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Role of vitamin D in regulating the neural stem cells of mouse model with multiple sclerosis

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Abstract. – OBJECTIVE: Multiple sclerosis (MS) is an autoimmune disease that results with a damaged myelin sheath as a result, there is an impairment of nerve impulse conduction. The medication for MS is able to delay its progression, but complete recovery is impossible. Recent studies with neural stem cells have promising results in treating as well as to recover the damaged nerves, but research on in vivo model system is limited in this aspect.

MATERIALS AND METHODS: Here we are able to successfully establish an MS mice model by injecting with myelin basic protein and we studied the neural stem cell response in supplement with vitamin D.

RESULTS: Through histology we provide strong evidence that the MS pathogenesis is reverted on response to vitamin D. We also identified through immunohistochemistry and western blotting that the vitamin D has the ability to trigger neural stem cells, and thereby it assist in recovery from MS. Further, their roles in preventing as well as delaying the MS development are also proven. The role of vitamin D has also cross checked with the help of tunnel assay.

CONCLUSIONS: Overall, our results conclude that the lesion associated apoptotic signals are reduced on administrated with vitamin D. The present data help to design a new therapeutic intervention to cure MS.

Key Words:
Multiple sclerosis, Vitamin D, Myelin basic protein, EAE.

Introduction

Multiple sclerosis (MS) occurs due to demyelination of neurons that results in impairment of communication that takes place in the brain and spinal cord column[1]. The overall process results in mental as well as physiological problems[2]. The most common symptom associated with MS is mental fatigue that results in loss of concentration and neurological related symptoms[3,4]. The MS progression initially causes axonal damage[5] and in the latter, it's likely to result into demyelination[6] especially in the cortical region of brain[7]. The initial phase of MS is related to relapsing condition and in advanced stages, it results into more complicated irreversible neurological damage[8]. Medication for MS can able to slow down its progression, but unable to cure them[9].

In this background neural stem cells (NSC) provide a promising option to reverse the damage that is caused by MS[10]. Recent studies[11,12] show that the transplantation of NSC provides therapeutic improvement in handling neurological disorder. Beyond this research now found that a diet rich in B vitamins, iodine and omega-3 fatty acids are helping to improve mitochondrial function, and thereby, provide a way for myelin repair to restore its activity[13]. Recent studies[14] show that the mother who is less exposure to sunlight at the time of pregnancy has a higher chance to give birth to a child who will be affected by MS. Vitamin D also plays a major role in preventing age related cancers[15], heart disease[16], insulin sensitivity[17], metabolic syndrome[18], type I diabetes and osteoporosis[19]. The vitamin D circulates in the body in the form of 25-hydroxyvitamin D and has a link with immune response by promoting the innate immunity at the same time. It also reduces the activity of humoral as well as cell-mediated immunity[20].

The relationship between cancer and vitamin D are extensively studied, but there aren't studies on MS, especially with animal model. In the present study using the MS mouse models we are able to find out the role of vitamin D in regulating the neural stem cells. By designing experiments we try to understand the link between vitamin D and

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neural stem stems and we demonstrate that vitamin D has positive effects on neural stem cells.

Materials and Methods

Mouse Model with Experimental Allergic Encephalomyelitis

Experimental Allergic Encephalomyelitis (EAE) is a useful model to display multiple sclerosis and it is widely used with rodent model system. EAE results in demyelination, inflammation and other neuropathlogical condition similar to that of MS[21]. The present study was carried out by developing EAE in eight weeks old female PL-SJLF1/J (PLSJL) mice, (Jackson Labs, Bar Harbor, ME, USA). The mice were subcutaneously immunized with specific doses of myelin basic protein (120 mg) by following the protocol as described previously[22]. Following immunization the mice started to show the clinical symptoms in the 2nd week. The mice were regularly monitored and categorized into 5 stages: stage 1 with a limp tail; stage 2 with hind limb weakness; stage 3 with one limb paralyzed; stage 4 with two limbs paralyzed; stage 5 with 2 or more limbs paralyzed or died. This categorization was based on the symptoms as evaluated by Cross et al[23]. The experimental protocols and animal use were monitored and approved by the institutional Animal Care Committee specifically made for this project.

Treatment Plan

Four sets of 8 weeks old mice were taken and each set was assigned with five mice. The first set of mice serves as a control without the induction of MS. The second set of mice was injected with myelin basic protein to induce MS. The third set of mice was pre-treated with vitamin D (100 ng/day) through food from the 4th week to 8th week before it was induced to form MS and the same diet was to continue as longer upto an 11th week. The fourth set of mice was post-treated with vitamin D (100 ng/day) after developing MS and it continued for up to 11th week. The 2nd set of mice showed clinical symptoms of stage 2 on 10th week and they were incubated for another week. At this point all the set of mice is sacrificed on 11th week for further examination.

Immunohistochemistry

For performing immunohistochemistry the brain tissues were initially fixed with 4% formalin and paraffin embedded. Following that the tissue were cut using microtome of thin size of 5 μm. The other steps are followed as described in Anderson et al[24]. Nonspecific binding was blocked by incubating the sections with 10% fetal calf serum in phosphate buffered saline (PBS) for 1 hour. The sections were then allowed to react with suitable primary antibody, anti-nestin antibody, Abcam (ab105389) (Cambridge, MA, USA) as per the manufactures protocol and after washing the slides were overlaid with suitable secondary antibody for 1 hour in a room temperature. The developed slides were then examined and documented.

Western Blot Analysis

The protein sample from normal and Vitamin D treated brain tissue are subjected to protein extraction and they were resolved in 12% SDS-PAGE gel as previously described[25]. The transferred membrane is then allowed to react with primary antibody of anti-nestin antibody, Abcam (ab105389) or Anti-Caspase-3 antibody, Santa Cruz Biotech (SC-7272) (Santa Cruz, CA, USA) and the protocol was followed as per the manufactures instruction. After washing the non specific binding, the membrane is incubated with secondary antibody that is specifically reactive with primary antibody. The membrane is then washed and developed to obtain the signal.

TUNEL Assay

The dissected brain from normal and Vitamin D treated tissue samples are subjected to apoptotic assay. As a sign of cell death the cell undergoing apoptosis cells shows characteristic DNA fragments which are detectable using caspase-3 antibody. The immunohistochemistry protocol was followed as described previously[26]. The tissue sections are incubated at 4º C for overnight with Anti-Caspase-3 antibody, Santa Cruz Biotech (SC-7272) and after washing, the slides are incubated with secondary antibody for 1 hour. The slides are then developed using 3,3′-diaminobenzidine (DAB) kit and mounted with coverslips for visualization under microscope.

Results

Histological Variation Between Normal, MS and Vitamin D Gained Brain Tissue

Histopathological differences between vitamin D treated as well as non- treated brain tissue are

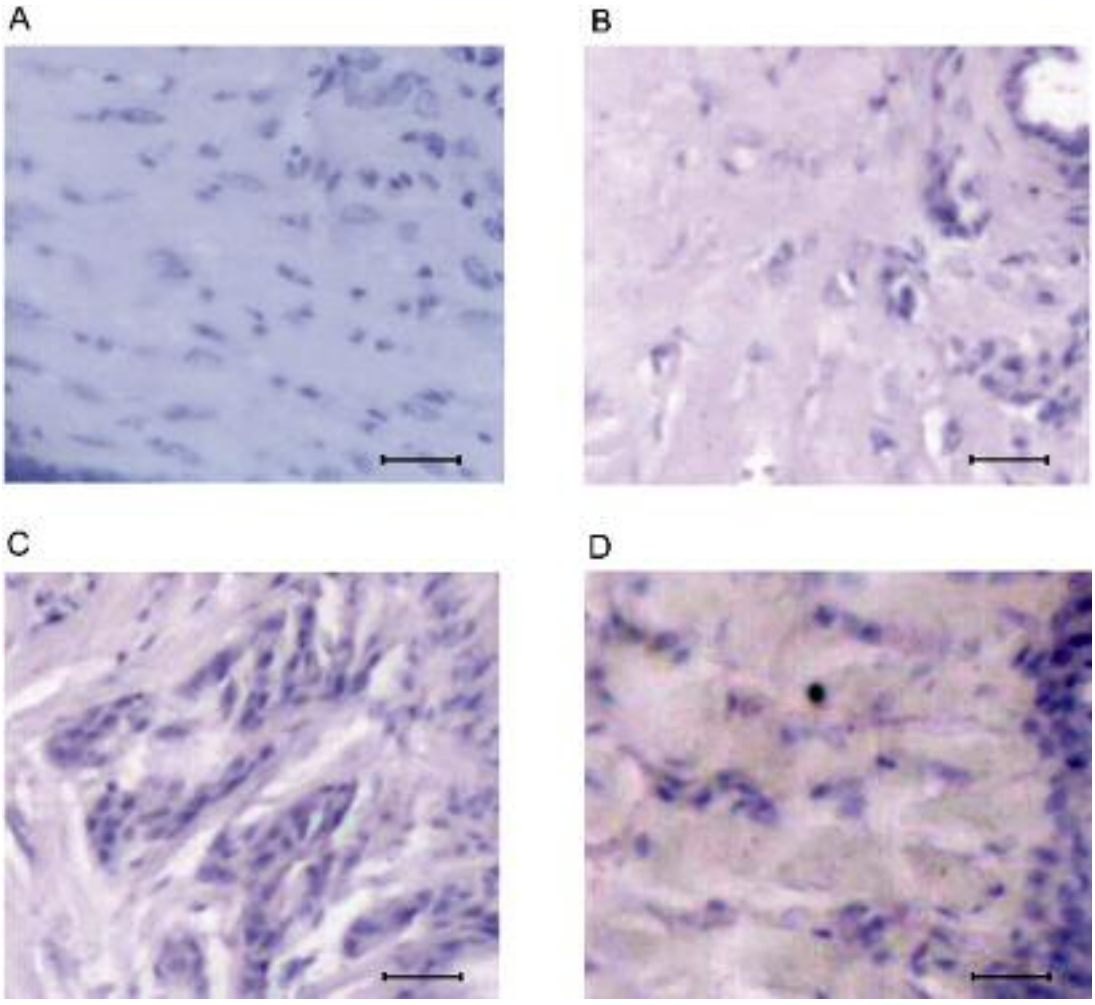

Figure 1. Histological variation between normal, MS and vitamin D gained brain tissue. ***A***, Histological section of normal brain tissue, which shows the axons that are evenly distributed with myelination. ***B***, Histology of MS tissue with axon loss and demyelination. ***C***, Mice pre-treated with vitamin D shows less damage to axon and its associated myelin sheath. ***D***, Mice post-treated with vitamin D shows marginal damage to axon and its associated myelin sheath. Scale Bar: 50 μm size.

carried out to understand the nature of the disease progression. The MRI imaging lacks pathological specificity hence histology is the best way to understand the disease pathology. The normal brain tissue (Set 1) has axons that are distributed evenly throughout the tissue with extensive gray matter (Figure 1A) but in the case of MS (Set 2) the axonal loss along with lesions are visualized and which also reflect the demyelination of grey matter (Figure 1B). The set 2 mice also show stage 2 clinical symptoms with hind limb weakness. The development of lesion suggests the disease progression along with clinical symptoms in set 2 mice. The vitamin D pre-treated mice (Set 3) shows fight back from developing MS without much affected axon but it shows limited damage in the form of lesion together with altered arrangement of tissue pattern (Figure 1C). The set 3 mice shows stage 1 clinical symptom with limp tail. The vitamin D post-treated mice (set 4) recovered from stage 2 clinical symptoms and it shows partial hind limb weakness. As a histological improvement the reappearance of axon body and remyelination can also take place (Figure 1D).

Role of Vitamin D in Regulating Neural Stem Cells

Neural stem cells have the ability to proliferate, self renewal and the ability to differentiation based on the response[27]. This ability of the neural stem cells proves to be an effective therapeutic

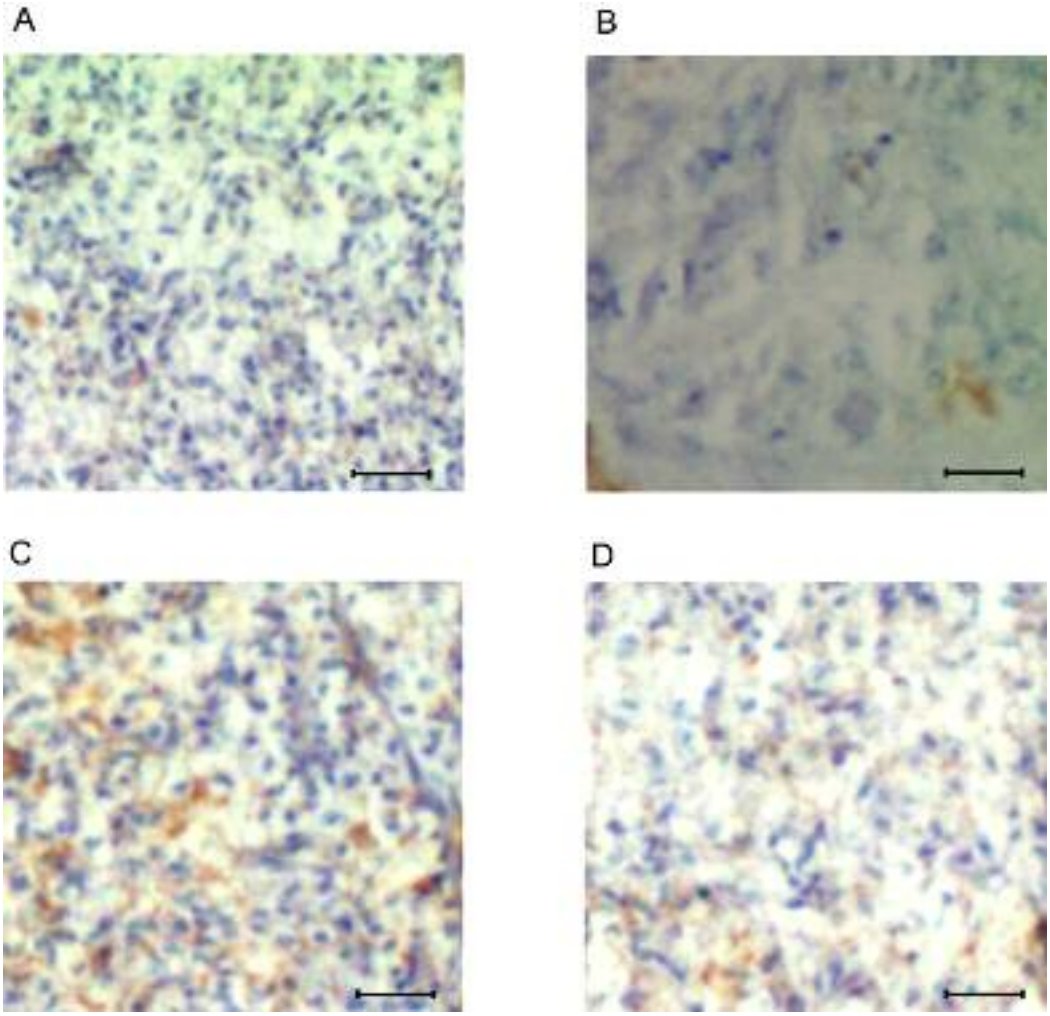

Figure 2. Immunohistological variation associated with neural stem cells in response to vitamin D. ***A***, Immunohistological section of normal brain tissue, which shows least expression of neural stem cells. ***B***, Immunohistological section of MS brain tissue, which shows limited expression of neural stem cells. ***C***, Mice pre-treated with vitamin D shows extensive expression of neural stem cells. ***D***, Mice post-treated with vitamin D shows marginal expression of neural stem cells. Scale Bar: 50 μm size.

use to treat neurological disorders. In the present investigation vitamin D was used to trigger the neural stem cell response. Immunohistochemistry was performed using anti-nestin antibody to track the neural stem cells. The control mice show the presence of limited neural stem cells that are distributed in their sections (Figure 2A). The mice developed with MS show restricted expression of nestin protein, which in counterpart implies the reduced neural stem cells in the section (Figure 2B). On the other hand, the mice pre-treated (Set 3 mice) with a vitamin D exhibit proliferated expression of neural stem cells (Figure 2C) which implies the supportive trigger of the repair process after developing initiative MS. Similarly the mice post-treated with vitamin D have also shown marginal proliferation of neural stem cells (Figure 2D) as a sign of recovery after developing stage 2 clinical symptoms.

Vitamin D Mediated Apoptosis Suppression

The most striking features of MS is development of tissue lesion that are associated with apoptosis[28]. Here we evaluate the role of vitamin D that may directly or indirectly promote tissue repair and as an outcome of that it shows reduced expression of apoptotic signal. The normal brain tissue of mice has slight signals for caspase-3 (Figure 3A) which may imply a normal cellular regulatory process. Perhaps its expression pattern shows significant elevation

in case of MS (Figure 3B). However, the mice pre-treated and post-treated with vitamin D show only marginal signals for caspase-3 protein (Figure 3C and D).

Immunoblot Analysis

The data obtained through immunohistochemistry was further verified using Western blot analysis. As a supportive and confirmation, the data obtained through Western blotting resemble and coincide with immunohistochemistry data. The proteins lysate isolated from different brain samples are subjected to Western blotting using anti-nestin and anti-apoptotic protein and their results are displayed as shown in the Figure 4A and B. The results with nestin antibody illustrate its elevated expression in vitamin D pre-treated and post-treated samples (Figure 4A), whereas apoptotic signals are upregulated in MS (Figure 4B). The data shows consistent with obtained immunohistochemistry data.

Discussion

The neuronal damage, particularly associated with demyelination is the major cause of MS[29]. The damage can be postponed using novel drugs, but the complete recovery from MS is a questionable[30]. Here we demonstrate that the administration of vitamin D was able to promote the proliferation of neural stem cells and it helps to res-

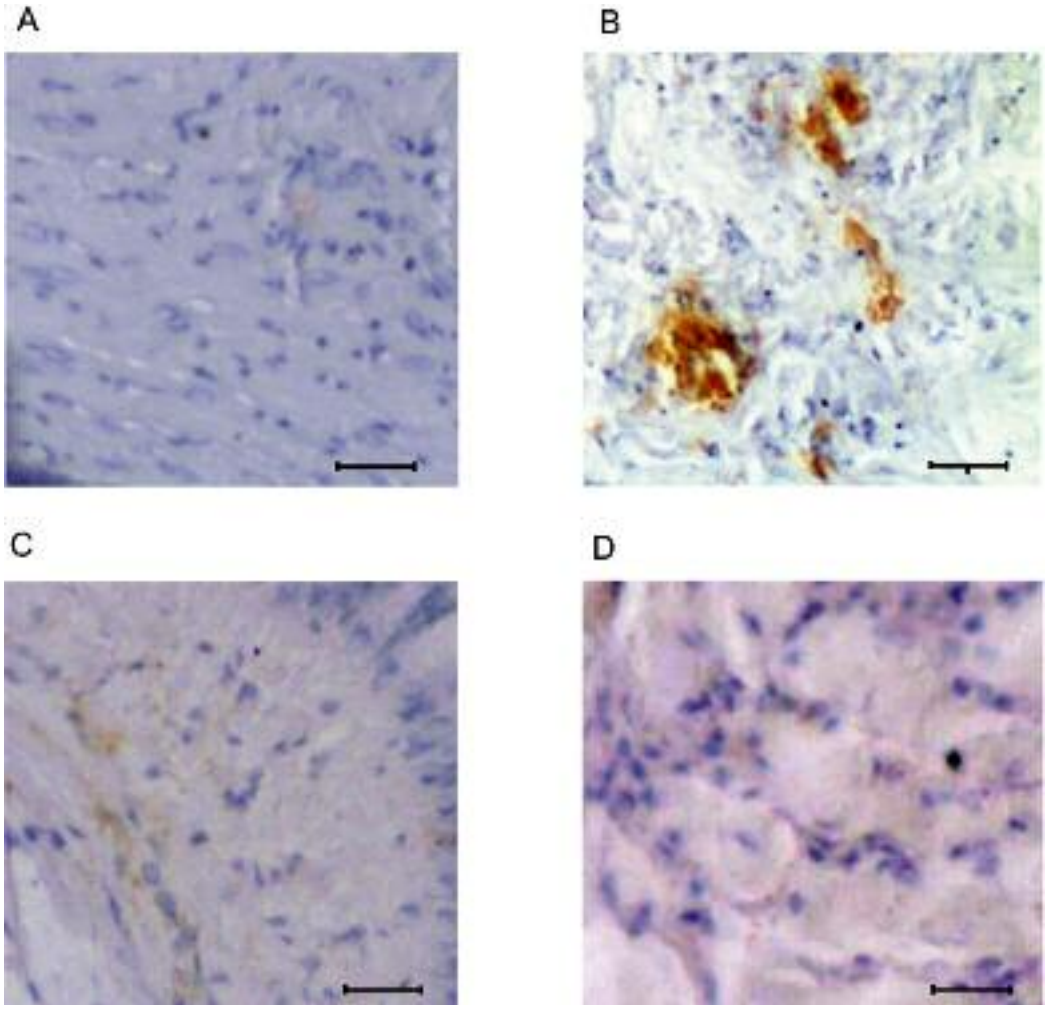

Figure 3. Immunohistological variation associated with apoptotic protein in response to vitamin D. ***A,*** Immunohistological section of normal brain tissue, which shows least expression of caspase-3 protein. ***B,*** Immunohistological section of MS brain tissue, which shows elevated expression of apoptotic protein. ***C,*** Mice pre-treated with vitamin D shows less expression of apoptotic protein. ***D,*** Mice post-treated with vitamin D shows marginal expression of apoptotic protein. Scale Bar: 50 μm size.

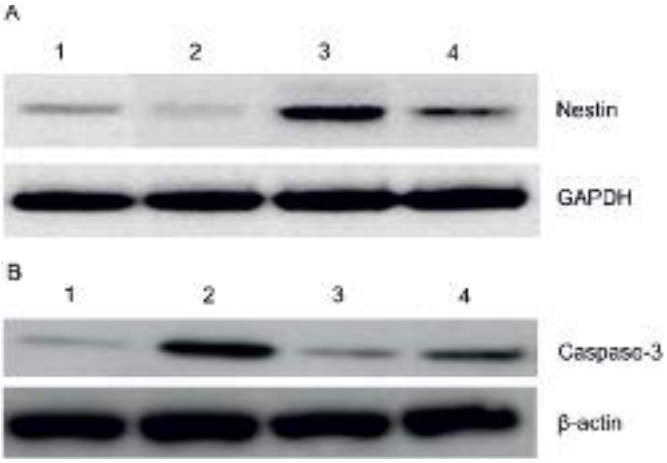

Figure 4. Validation of Neural stem cells and apoptotic protein expression by western blotting. ***A,*** Lane 1-4 represents the nestin protein expression in normal, MS, Mice pre-treated with vitamin D and Mice post-treated with vitamin D brain tissue samples. For control purposes GAPDH (Glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control. ***B,*** Lane 1-4 represents the caspase-3 protein expression in normal, MS, Mice pre-treated with vitamin D and Mice post-treated with vitamin D brain tissue samples. For control purposes β-actin was used as a loading control.

cue the damage which is evident with improvement in clinical symptoms. Histopathological as well as immunohistochemistry provides a better opportunities to test the recent hypothesis in this field because it helps to analyze different pathological conditions which are difficult with other imaging techniques[31].

In vitro studies with vitamin D prove its role in controlling cell growth, cell survival, differentiation ability, anti-proliferative effect, DNA damage hindering ability and it has a therapeutic role in breast, colon and skin cancer[15], but the integrity of the data obtained from *in vitro* studies to *in vivo* systems are not extensively studied. Here we successfully established mice model with MS to study the histological variation between vitamin D treated and control tissue (Figure 1A-D) that helps to understand the MS pathogenesis through which it is able to design new therapeutic models. From histological data, it is concluded that the pathological heterogeneity varies as a treatment procedure differ when it is compared with control and MS tissue.

Neural stem cell is the basis for tissue repair after developing with MS because it has the ability to proliferate and differentiate into other forms of cells in neuron[32]. In the present study the effect of vitamin D on neural stem cells are analyzed and we come up with interesting results. The pretreatment with vitamin D shows positive effect in triggering neural stem cells and, thereby, prevent the development of complex stage of MS when comparing with the positive control (Figure 2A-D). Others important features of MS are the local apoptosis that are associated with MS pathogenesis[33]. As an indirect evidence, the medication with vitamin D helps to prove that it hinders the apoptosis signals when compared with suitable controls (Figure 3A-D). Furthermore, the data obtained with immunohistochemistry are consistent with Western blot data (Figure 4A-B).

Conclusions

From our study we are able to point out that, MS progress with axonal loss and demyelination, and its progression get prevented or reverted when treated it is pre or post treated with vitamin D. It also revealed that the vitamin D has the ability to trigger neural stem cells and to suppress apoptosis. The results obtained help to design new therapeutic strategies with interesting outcomes.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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