $P<0.05$ and $P<0.05$, respectively). Compared to the LPC- treated group, administration of vitamin D3 for 21 days significantly decreased the trial time on the 19th, 20th and 21st days ($P<0.001$, $P<0.05$ and $P<0.05$, respectively) (Figure 4B).

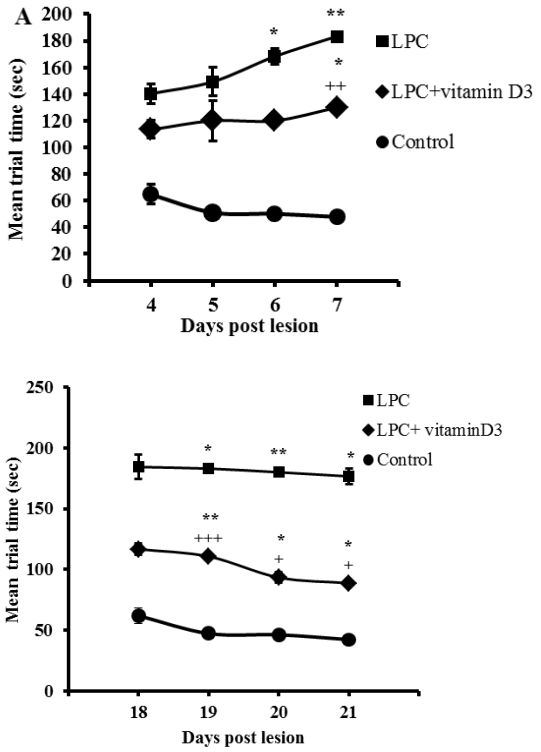


Figure 4. Effect of vitamin D3 on the trial time on (a) 4-7th and (b) 18-21st days after local injection of lysophosphatidyl choline (LPC) (Mean± SEM). Groups indicated with* and ** have *P*<0.05 and *P*<0.01 compared to the control groups (i.e., animals received no surgery and treatment, saline, sesame oil and vitamin D3 treated rats), respectively. Group indicated with +, ++ and +++ have *P*<0.05, *P*<0.01 and *P*<0.001 compared to the LPC- treated rats, respectively

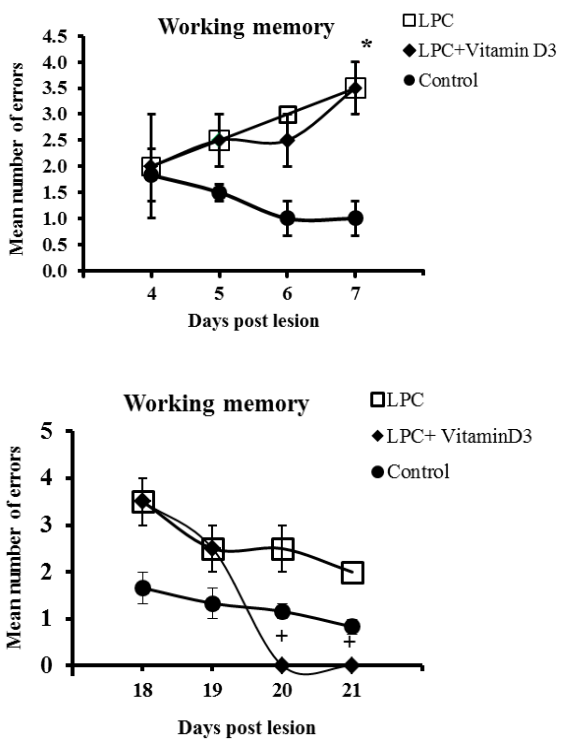


Figure 5. Effect of vitamin D3 on the working memory errors on (a) 4-7th and (b) 18-21st days after local injection of lysophosphatidyl choline (LPC) (Mean± SEM). Groups indicated with* have *P*<0.05 compared to the control groups (i.e., animals received no surgery and treatment, saline, sesame oil and vitamin D3 treated rats), respectively. Group indicated with + have *P*<0.05 compared to the LPC- treated rats

The results indicated that in LPC- treated animals on the 7th days, working memory errors was significantly higher than that in control animals (*P*<0.05). In vitamin D3- treated animals for 7 days, there was no significant difference compared to the control and LPC- treated animals (Figure 5A). On days 18-21, there was no significant difference in LPC- treated and vitamin D3- treated animals compared to the control animals. Administration of vitamin D3 for 21 days significantly decreased the working memory errors compared to the LPC-treated group on the 20th and 21st days (both *P*<0.05, Figure 5B).

As shown in Figure 6A, reference memory errors in LPC- treated animals on the 4th, 5th, 6th and 7th days (*P*<0.05, *P*<0.05, *P*<0.05 and *P*<0.001, respectively) and in vitamin D3- treated animals on the 7th days (*P*<0.05) was significantly higher than that in control animals. Administration of vitamin D3 for 7 days significantly decreased the reference memory errors on the 7th days (*P*<0.05) compared to the LPC-treated group. The reference memory errors in LPC- treated animals on the 20th and 21st days was significantly higher than that in control animals (*P*<0.01 and *P*<0.05, respectively) (Figure 6B). There was no significant difference among control and vitamin D3- treated animals. While administration of vitamin D3 for 21 days significantly decreased the reference memory errors on the 20th and 21st days (both *P*<0.05) compared to the LPC- treated group (Figure 6B).

Discussion

Several studies have proposed that demyelinating insults occur in CNS gray matter of MS patients. Hippocampal formation is known as one of the important gray matters, which are reported to be affected by MS (30). The purpose of the present study was to investigate the effect of vitamin D3 on the behavioral process and oxidative stress of rat hippocampal formation following local injection of LPC. Several studies on experimental autoimmune encephalomyelitis (EAE), which is an animal model of MS, have illustrated that supplementation with active vitamin D blocks the onset and progression of EAE (18, 19). Thus, theoretically, vitamin D might be an

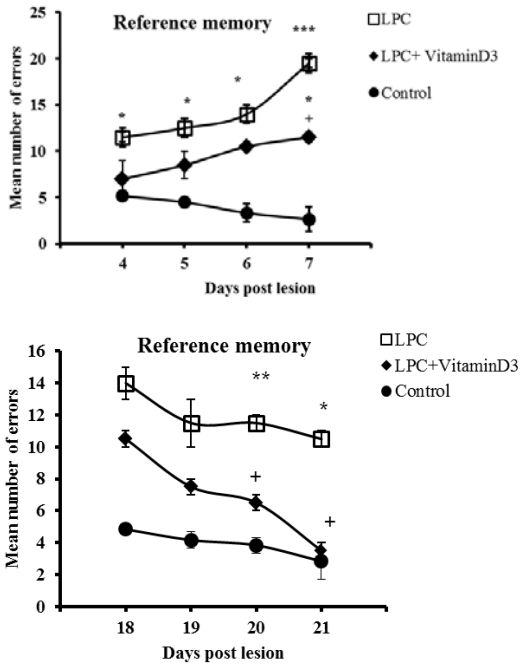


Figure 6. Effect of vitamin D3 on the reference memory errors on (a) 4-7th and (b) 18-21st days after local injection of lysophosphatidyl choline (LPC) (Mean± SEM). Groups indicated with*, **and *** have *P*<0.05, *P*<0.01 and *P*<0.001 compared to the control groups (i.e., animals received no surgery and treatment, saline, sesame oil and vitamin D3 treated rats), respectively. Group indicated with + have *P*<0.05 compared to the LPC- treated rats

appropriate candidate to alleviate the MS progression.

In this study, our results showed that LPC injection into the CA1 area caused local demyelination. This finding was similar to previous reported results on demyelination caused by LPC administration in the juvenile ventral hippocampus (31) and optic chiasm in rat (20, 21). Although different mechanisms may contribute to the demyelination and neurodegeneration in MS, it became clear in recent years that oxidative stress plays the greatest role in the process (32). Oxidative stress may cause selective oligodendrocyte death, and thereby demyelination (33). Furthermore it is involved in the cascade of events leading to neuronal cell death (34). Various oxidative stress markers have been investigated in MS and the oxidative stress has been reported to be generally increased in MS patients (35). Previous studies have suggested that lipid peroxidation plays a role in the inflammatory processes and in the pathogenesis of MS (9). MDA is the breakdown product of the most important chain reactions leading to the oxidation of polyunsaturated fatty acids, and therefore serves as a reliable marker of oxidative stress-mediated lipid peroxidation (36). It has been demonstrated that the serum lipid peroxidation rates and MDA levels are higher in MS patients (9). Previous studies have reported significant elevations of blood MDA levels in MS patients (37, 38). Also, it was shown that intracerebral injection of ethidiumbromide (demyelinating agent) caused increased MDA in cortex, hippocampus and striatum (39). In accordance with the previous studies, in this study we have observed that the MDA levels are increased in the hippocampus tissue of experimental groups 7 and 21 days post lesion compared with the control group.

The interaction of vitamin D3 with ROS is also important for neuroprotection. Various studies indicated that vitamin D3 possess an antioxidative property (40, 41). In our study, results have shown that administration of vitamin D3 for 7 and 21 days post lesion significantly decreased levels of MDA compared with the LPC- receiving groups. This positive action of vitamins D3 against lipids peroxidation may be caused by its protective and antioxidative effects in brain.

Pro-oxidants like H_2O_2 inhibit expression of myelin genes in human primary oligodendrocytes through cellular redox alterations (42). A number of studies have shown beneficial effects of CAT, a H_2O_2 scavenger, treatment on EAE (43). In this study, the positive effects of vitamins D3 on oxidative stress were also verified by the CAT activities assessment. Subsequent to local injection of LPC, in 7 and 21 days post lesion CAT activity was less than that in control group. Increase in the CAT activities in animals treated with vitamin D3 for 7 days was non-significant. While in 21 days following vitamin D3 administration, CAT activity was significantly higher than that in animals treated with LPC. These results are in accordance with other studies that demonstrated the antioxidative effects of vitamins D3 in dentate gyrus (44) and mesencephalic tissue (45). Since ROS plays a pivotal role in the initial phase as well as the chronic stage of MS, antioxidant therapy might be an attractive approach to limit disease progression (46).

The prevalence of cognitive impairment in MS ranges from 40 to 65% (47). The most common cognitive deficits are reduced speed of data processing, decreased memory and impaired spatial perception (47). There is wide agreement that spatial memory is dependent on the integrity of the hippocampus (48). So that, it has been previously shown that working memory and performance were significantly impaired by bilateral dorsal hippocampal lesions induced by quinnolinic acid (24). Oxidative stress has been implicated in the pathophysiology of several neurodegenerative disorders characterized by progressive cognitive deficits (49). Also, the results of a study showed that ROS generation is associated with impaired memory consolidation post EAE induction (50). Several studies have shown spatial learning and memory

impairment after EAE induction in the Morris water maze and Banes maze (51, 52).

Consistent with these studies, our data clearly indicated that 7 and 21 days post lesion, an increase in trial time, working and reference memory errors was observed in animals treated with LPC compared to the control group. Therefore, spatial memory was significantly impaired by bilateral CA1 lesions. This finding can be caused by demyelination of CA1 area subsequent to local injection of LPC, which leads to impaired spatial memory. Administration of vitamin D3 for 7 and 21 days reversed this effect compared to the LPC- treated group. The results of our study, was similar to other findings based on the effect of 1,25(OH)2D3 supplementation on the maze performance of the animals (53). Consistent to our results, valuable roles of vitamin D in cognitive function have been proposed (54). Also, some reports have demonstrated the effects of vitamin D on learning (55) and behavior (56). Although the antioxidant vitamin D is recommended as a supplement for different therapeutic regimes of MS (18), yet the exact mechanism of how vitamin D may be linked to MS is not clear. Neuroprotection through antioxidative agents (57) could be probable mechanism. One limitation of this study is that the extent of demyelination induced by LPC injection and the myelination intensity has not been assessed.

Conclusion

Findings in the present study indicates that demyelination due to the local injection of LPC into the rat hippocampus is associated with increased oxidative stress. The study suggests an antioxidant effect for the vitamin D3. Based on our results, it seems that treatment with vitamin D3 is able to reduce spatial learning and memory deficits, through its antioxidative effects in an experimental model of MS. This result suggests that vitamin D therapy may help to prevent the development of MS and could be a useful addition to the therapy. However, evaluation of beneficial effects of vitamin D3 on the spatial memory and its role in preventing oxidative stress in MS patients requires much more extensive clinical studies.

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