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EXPERIMENTAL ARTICLES

Analysis of Molecular Events Associated with Adult Rat Dorsal Hippocampus Demyelination Following Treatment with Vitamin D3[1]

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Abstract—Demyelination is the pathological hallmark of multiple sclerosis (MS) lesions. Considering the involvement of hippocampus in MS, we aim to evaluate the effect of vitamin D3 on molecular events in the dorsal hippocampus (CA1 area) following the induction of experimental demyelination in rats. All experiments were carried out on adult male Wistar rats. For demyelination induction, 2 μL lysophosphatidyl choline (LPC) was injected into the CA1 area of rat brain using stereotaxic surgery. Animals were treated with vitamin D3 dissolved in sesame oil at doses of 5 μg/kg intra-peritoneally for 7, 14 and 21 days post receiving LPC. The hippocampus tissue was then removed to measuring the expression of Olig2 (marker of OPCs), GFAP (marker of astrocyte) and Nogo-A (axonal growth inhibitor) genes into lesion. RT-PCR analysis indicated that following hippocampus demyelination Olig2, GFAP and Nogo-A genes expression were significantly increased on days 7, 14 and 21 post lesion. While administration of vitamin D3 for 7, 14 and 21 days post lesion significantly caused a decrease in Olig2, GFAP and Nogo-A genes expression. Our results indicated the positive effect of vitamins D3 on process of remyelination by enhancing oligodendrocyte precursors' recruitment and decreasing of inhibitory genes such as Nogo-A and GFAP in the context of demyelinating diseases like MS.

Keywords: multiple sclerosis, demyelination, remyelination, hippocampus, vitamin D3

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INTRODUCTION

Multiple sclerosis (MS) is a disease characterized by demyelinating lesions and variable remyelination in the CNS [1]. Chronic demyelination predisposes axons to degeneration, an event that is thought to be the major cause of progressive functional decline [2]. Experimental study has shown that remyelination protects axons from demyelination-associated degeneration [3]. The current consensus is that remyelination is generally mediated by a population of precursor cells which are most frequently referred to as oligodendrocyte precursor cells (OPCs) [4]. These cells are identified by the expression of a number of markers such as oligodendrocyte transcription factor 2 (Olig2) [5]. Astrocytes are acutely sensitive to disturbances in their environment and up-regulated a panel of genes such as glial fibrillary acidic protein (GFAP) [6]. In recent years Nogo-A has evolved from being one of the most important axonal regrowth inhibitors present in CNS. Therapeutically, targeting the Nogo-A protein has been tried in an attempt to promote regeneration in spinal cord injury models [7].

Various animal model systems have been developed to study different aspects of remyelination. A common model used to study the remyelination involves focal injections of toxins such as lysophosphatidyl choline (LPC) into the brain. This induces a reproducible demyelination in predefined areas of the CNS at the site of injection [8].

The etiology of MS is still unknown but the disease is associated with certain environmental and hereditary risk factors [9]. Vitamin D is emerging as a probably important environmental factor in MS [10]. Studies have repeatedly found associations between vitamin D levels and disease activity [11, 12] and several authors have suggested that supplementation of vitamin D should be recommended to MS patients [13, 14]. Thus, vitamin D might be an appropriate candidate to alleviate the MS progression.

In MS demyelination not only affects the white matter, but also the grey matter of the brain including the hippocampus [15]. By our knowledge, this is the first study that examines the effect of vitamin D3 on the expression levels of Olig2 (marker of OPCs), GFAP (marker of astrocyte) and Nogo-A (axonal growth inhibitor) in the LPC model in CA1 area of hippocampus in rats.

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The sequences of primers were used for PCR amplification of Olig2, GFAP, Nogo-A and GAPDH

Gene	Primer	Primers sequence
Olig2	Forward	5'-CTCTTTGGCTGAACACTCCAA-3'
	Reverse	5'-CCCAAAAAGGATTCCGTTCCA-3'
GFAP	Forward	5'-CTCGTGTGGATCTGGAGAGGAA-3'
	Reverse	5'-GCCCTCCAGCAATTTCCTGTAG-3'
Nogo-A	Forward	5'-GTCTCTGCTTTG GAACCTCAGA-3'
	Reverse	5'-AAACACCCACATCAACAC TGCA-3'
GAPDH	Forward	5'-AGAACATCATCCCTGCATCC-3'
	Reverse	5'-AGCCGTATTCATTGTCATACC-3'

MATERIALS AND METHODS

Animals. All experiments were carried out on adult male Wistar rats with weighting ranging from 200–250 g (Pastor Institute, Karaj, Iran). Animals were housed four per cage under a 12-h 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 9 groups; each group contained eight animals. Group 1: control animals received no surgery and treatment; Group 2: animals received 2 μL saline by stereotaxic intra-hippocampal injection in CA1 area; Group 3: animals received 150 μL sesame oil intra-peritoneally; Groups 4, 5, 6: animals received 2 μL lysophosphatidyl choline (LPC) by stereotaxic intra-hippocampal injection in CA1 area (they were evaluated 7, 14, and 21 days after injection LPC); Group 7, 8, 9: animals were treated with vitamin D3 dissolved in sesame oil at doses of 5 μg/kg (from DSM Nutritional Products, Village-Neuf, France) [16, 17] intra-peritoneally for 7, 14, and 21 days post receiving LPC.

Stereotaxic LPC microinjection. After 1 week of acclimatization, rats were anesthetized with a mixture of ketamine hydrochloride (Sigma, Germany) and xylazine (10 and 2 mg/kg intra-peritoneal, respectively) [18] 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 0.9% saline [19] 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) [20] by using a 5 μL Hamilton syringe. 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.

Gene expression study. The hippocampus tissue were extracted after scarifying the anesthetized animals and immediately preserved in liquid nitrogen. In order to determine Olig2, GFAP, Nogo-A and GAPDH gene expression levels total RNA was isolated based on isothiocyanate–phenol–chloroform protocol [21], using RNX$^+$ reagent (Cinna Gen, Tehran, Iran) according to the manufacturer's instructions. The final total RNA pellet was suspended in 30 μL of DEPC (diethylpyrocarbonate, Fermentas)-treated water. Five μL of total RNA was used for spectrophotometric determination of the RNA concentration at 260 nm. For each sample cDNA was prepared using 1 μg of total RNA using olig dT primer and M MuLV reverse transcriptase (Fermentas). PCR was performed using 1 μL synthesized cDNA as template, specific primers and 2× PCR Master Mix (CinnaGen, Tehran, Iran) based on the manufacturer's instruction. Primer sequences for Olig2, GFAP, Nogo-A, and GAPDH (housekeeping gene), designed on the basis of the published sequences in Gen Bank (table). Segments of Olig2, GFAP, Nogo-A, and GAPDH cDNAs were amplified for 32, 25, 26 and 25 cycles, respectively. The reaction parameters were adjusted to obtain a condition with linear relation between the number of PCR cycles and PCR products and with linear relation between the initial amount of cDNA template and PCR product. Ten microliters of amplified products was run on 1.5% agarose gel (Roche, Germany). Agarose gels were stained by ethidium bromide (0.5 μg mL^{-1}) and visualized under a UV light. A 50 bp DNA ladder (Gene Ruler 50–1000 bp, Fermentas) was used as a molecular size marker. Semi-quantitative analysis of PCR products were done by band densitometry using a computerized image analyzing system (Carestream Gel Pro 212 Imager, USA). Eight gels were analyzed.

Tissue processing and histology. Additional animals (each group contained three animals) for tissue processing and histology were re-anesthetized on days 7, 14 and 21 post lesion and were perfused intracardially with 0.1 M phosphate buffer 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 h, and 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 h. Adequate contrast was made by transient immersion of preparations in 0.05% lithium carbonate and 70% alcohol. After distilledwater washes, the sections were counter stained 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 [22, 23], and the sites of demyelination were verified. Only animals whose demyelination sites were exactly placed in the CA1 area were used for data analysis (Fig. 1).

Statistical analysis. The results are expressed as mean ± SEM. The values of band density achieved from gel analysis and band densitometry were normalized to GAPDH for each sample. The averages obtained for different groups were normalized to control level, then were compared by using two way ANOVA, followed by the Tukey post-hoc. For all analysis, $p < 0.05$ was considered significant.

RESULTS

Changes in genes expression. To evaluate demyelination and remyelination possesses following LPC-induced experimental demyelination in rat hippocampus, we accomplished several gene expression studies on the lesion site at 7, 14 and 21 days post lesion using semi-quantitative RT-PCR. Gel documentations revealed bands with different densities for Olig2, GFAP, Nogo-A in the experiment time course. Since there was no difference between the animals received no surgery and treatment, saline and sesame oil groups the data for these three groups were merged and mentioned as control data.

Olig2 gene expression. Two-way ANOVA on Olig2 gene expression on day 7 post-lesion (Fig. 2) revealed significant effects ($F_{2,21} = 224.49$, $p = 0.000$). Between-group comparisons indicated that level of Olig2 expression as a marker of OPCs recruitment were began to increase on day 7 in LPC- treated animals and vitamin D3 treated animals post-lesion compared with control group (both $p < 0.001$; Fig. 2). Application of vitamin D3 for 7 days produced a trend to decrease the expression level of Olig2 and the effect of vitamin D3 was statistically significant compared with LPC-treated animals ($p < 0.001$; Fig. 2).

On day 14 post-lesion Olig2 gene expression ($F_{2,21} = 298.21$, $p = 0.000$) was significantly higher than that in LPC-treated animals and vitamin D3 treated animals compared with control group (both $p < 0.001$; Fig. 2). Treatment of animals with vitamin D3 for 14 days post-lesion decreased the expression of Olig2 compared with LPC- treated animals ($p < 0.001$; Fig. 2).

Although Olig2 gene expression level was then decreased on day 21 post-lesion ($F_{2,21} = 412.50$, $p = 0.000$), it still remained higher than that of control group (both $p < 0.001$; Fig. 2). Also following 21 days exposure to vitamin D3 the expression level of Olig2 significantly decreased compared to the LPC-treated animals ($p < 0.001$; Fig. 2).

GFAP gene expression. GFAP mRNA levels was increased 7 days post-lesion ($F_{2,21} = 1132.83$, $P = 0.000$) in LPC-treated animals and vitamin D3 treated animals compared with control group (both $p < 0.001$; Fig. 3). As is shown in Fig. 3, administration of vitamin D3 during 7 days did not show any significant effect on GFAP mRNA levels compared to the LPC-treated rats.

On day 14 post-lesion GFAP gene expression ($F_{2,21} = 402.85$, $p = 0.000$) was significantly higher than that in LPC- treated animals and vitamin D3 treated animals compared with control group (both $p < 0.001$; Fig. 3). Treatment of animals with vitamin D3 for 14 days post-lesion decreased the expression of GFAP compared with LPC- treated animals ($p < 0.001$; Fig. 3).

Although GFAP gene expression level was then decreased on day 21 post-lesion ($F_{2,21} = 796.01$, $p = 0.000$), it still remained higher than that of control group (both $p < 0.001$; Fig. 3). Following 21 days exposure to vitamin D3, the expression level of GFAP significantly decreased compared to the LPC-treated animals ($p < 0.001$; Fig. 3).

Nogo-A gene expression. Between-group comparisons indicated that levels of Nogo-A expression were began to increase on day 7 ($F_{2,21} = 824.18$, $p = 0.000$) in LPC-treated animals and vitamin D3 treated animals post-lesion compared with control group (both $p < 0.001$; Fig. 4). Application of vitamin D3 for 7 days produced a trend to decrease the expression level of Nogo-A and the effect of vitamin D3 was statistically significant compared with LPC-treated animals ($p < 0.001$; Fig. 4).

On day 14 post-lesion Nogo-A gene expression ($F_{2,21} = 630.18$, $p = 0.000$) was significantly higher than that in LPC-treated animals and vitamin D3 treated animals compared with control group (both $p < 0.001$; Fig. 4). Treatment of animals with vitamin D3 for 14 days post-lesion decreased the expression of Nogo-A compared with LPC- treated animals ($p < 0.001$; Fig. 4).

Although Nogo-A gene expression level was then decreased on day 21 post-lesion ($F_{2,21} = 1265.13$, $p = 0.000$), it still remained higher than that of control group (both $p < 0.001$; Fig. 4). Also following 21 days

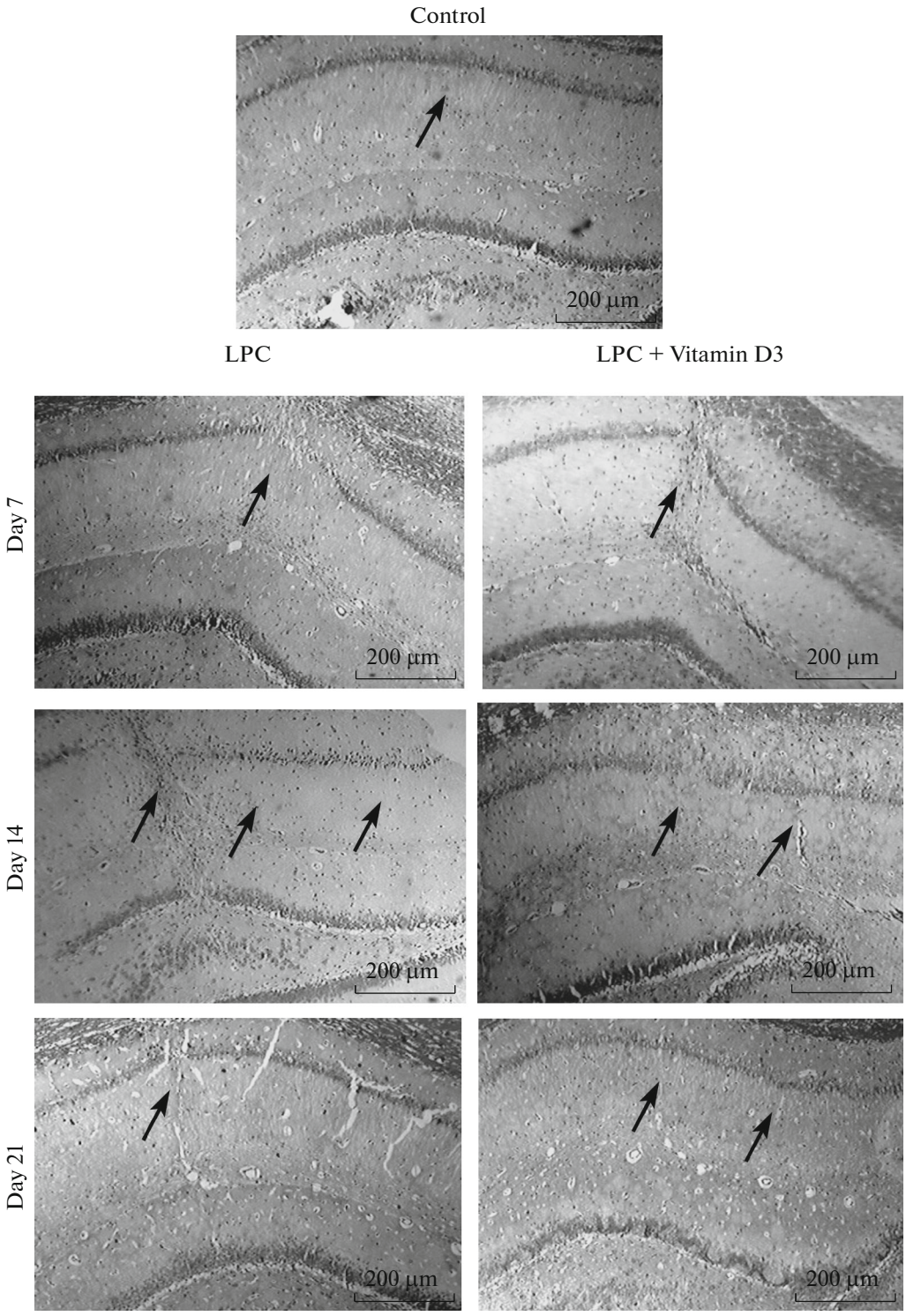

Fig. 1. Representative light micrographs of the coronal sections of the dorsal hippocampus (CA1 area) after myelin specific staining using luxol fast blue and cresyl fast violet for control group (i.e., animals received no surgery and treatment, saline and sesame oil treated rats); LPC-treated groups and vitamin D3- treated groups on 7th, 14th and 21st days post lesion. Arrows show demyelinated sites. Magnification: 100×; Scale bar: 200 μm.

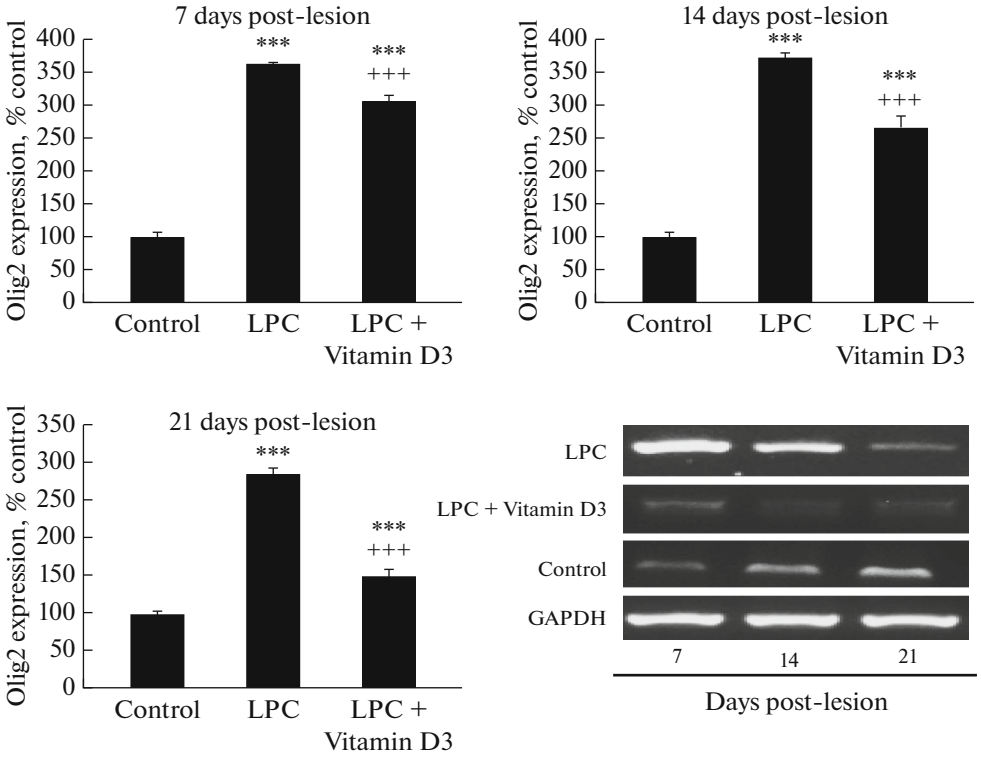

Fig. 2. Effect of vitamin D3 on the expression of Olig2 gene using semi-quantitative RT-PCR on days 7, 14 and 21 post-LPC injection into the hippocampus (Mean ± SEM). *** $p<0.001$ as compared to the control groups (i.e., animals received no surgery and treatment, saline and sesame oil treated rats), respectively. +++ $p < 0.001$ as compared to the LPC-treated rats. Shows representative bands for Olig2 gene.

exposure to vitamin D3 the expression level of Nogo-A significantly decreased compared to the LPC-treated animals ($p < 0.001$; Fig. 4).

DISCUSSION

Studies suggest that the neuronal system, including the hippocampal subfields and glial cells, is the potential target of vitamin D [24, 25]. Most of the biological effects of the vitamin D are mediated by vitamin D receptor. This receptor is expressed by hippocampus neurons and glial cells [26]. The suggestion that vitamin D may have a protective effect on MS is not new. It has been previously hypothesized that vitamin D is a natural inhibitor of MS [27]. In this study we have investigated the model of demyelination following local injection of LPC in CA1 area of hippocampus in terms of the changes in expression of mRNA of the Olig2, GFAP and Nogo-A genes. A local injection of LPC into the brain induces demyelination, as seen in the hippocampus [28] and optic chiasm in rat [18, 19].

There is increasing evidence that neurogenesis occurs in the adult brain, but axonal regeneration in immune mediated encephalomyelitis (EAE) and MS still requires the mobilization and recruitment of neural precursor cells to the lesion site [29]. It is generally believed that remyelination is mediated through OPCs in the adult CNS [30]. Within the adult CNS, a quiescent population of OPCs becomes activated in response to demyelination, such as occurs in MS [31], and responds by proliferation and migration, and finally differentiation into myelin sheath forming oligodendrocytes [32]. Olig2 plays essential roles in oligodendrocyte specification and differentiation [33] and is used in different studies as OPCs marker [34, 35]. Changes in the expression of Olig2 show the relative number of activated OPCs in the site of demyelination [31]. Our result showed an increase in the level of Olig2 mRNA on day 7, 14 and 21 post-lesion in LPC-treated rats. Thus, increase in Olig2 gene expression observed in our study could reflect existence of oligodendrogenesis potential in the demyelinated CA1 area. Consistent with our results, several studies have reported that the mRNA of Olig2 increased following demyelination induction [18, 19, 31, 36]. This elevated quantity of expression Olig2 in LPC-treated rats was significantly attenuated by vitamin D3. Reversed expression of Olig2 after treatment with vitamin D3 on days 7, 14 and 21 may be due to differentiation of progenitor cells to adult oligodendrocytes that express a lower level of this transcription factor. It has been previously hypothesized that vita-

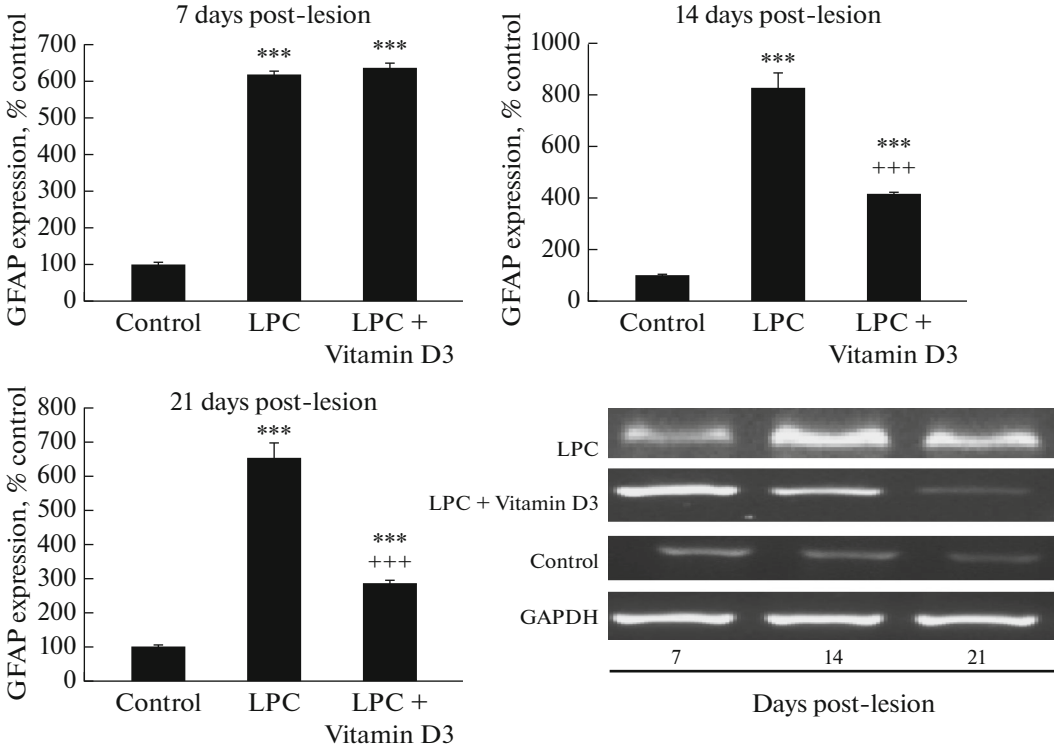

Fig. 3. Effect of vitamin D3 on the expression of GFAP gene using semi-quantitative RT-PCR on days 7, 14 and 21 post-LPC injection into the hippocampus (Mean ± SEM). ***$p < 0.001$ as compared to the control groups (i.e., animals received no surgery and treatment, saline and sesame oil treated rats), respectively. +++$p < 0.001$ as compared to the LPC-treated rats. Shows representative bands for GFAP gene.

min D could alleviate MS symptoms by protecting myelin through activation of oligodendrocytes [37].

In the CNS following injury astrocytes become reactive and respond in a typical manner, termed astrogliosis. Astrogliosis is characterized by up-regulation of GFAP [38, 39]. Zaaraoui et al. have proposed that GFAP negatively correlate with myelin content during remyelination [40]. Studies have shown that the following EAE disease [41], local demyelination of the optic chiasm by LPC [18, 19], cuprizone treatment in regions of the hippocampus [42] and corpus callosum [43] the amount of GFAP gene expression, signaling the formation of reactive astrocytes, was significantly increased post lesion. In our study GFAP gene expression level was increased in response to the LPC-induced demyelination in the CA1 area of hippocampus. This could indicate an increased astrocyte activity in the lesion area. Spach and colleagues in EAE disease [41] and also Nystad and colleagues in the cuprizone model of demyelination [43] have reported that the GFAP levels decreased with 1,25-$(OH)_2$ D3 treatment, indicating a return of the reactive astrocytes to their homeostatic state. Consistent with these studies, our data clearly indicated that the vitamin D3 affected on astrocyte activities. So that, in our case we have observed that the GFAP expression levels decreased in the hippocampus tissue of experimental groups after treatment with vitamin D3. Also, several previous studies showed that the 1,25-$(OH)_2$ D3 likely enhanced astrocyte survival [44, 45].

One of the major challenges in treating MS is that regeneration in the CNS inherently fails. The axon regenerative failure is associated with the formation of the glial scar and the presence of inhibitory molecules [46]. Among the several inhibitors that have been characterized, Nogo-A is a prominent myelin-derived inhibitor of axonal outgrowth. Nogo-A mRNA is expressed quite selectively in oligodendrocytes and certain neurons with little peripheral expression [47]. An interaction of Nogo on the oligodendrocyte surface with Nogo-66 Receptor (NgR) on axons has been suggested to play an important role in limiting axonal growth [48].

Looking at the role of Nogo-A in MS, an increase in the expression of Nogo-A by oligodendrocytes was found in chronic active demyelinating plaques [49]. In adult animals Nogo-A mRNA was predominantly expressed in the pyramidal cell layers of the CA1 to CA3 region, and to a lesser extent in the dentate gyrus. Expression of Nogo-A in oligodendrocytes has been reported to be up-regulated in active demyelinating lesions of MS [50]. Consistent with these studies, in

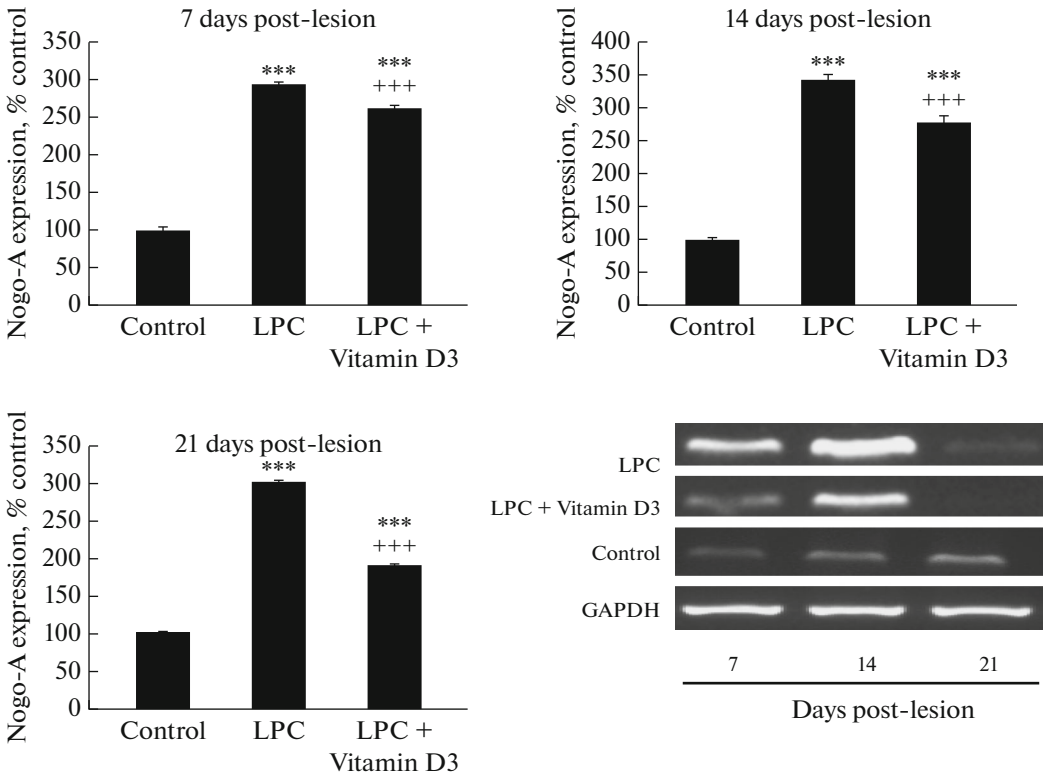

Fig. 4. Effect of vitamin D3 on the expression of Nogo-A gene using semi-quantitative RT-PCR on days 7, 14 and 21 post-LPC injection into the hippocampus (Mean ± SEM). ***$p < 0.001$ as compared to the control groups (i.e., animals received no surgery and treatment treatment, saline and sesame oil treated rats), respectively. +++$p < 0.001$ as compared to the LPC-treated rats. Shows representative bands for Nogo-A gene.

our case, we observed a significant increase in Nogo-A expression in LPC-treated rats on days 7, 14 and 21 post lesion. However, the administration of vitamin D3 for 7, 14 and 21 days caused a decrease in Nogo-A expression post lesion. Studies have demonstrated that ablation of Nogo-A activity attenuates clinical symptoms, demyelination and axonal damage [51, 52]. Therefore in our case vitamin D3 might be capable of increasing axonal regeneration by reducing the expression of Nogo-A gene.

However, very little is known about the role of vitamin D during myelination, accumulating evidence indicate that vitamin D reduces demyelination [10, 43, 53] and might be capable of increasing the remyelination process [54]. In our previous study, the effects of vitamin D on the process of anxiety in a model of demyelination have been shown [23]. Also, vitamin D is involved in brain differentiation and neuroprotection, which are relevant to MS pathogenesis [55]. Neuroprotection through anti-oxidative [23, 56], neuronal calcium regulation, enhanced nerve conduction and detoxification [57] can be probable mechanisms, although yet the exact role is not completely understood in basic and clinic. Therefore, the positive effect of vitamin D3 on myelin repair may be contributed to the neuronal protective roles of the several factors regulated by vitamin D3.

The results and the assays in this study only cover the changes in the mRNA levels, so further confirmation by other methods is required.

CONCLUSIONS

Using a toxin-induced model of demyelination, we found that the vitamin D3 could have a positive effect on the remyelination process, possibly through a stimulating effect on oligodendrocyte progenitor cells and their differentiation to myelinating cells, astrocyte activation and axonal regeneration. Hence, it suggests that vitamin D therapy may help prevent the development of MS and could be a useful addition to the therapy. The potential of vitamin D3 to stimulate remyelination should be investigated further, with functional data to clarify the exact mechanism for this effect.

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