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Immunomodulatory effects of Calcitriol in acute spinal cord injury in rats

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ABSTRACT

Pharmacological therapy options for spinal cord injury (SCI) in acute phase have so far been limited, thus we focused on Calcitriol, FDA-approved biologically active form of vitamin D whose neuroprotective effects are increasingly recognized, to ameliorating damage following acute SCI in rats. Calcitriol (1 μg/kg) treatment for 7 consecutive days after SCI was compared SCI control and Sham control rat groups. Calcitriol-treated group had significantly improved outcome in standard functional recovery evaluation test (BBB) 12 weeks after SCI compared to SCI control, which was confirmed by increased ventral horn motor neurons in Calcitriol-treated group. In addition, proliferation test performed on lymphocytes from spleen and lymph nodes one week after SCI showed that calcitriol injection has a significant regulatory effect on Division Index (DI) in response to MBP stimulation compared to control SCI groups, which was associated with significant reduction in IFN-γ and IL-17A secretion and leukocyte infiltration into injury site. Along with confirmation of immunoregulatory aspects of Calcitriol treatment against myelin antigens in SCI, this study has shown that reducing the extent of progressive tissue loss by Calcitriol therapy in acute phase, could result in better recovery after SCI.

1. Introduction

There has been growing attention in application of immunotherapeutic agents for acute-phase treatment to improve motor function after spinal cord injury (SCI), however, just limited approaches applied in clinical studies and therapies still have inadequate efficacy [1]. Lymphocytes form the cellular basis of adaptive immunity, which has been proved to play a crucial role during pathogenesis and following tissue repair after spinal cord injury [2]. It has been shown that restricting T lymphocyte proliferation and activity through immunosuppressant drugs such as FK506 and Cyclosporine-A could enhance motor recovery after SCI [3,4], as well as immunodeficient nude rats and mice demonstrated better motor outcome after SCI [5,6]. Naive T lymphocytes need to be primed and differentiated at the peripheral lymphoid organs into effector T-helper (Th) cells subtypes to gaining access to central nervous system (CNS). These subtypes are categorized principally by their lineage-specific cytokines; for example, Th1 cells produce IFN-γ, Th2 cells generate IL-4, and Th10 cells produce IL-10, while Th17 cells generate IL-17 [7–9]. Furthermore, beneficial and detrimental role of IFN-γ, IL-4, IL-10 and IL-17 on SCI repair and functional recovery have been shown [10–13]. Accordingly, assessment of immunotherapeutic agents as well as understanding the mechanisms of action will advance the development of novel therapies to avoid or diminish long-term disabilities after SCI.

Calcitriol (1 alpha-dihydroxivitamin D3), the most biologically active metabolite of vitamin D3, is well known for its classical hormonal action related to the regulation of calcium homeostasis and bone health, However, the discovery that Calcitriol or vitamin D receptor (VDR) is expressed on immune cells, made it clear that the effects of Calcitriol can go beyond its traditional role [14]. In recent years, several studies have been performed to examine the therapeutic value of Calcitriol against autoimmune and progressive neurodegenerative disease such as multiple sclerosis and Parkinson's disease, respectively. [15,16]. Additionally, many investigations have been focused on Calcitriol as a potential therapeutic agent for traumatic brain injury (TBI), cerebral ischemic injury and cerebral malaria according to anti-inflammatory and neuroprotective effects with improved function recovery [17–19]; however, there are just few studies assessing Calcitriol effects on spinal cord injury in acute phase not unraveling therapeutic mechanisms [20–22].

Calcitriol is a potent adoptive immune system regulator, which the importance of the T lymphocytes as a direct target of Calcitriol has been

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demonstrated in experimental autoimmune disease that Calcitriol was not able to ameliorate EAE when the VDR was absent in T cells in chimeric mice [23]. It has been illustrated the effects of Calcitriol vary among the various $CD4^+$ Th cell subsets [24] and in many experimental models Calcitriol not only acts as an immunomodulatory agent by inhibiting Th1 cells function, but also regulating Th2, T regulatory (Tregs) and Th17 lymphocytes [25]. Furthermore, based on in vitro results, Calcitriol can inhibit T cell proliferation and differentiation and modulates their cytokine production in a dose-dependent fashion [26–28]. Although the regulatory roles of Calcitriol have been reported in many disease models, the immunomodulatory effects of Calcitriol treatment in acute phase after spinal cord injury have not been considered. The acute phase initiates immediately following SCI including blood brain barrier (BBB) disruption, glutamate excitotoxicity, free radical formation, lipid peroxidation, inflammation, and necrotic cell death [29]. Besides, it has been demonstrated SCI can begin T-lymphocyte priming and reactions to CNS antigens, specifically, highly responsive to MBP in secondary lymphoid tissues during acute phase and first week after SCI that passive transfer of these SCI-sensitized T cells isolated within seven days from lymph nodes and spleen of spinal cord injured rats into naive rats could cause mild and transient paralysis and neuroinflammation [30]. Therefore, in this study, we evaluate immunoregulatory impact of Calcitriol on cell-mediated auto immune response directed against myelin sheath self-antigens after spinal cord injury by analyzing cytokine production patterns in peripheral lymphoid organs including spleen and lymph nodes.

In this study, we have utilized a clinically relevant spinal cord injury contusion model [31] to explore the effect of Calcitriol treatment in acute phase after spinal cord injury. We demonstrated that intraperitoneal injection of Calcitriol enhanced locomotor recovery, which was accompanied by ameliorated lesion pathology with higher spinal neuron survival in acute phase after spinal cord injury. Using carboxyfluorescein diester amine (CFSE) flow cytometry analysis, we found that Calcitriol in acute phase after SCI suppressed autoimmunity against MBP with reducing proliferative responses. We confirmed that Calcitriol can inhibit deleterious response occurred against MBP after SCI that influenced cytokine secretion patterns in peripheral lymphoid organs. Our data recommended that Calcitriol immunomodulatory effects in acute phase after spinal cord injury could be neuroprotective by limiting neurodestructive proinflammatory cytokines of T cells.

2. Materials and methods

2.1. Animals

Female Sprague-Dawley rats 11 weeks were obtained from Razi Institute (Tehran, Iran). The animals were kept in an environment with a 12 h' dark/light phase at the standard condition of temperature (21 ± 1 °C) and controlled humidity. The rats were given ad libitum access to food and water over the study period and each wire cage housed one rat. All surgical and postoperative care procedures were performed in accordance with The Animal Ethics Committee of Shiraz University, Shiraz, Iran.

2.2. Experimental groups

Sprague-Dawley (SD) rats were randomly divided into three groups: Sham (laminectomy alone without spinal cord injury, n = 12); spinal cord injured animals without treatment (untreated-SCI) (n = 12) and Calcitriol treated for 7 consecutive days after SCI (1 μg/kg, n = 12). Half of the animals from each group were euthanized seven days post injury and others at 12 weeks after SCI were perfused.

2.3. Calcitriol acetate and MBP

An Injectable form of Calcitriol (Calcijex ®, Abbott Laboratories, Liscate, Italy) was administered intraperitoneally. Myelin basic protein peptide 68–86 (MBP 68–86) was synthesized by Pepmic Co. Ltd., China.

2.4. Surgical procedure

All rats were anesthetized with a mixture of xylazine (100–150 mg/kg) and ketamine (60–90 mg/kg) by intraperitoneal (IP) injection, and laminectomy at the T9-T10 thoracic level exposed the spinal cord. The vertebral column was stabilized using clamps on T8 and T11 vertebrae, and rats received injury by dropping a 10-g weight (a 2-mm metal rod) from 12.5 mm height centered above exposed spinal cord. The impact rod was immediately removed following the injury, and the muscle layers and skin were sutured separately. The animals were placed on heating pads while anesthetized and monitored during recovery. The animals were housed in individual cages and bladders were voided manually daily until they recovered their function. 50 mg/kg of an antibiotic (Cefazolin, Jaber Ibn Hayan Co., Tehran) was given for seven days after surgery to prevent infection. After surgery, 6 ml of sterile saline was administered subcutaneously for three days. Rats always had access to food and water, with some food pellets placed on the bottom of each cage for easier access. The sham-operated rats underwent identical to those of the SCI animals, including anesthesia and laminectomy, but without impact injury.

2.5. Open field locomotor test

Basso, Beattie, and Bresnahan (BBB) test was used to evaluate functional outcome after spinal cord injury [32]. The rats were tested two and seven days after SCI, after which their assessment was performed once a week until 12 weeks after SCI by observers blinded to experimental treatment. Using BBB locomotor rating scale, specific components of functional behavior were analyzed such as joint movement, paw placement, stepping, coordination, toe clearance, trunk and abdomen position. In this test, a score of 21 is an indication of normal hind limb locomotor function (complete mobility) and a score of 0 is a sign of lack of natural hind limb movement (complete immobility).

2.6. Tissue processing for histological evaluation

Rats were deeply anesthetized with intraperitoneal injections of ketamine (80 mg/kg) and xylazine (10 mg/kg) 12 weeks after spinal cord injury. For intracardiac perfusion, each rat was put in its prone position. We opened the chest cavity, the left ventricle (cardiac apex) was perfused with butterfly angiocath perfusion catheter and cut open the right atrium. After injection of 1 mL 1% lidocaine to dilate blood vessels, we perfused 200 ml of saline to eliminate blood, followed by 300 ml of 4% paraformaldehyde. The spinal cord with vertebrae was then removed and left in 4% paraformaldehyde overnight at 4 °C and in the following day, the spinal cord was isolated from the vertebrae. For spinal cord samples, 1-cm lengths of spinal cord centered on the injury site were cut. After fixation, tissues were dehydrated using a graded series of ethanol solutions, cleared in xylene, and embedded in paraffin blocks for sectioning. Spinal cord samples were serially sectioned transversally with a rotary microtome at 5-μm thickness and mounted on slides.

2.7. Luxol Fast Blue (LFB)-Cresyl Violet staining

The sections were deparaffinized in xylene and rehydrated in 100% and then 95% ethanol.

For white matter staining, sections were incubated in 0.1% Luxol Fast Blue (*Sigma*, St. Louis, MO) diluted in 95% ethanol with 10% acetic acid at 56 °C for 24 h, rinsed in 95% ethanol and then distilled water, differentiated in 0.05% lithium carbonate, rinsed in 70% ethanol followed by distilled water, counterstained in 0.01% Cresyl Violet (*Sigma*, St. Louis, MO) solution for 40 s, rinsed in distilled water, differentiated

the slides in 95% ethanol, rinsed them in 100% ethanol, cleared with xylene, and cover-slipped with mounting media.

2.8. Lesion volume estimation

Stained sections were photographed with a microscope (Nikon, Japan). Spared white matter sites were outlined and quantified using ImageJ software (NIH, USA). Sections with the least spared white matter were selected the injury epicenter. The percentage of cavity in 5000 μm length of the injured spinal cord (including rostral and caudal regions from the injury epicenter) was examined. The percentage of cavity and spared tissue were calculated for 35 sections using Cavalieri's method [33] and serial summation of the spared tissue and volumes of cavity yielded the total volume of the spared tissue. In the SCI, Calcitriol treated and untreated SCI groups, the area of the lesion was determined by the presence of necrosis, inflammatory cells, cavity and cyst formation.

2.9. Motoneuron quantification

Spinal cord sections were photographed with a microscope (Nikon, Japan). Cell bodies of motor neuron in ventral horn were outlined using ImageJ software, and ventral horn was defined as the area of gray matter ventral to the central canal. Motor neurons were quantified by counting clearly identifiable nucleus and a cell soma larger than 40 μm in dimeter at the lesion epicenter, 1-mm and 2-mm rostral and caudal of epicenter. Sections with minimal spared white matter were designated the injury epicenter.

2.10. CFSE assay

Cells were pooled from excised inguinal, lumbar, and popliteal lymph nodes and isolated from spleen within seven days after SCI from all groups. The cells were cultured in flat-bottomed wells in RPMI-1640 medium (GIBCO, New York) that was supplemented with 10% fetal bovine serum (Gibco, New York) on a 96-well microtiter plate. Cells (2×10^5 cells per well) were cultured for 72 h in an antigen-free medium (unstimulated control) or together with MBP (50 μg/ml) at 37 °C in 5% CO_2. After twice rinsing with RPMI-1640, the cells were labeled with carboxyfluorescein diester amine (CFSE) (CellTrace TM CFSE Cell Proliferation Kit, Invitrogen, Molecular Probes, USA). CFSE was used at a final concentration of 1 μM. The cells were then incubated for 72 h at 37 °C and unstained cells were used to discern the background autofluorescence. The unstimulated cells from lymph nodes and spleen used as a reference to fix the zero point of the peak for the undivided population for FlowJo software (FlowJo, Ashland, OR, USA) analysis. The area of lymphocytes was gated for analysis according to light scattering characteristics (size/granularity) of these cells. Ten thousand events were collected for each sample on FACS CanII instrument (BD Company, USA) and data were evaluated by FlowJo software.

2.11. Cytokine analysis

ELISA kits (DuoSet; R&D System, Ooxon, UK) were utilized to analyze the concentrations of either IL-4 (lower and upper quantitation limits were 15.625 and 1000 pg/ml, respectively), IFN-γ (lower and upper quantitation limits were 39.1 and 2500 pg/ml, respectively; samples were diluted 1:30), or IL-10 (lower and upper quantitation limits were 62.5 and 4000 pg/ml, respectively) and an ELISA kit (ELISA Ready-Set-Go; eBioscience, San Diego, CA) was used for IL-17A (lower and upper quantitation limits were 1.5625 and 200 pg/ml, respectively) measurement. Mononuclear cells isolated from spleen and lymph nodes of experimental groups one week after spinal cord injury were stimulated with MBP (50 μg/ml) in 96-well plates for 72 h and then the supernatants were assessed by ELISA kits as described by the manufacturer.

2.12. Hematoxylin and Eosin (H&E) staining

The sections were deparaffinized in xylene and rehydrated in 100% and then 95% ethanol. In order to stain the nuclei, we incubated sections in Gill Hematoxylin (*Sigma*, St. Louis, MO) for 12 min, rinsed in running tap water and then differentiated in Bluing reagent, rinsed in distilled water followed by 95% ethanol, counterstained in Eosin Y solution (*Sigma*, St. Louis, MO) solution for 20 s, rinsed in distilled water, differentiated the slides in 95% ethanol, rinsed them in 100% ethanol, cleared with xylene, and cover-slipped with mounting media.

2.13. Quantitative analysis of leukocyte infiltration

Sections cut (3 sections/animal) at the impact site (epicenter) and adjacent sections immediately rostral/caudal to the epicenter were photographed with a microscope (Nikon, Japan) at high-power (40×). Subsequently, leukocytes were manually counted throughout the entire cross-section within the parenchyma using ImageJ software and analyzed from each animal/group then were averaged.

2.14. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software Inc., San Diego, CA). Data distribution was analyzed by Kolmogorov–Smirnov test. Two-way ANOVA and Tukey's post hoc test were used for BBB score comparison. One-way ANOVA and Tukey's post hoc test were performed for differences in other values. Differences in means of the leukocyte infiltration were compared by means of the independent samples *t*-test. Data were presented as mean ± SD for BBB scores and leukocyte infiltration and other values were reported as mean ± SEM. p values < 0.05 were considered as statistically significant.

3. Results

3.1. BBB score outcome

To examine whether the Calcitriol treatment promote functional recovery after spinal cord injury, standard motor function BBB score was analyzed from all experimental groups during 12 weeks after spinal cord injury (Fig. 1).

The statistical analysis revealed that the BBB score in Calcitriol treatment group (2 weeks: 11 ± 0.8; 3 weeks: 11.3 ± 1.3; 4 weeks: 12.3 ± 1.6) significantly increased compared to SCI control group (2 weeks: 9.5 ± 0.8; 3 weeks: 9.9 ± 0.9; 4 weeks: 11 ± 1.3) from two-week to four-week after SCI ($p < 0.05$) (Fig. 1); however, no significant difference was seen among the untreated-SCI group (2 days: 1.5 ± 0.5; 1 week: 8.2 ± 0.5) and Calcitriol-treated groups (2 days: 1.9 ± 0.4; 1 week: 9.3 ± 0.6) two days and one-week post injury (Fig. 1).

The rat hindlimb motor function of the Calcitriol treatment (5 weeks: 12.4 ± 1.6; 6 weeks: 12.5 ± 1.7; 7 weeks: 12.9 ± 1.1) and SCI control groups (5 weeks: 11.2 ± 1.5; 6 weeks: 11.7 ± 1.2; 7 weeks: 11.9 ± 0.9) exhibited no statistically significant difference in recovery between 5 and 7 weeks after the injury; whereas the BBB scores was significantly higher in the Calcitriol-treated group (8 weeks: 13.4 ± 1.2; 12 weeks: 13.4 ± 1.2) compared with those of the SCI control group (8 weeks: 12 ± 1; 12 weeks: 12 ± 1) in 8-week after SCI ($p < 0.05$); which significant difference in the improvements were maintained until 12 weeks after spinal cord injury. Thus, Calcitriol treatment improved motor function recovery after contusion SCI (Fig. 1).

3.2. Effects of Calcitriol treatment on cavity percentage of lesion volume

To determine the effect of Calcitriol treatment on histological results

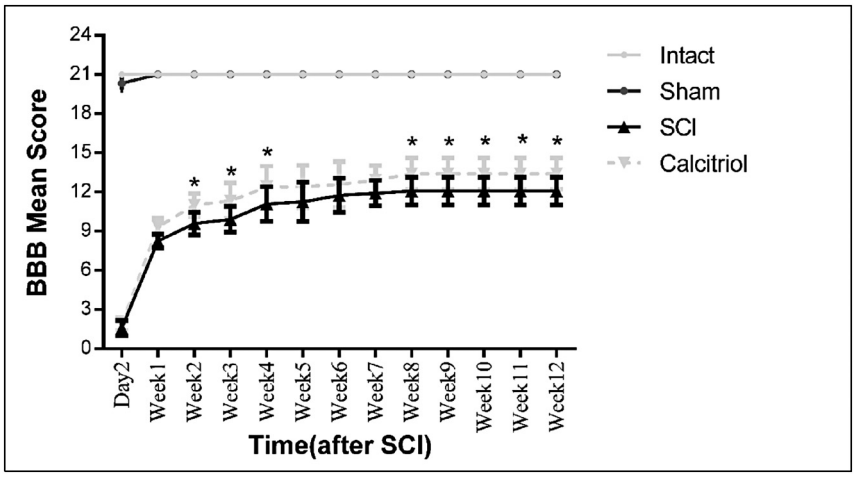


Fig. 1. Calcitriol treatment in acute phase after spinal cord injury has improved locomotion function. BBB scores ranged from 0 to 21 points. The minimum point (0) indicated complete paralysis and the maximum point represented normal function. Data were expressed as the mean ± SD of six rats for each group. Asterisk indicates significant differences (Two-way ANOVA, Tukey's post hoc test) *$p < 0.05$, Calcitriol 1 μg/kg vs. SCI. BBB: The Basso, Beattie & Bresnahan.

after spinal cord injury, we measured the cavity percentage of lesion volume in experimental groups after 12 weeks of spinal cord injury by Luxol Fast Blue-Cresyl Violet staining. The percentage of cavity in 5000 μm length of the injured spinal cord was analyzed with image J software.

As shown in Fig. 2A (left column), the lesion cavity was increased in SCI control group compared to Sham control. On the contrary, no significant difference was found between Calcitriol treatment and SCI control groups (Fig. 2B); which indicates that Calcitriol treatment had no effect on lesion volume in acute phase after spinal cord injury.

3.3. Motoneuron loss outcome

To evaluate motoneuronal loss, we counted motoneurons using image J software in transverse sections of ventral horn at the lesion epicenter and 2 mm rostral and caudal of epicenter within 12 weeks after spinal cord injury by Luxol Fast Blue-Cresyl Violet staining (Fig. 2A right column).

We found that Calcitriol treatment results in significant increase in spared motor neurons at lesion epicenter and 1 mm rostral and caudal to epicenter compared to SCI control group ($p < 0.05$) (Fig. 2 C); There was no significant difference in motoneuron loss at 2 mm rostral and caudal to epicenter among Calcitriol treatment and SCI control groups. The results revealed that Calcitriol treatment could limit motoneuronal loss after spinal cord injury.

3.4. Proliferative response to Myelin basic protein (MBP)

To identify the mechanisms involved in positive effect of Calcitriol treatment following spinal cord injury, the proliferative efficacy of spleens and lymph node lymphocytes in response to Myelin basic protein (MBP) was assessed one week after spinal cord injury by CFSE assay in experimental groups (Fig. 3A, B).

Quantitative analysis of division index showed a significant decrease in response to MBP stimulation in Calcitriol treatment compared to SCI control groups ($p < 0.05$) in spleen (Fig. 3C). Moreover, a similar pattern of response was demonstrated in cells isolated from lymph nodes in which division index increased against MBP in Calcitriol treatment rats in comparison with SCI control ($p < 0.05$) (Fig. 3D). The obtained data showed that Calcitriol treatment in acute phase after spinal cord injury diminished lymphocyte reactivity to MBP.

Moreover, CFSE results showed that division index was significantly increased against MBP in SCI control group compared with non-injured sham group in both spleen and lymph node cells ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 3C, D), indicating enhanced autoreactivity to MBP after spinal cord injury.

3.5. IFN-γ secretion in response to Myelin basic protein (MBP)

IFN-γ, a key cytokine in multiple immune processes and pathologies of neurotraumatic injury, produced by CD4 *Th1* and CD8 cytotoxic T lymphocyte (CTL) effectors [34]. To measure IFN-γ concentration, mononuclear cells from spleen and lymph nodes of experimental rats, which were collected after one week of spinal cord injury, were co-cultured with MBP for 72 h and then the supernatants were tested by Sandwich ELISA to examine changes in cytokine secretion.

Our results showed that IFN-γ secretion was significantly decreased against MBP in spleen and lymph node cells after spinal cord injury in Calcitriol-treated group in comparison with SCI control group ($p < 0.05$) (Fig. 4A, B). Moreover, ELISA results revealed significant increase in response to MBP in SCI control group compared to non-injured sham group in spleen and lymph node cells ($p < 0.01$, respectively) (Fig. 5A, B). In general, we found that spinal cord injury enhanced IFN-γ secretion against MBP, whereas Calcitriol treatment can decrease this response.

3.6. IL-4 secretion in response to Myelin basic protein (MBP)

Considering the importance of IL-4 effects in spinal cord injury [11], we assessed IL-4 concentration in supernatants of spleen and lymph node cell cultures stimulated with MBP. Calcitriol treatment group showed significant increase in IL-4 production compared to SCI control group in lymph node cells in response to MBP ($p < 0.05$); however, there was no significant difference between Calcitriol treatment group and SCI control group in spleen in response to MBP (Fig. 5A, B). Our data demonstrated that Calcitriol treatment could reduce IL-4 production after spinal cord injury within the first week after SCI, however spinal cord injury had no effect on IL-4 production in response to MBP.

3.7. IL-17 secretion in response to Myelin basic protein (MBP)

IL-17 is an important proinflammatory cytokine, which is mainly produced by Th17 cells and plays a critical role in the induction of neuroinflammation after spinal cord injury [35]. ELISA results showed significant decrease in response to MBP in Calcitriol treated group compared with SCI control group in both spleen and lymph node cells culture ($p < 0.05$) (Fig. 6A, B). Our data revealed that IL-17A secretion against MBP was augmented after spinal cord injury as SCI control group showed significant increase in IL-17A production compared to non-injured sham group ($p < 0.01$) (Fig. 6A, B). In conclusion, our results revealed that Calcitriol treatment could decrease IL-17A production in acute phase after spinal cord injury.

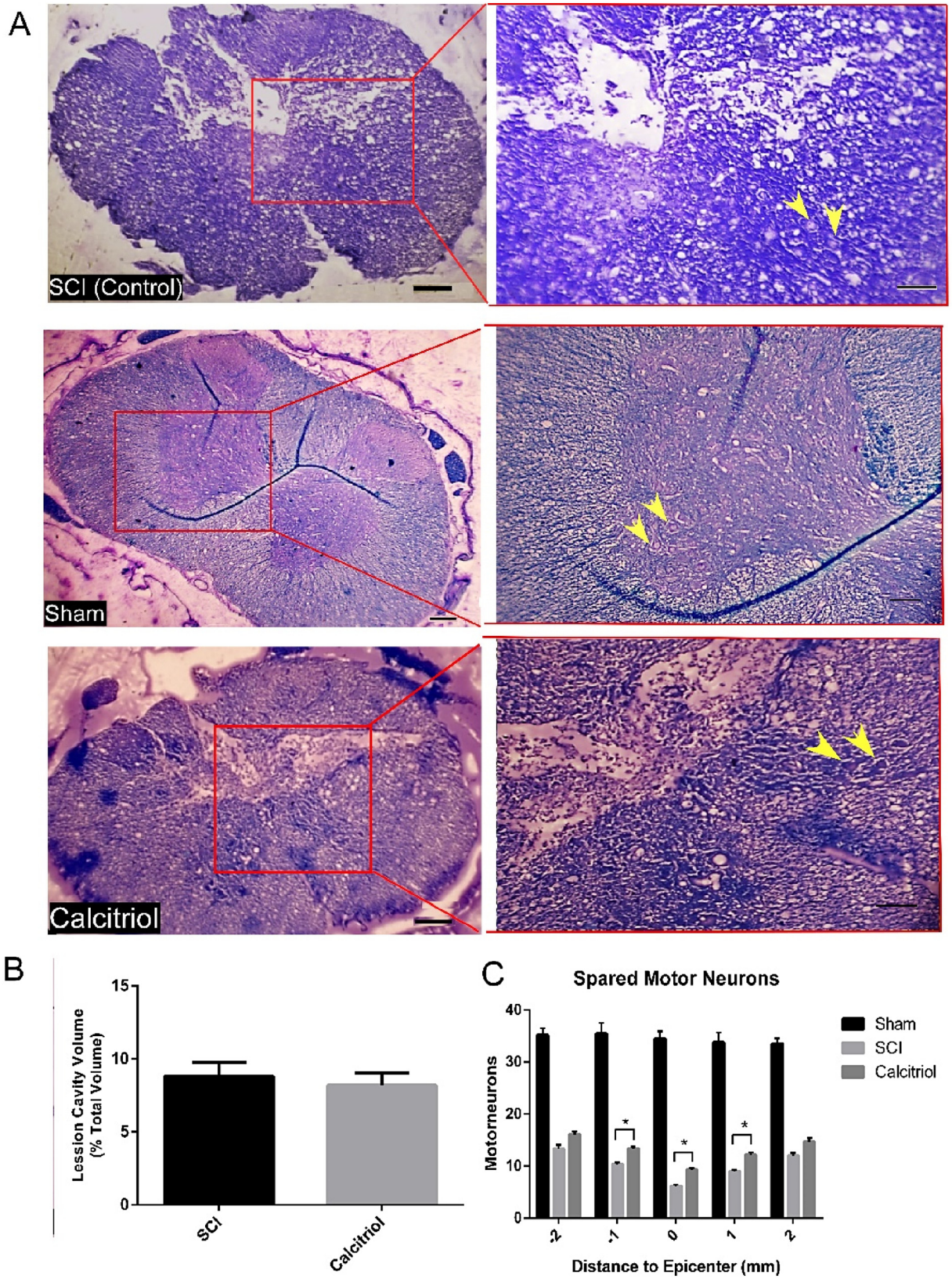


Fig. 2. Calcitriol treatment caused increased ventral horn motor neurons with no effect on lesion cavity volume after spinal cord injury. A: Representative images showing transverse section of spinal cord, the lesion cavity, and ventral horn gray matter from spinal cord at injury level of all groups evaluated in this study at 12 weeks after spinal cord injury by Luxol Fast Blue-Cresyl Violet staining. Yellow arrows illustrate the ventral horn motor neurons. Scale bar represent 100 μm and 50 μm in left and right columns, respectively. B: Bar diagram shows the average percentage (n = 6 rat/group) of lesion cavity volume of experimental groups at 12 weeks after spinal cord injury. C: Bar diagram represents the average (n = 6 rat/group) numbers of ventral horn motor neurons in gray matter from spinal cord groups at 12 weeks after spinal cord injury. The motor neurons quantified at the lesion epicenter, 2 mm rostral and caudal of epicenter. Data are expressed as mean ± SEM. Asterisk indicates significant differences (one-way ANOVA, Tukey's post hoc test). (*$p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

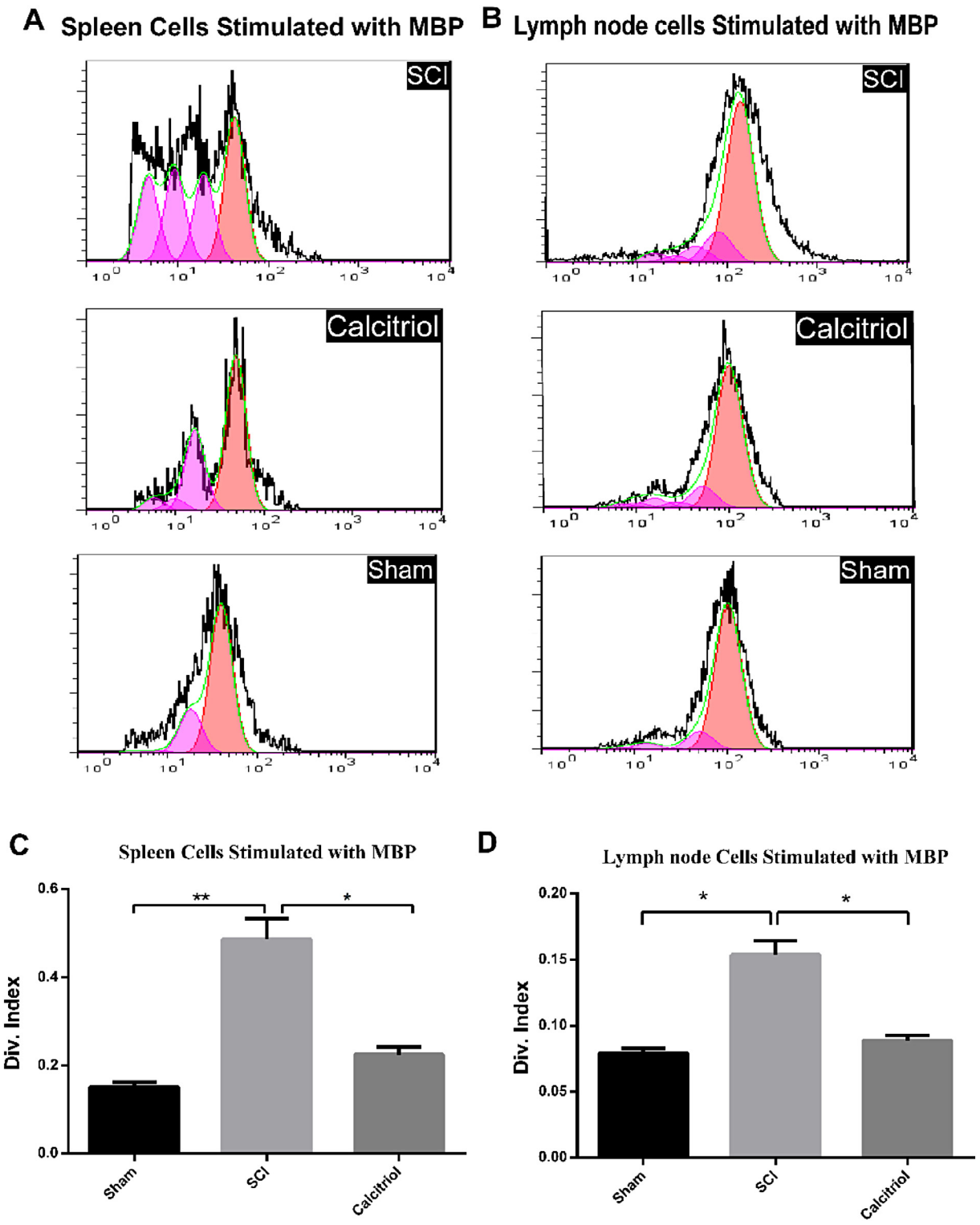


Fig. 3. Decreased proliferative activity of spleen and lymph node cells in response to MPB stimulation in CFSE proliferation assay after Calcitriol treatment in acute phase one week after spinal cord injury. A, B: Representative *flow cytometric analysis of CFSE*-labeled lymphocytes of spleen and lymph node cells. Cells were isolated from Calcitriol treatment group, spinal cord injury control group (SCI), and surgery control group (Sham) at one week after spinal cord injury, were stained with CFSE and subjected to proliferation in a 96-well plate stimulated with MBP (50 μg/ml) for 72 h. Representative histograms indicating the multiple peaks at consecutive generations of divisions are shown. C, D: Bar diagram shows the average of division index in Calcitriol, SCI, and Sham groups (n = 6 rat/groups). CFSE cell proliferation data were quantitatively analyzed by FlowJo software. Data are expressed as mean ± SEM. Asterisk indicates significant differences (one-way ANOVA, Tukey's post hoc test). (*$p < 0.05$, **$p < 0.01$). MBP: Myelin basic protein.

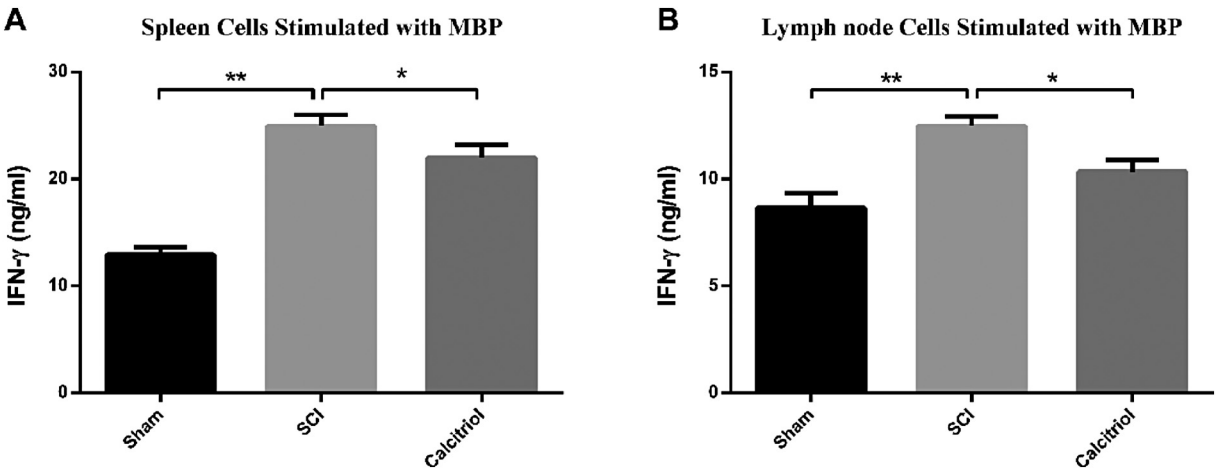


Fig. 4. Calcitriol treatment reduced IFN-γ production by spleen and lymph nodes cells against MBP after spinal cord injury. A, B: Bar diagrams show the average of IFN-γ concentration in culture supernatants secreted by mononuclear cells isolated from spleen and lymph nodes one week after spinal cord injury and stimulated with MBP (50 μg/ml) in 96-well plates for 72 h as determined by Sandwich ELISA. Values represent the mean ± SEM of six rats for each group. Asterisk indicates significant differences (one-way ANOVA, Tukey's post hoc test). (*$p < 0.05$, **$p < 0.01$).

3.8. Secretion of IL-10 in response to Myelin basic protein (MBP)

The evidence suggest that IL-10, as a potent anti-inflammatory cytokine, plays an important role in spinal cord injury and represents a therapeutic target for improving recovery following SCI [13]; therefore, in this study, IL-10 was measured by EILISA assay in spleen and lymph node responder cells to MBP stimulation in vitro.

Overall, no significant difference was observed among experimental groups using Tukey's post hoc multiple comparison test (Fig. 7A, B). Our results showed that Calcitriol treatment could not change IL-10 production in acute phase subsequent to spinal cord injury in response to MBP.

3.9. Leukocyte infiltration after SCI

To evaluate leukocyte infiltration toward the site of injury, we counted leukocytes using image J software in transverse sections at the lesion epicenter within 7 days after spinal cord injury by H&E staining (Fig. 8A).

Our findings demonstrate that Calcitriol treatment results in significant decrease in leukocyte recruitment at lesion area compared to SCI control group ($p < 0.05$) (Fig. 8B); The results revealed that the pharmacological attenuation by Calcitriol treatment could regulate leukocyte trafficking into inflammatory sites after spinal cord injury.

4. Discussion

Despite the promising therapeutic effects of many agents, only methylprednisolone was shown to be effective in treatment in large-scale clinical studies in acute phase following spinal cord injury [36,37]. As a result, to prevent and minimize post-traumatic deleterious events after SCI, looking for molecules with neuroprotective and neuroregenerative properties, preferably FDA-approved, is important for acute-phase treatment of SCI. Calcitriol, the endogenously produced hormone with favorable safety profiles, has demonstrated neuroprotective properties with promising results in multiple models of neurodegenerative and neuroimmune diseases [38]. Furthermore, Current literature emphasized a multidirectional mechanism of action of Calcitriol, with a special focus on its immunomodulatory properties, as a result of which Calcitriol receptor (VDR) expression on immune cells has been demonstrated [39]; however, there is just few studies assessing therapeutic benefit of Calcitriol treatment after spinal cord injury

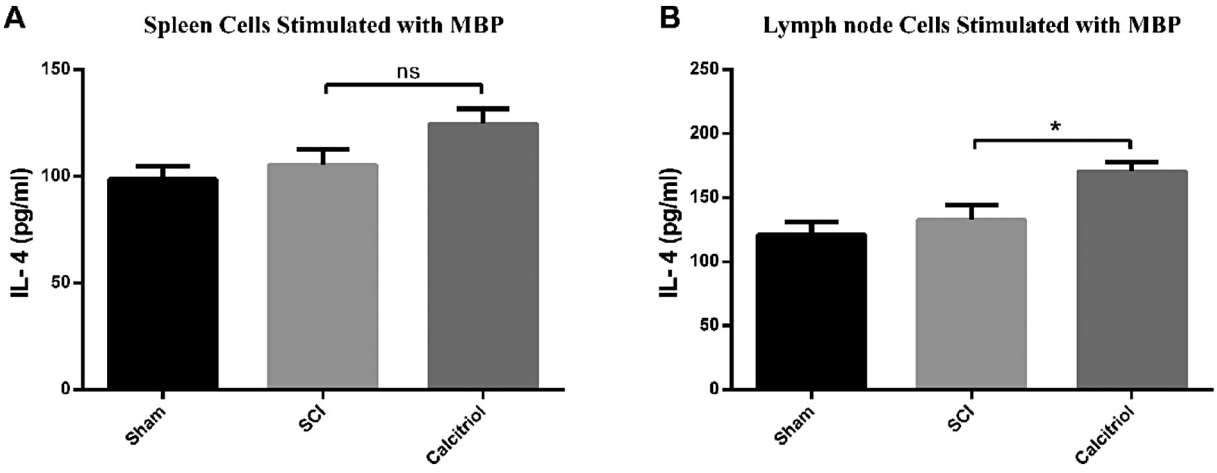


Fig. 5. Increased IL-4 secretion of lymph nodes cells in response to MBP in Calcitriol treatment group in acute phase after spinal cord injury. A, B: Representative bar diagrams indicating the average of IL-4 levels in culture supernatants produced by mononuclear cells isolated from spleen and lymph nodes one week after spinal cord injury and stimulated with MBP (50 μg/ml) in 96-well plates for 72 h as measured by ELISA. Data are expressed as the mean ± SEM of six rats for each group. Asterisk indicates significant differences (one-way ANOVA, Tukey's post hoc test). (*$p < 0.05$).

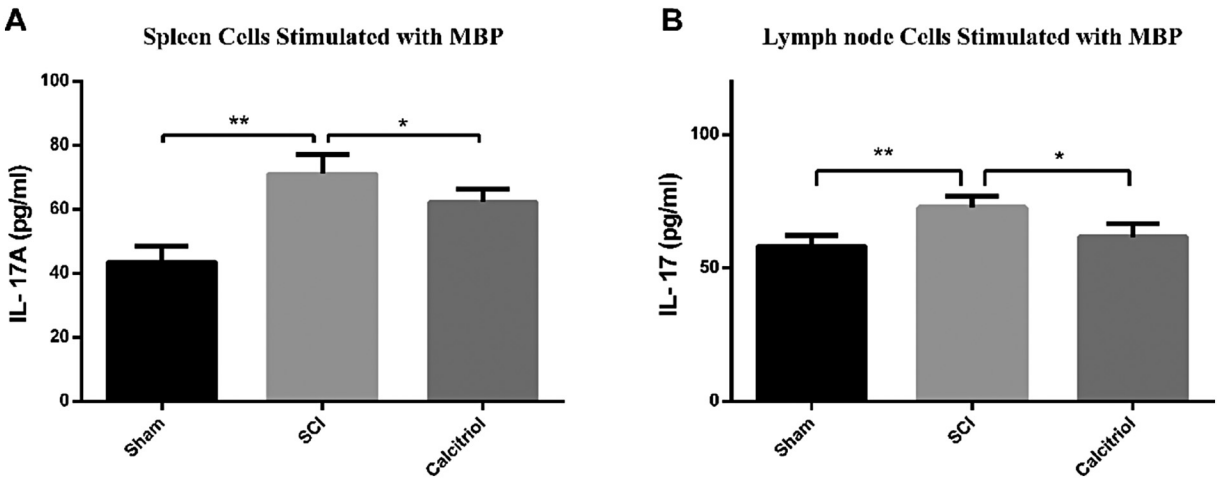


Fig. 6. Decreased IL-17A secretion after spinal cord injury against MBP in cells isolated from spleen and lymph nodes treated with Calcitriol in acute phase after spinal cord injury. A, B: Bar diagrams show the average of IL-17A concentrations in culture supernatants secreted by mononuclear cells isolated from spleen and lymph nodes one week after spinal cord injury that were stimulated with MBP (50 μg/ml) in 96-well plates for 72 h as determined by ELISA. Data are expressed as mean ± SEM of six rats for each group. Asterisk indicates significant differences (one-way ANOVA, Tukey's post hoc test, $*p < 0.05$, $**p < 0.01$).

with no assessment of immune response [20–22]. Therefore, evaluation of Calcitriol treatment in acute phase after spinal cord injury as well as identification of its immunomodulatory effects and mechanism of action will facilitate the development of novel treatments to prevent or reduce long-term disabilities that result from SCI.

In this study, we investigated the effect of treatment with high dose Calcitriol in acute phase after spinal cord injury intraperitoneally for 7 consecutive days in rats. Standard motor function evaluation test (BBB) was performed for 12 weeks and histological assessment was conducted on tissue spinal cord sections. We found that Calcitriol treatment after SCI was associated with improved spontaneous functional recovery following SCI with increased in the number of preserved motor neurons confirming the neuroprotective effects. In agreement with our data, which indicate positive effect of Calcitriol treatment after spinal cord injury, it has been demonstrated that spinal cord injured rats treated intraperitoneally with Calcitriol during 7 day after injury, have improved locomotor recovery associated with reduced oxidative stress, apoptosis and neuronal loss [21]. Moreover, our findings are in line with one of previous studies showing that early delivery of Cholecalciferol (unhydroxylated form of vitamin D3) improves locomotion and increases the number of spared axons after spinal cord jury [20].

To identify the probable mechanisms, secondary lymphoid organs, which include lymph nodes and spleen, were isolated from other sets of animals one week after spinal cord injury and proliferative responses as well as cytokine production were measured in response to MBP. It has been demonstrated the negative effect of autoimmunity against MBP after spinal cord injury derived from MBP-reactive T-cell lines [40,41]. In this study, our results revealed that Calcitriol treatment decreased proliferative response against MBP accompanied with change in cytokine production. In parallel to our findings, previous studies also showed that Calcitriol inhibits the proliferation of activated lymphocytes in a dose-dependent fashion in vitro and in vivo, however, proliferation of naïve CD45RA+ T cells were affected less than CD45R0+ activated T lymphocyte and memory T cells [26,27,42]. Additionally, naive and activated T lymphocytes vary significantly in VDR expression; resting T lymphocytes expressed < 1000 VDR per cell and activation amplified this number 10-fold [43,44], highlighting the importance of inflammatory conditions in acute phase after SCI which Impact on immunomodulatory effect of Calcitriol.

In this study, our results revealed that Calcitriol treatment in acute phase after SCI could result in decreased IFN-γ secretion in isolated mononuclear cells against MBP, whereas IL-4 production could be

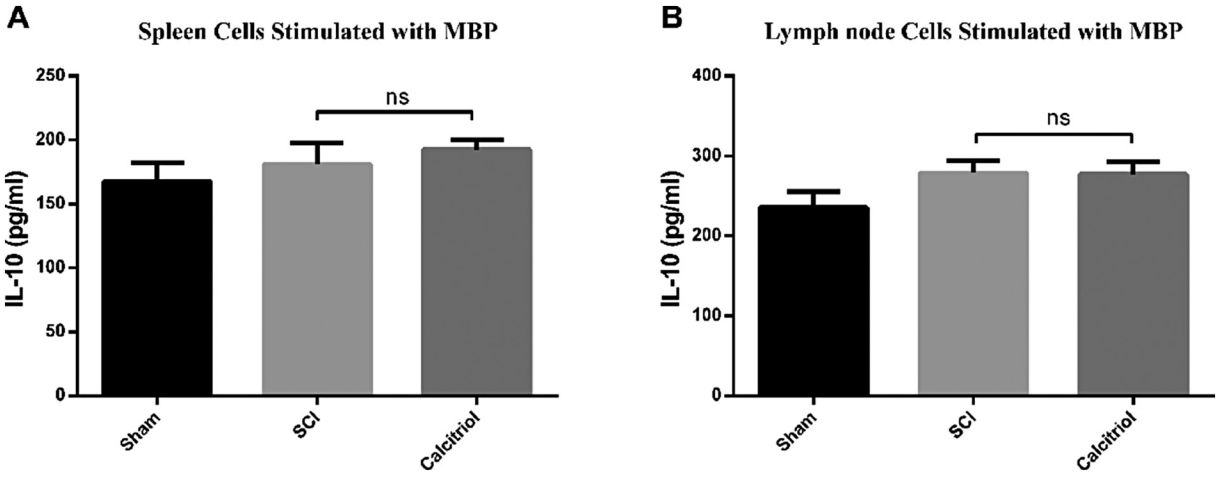
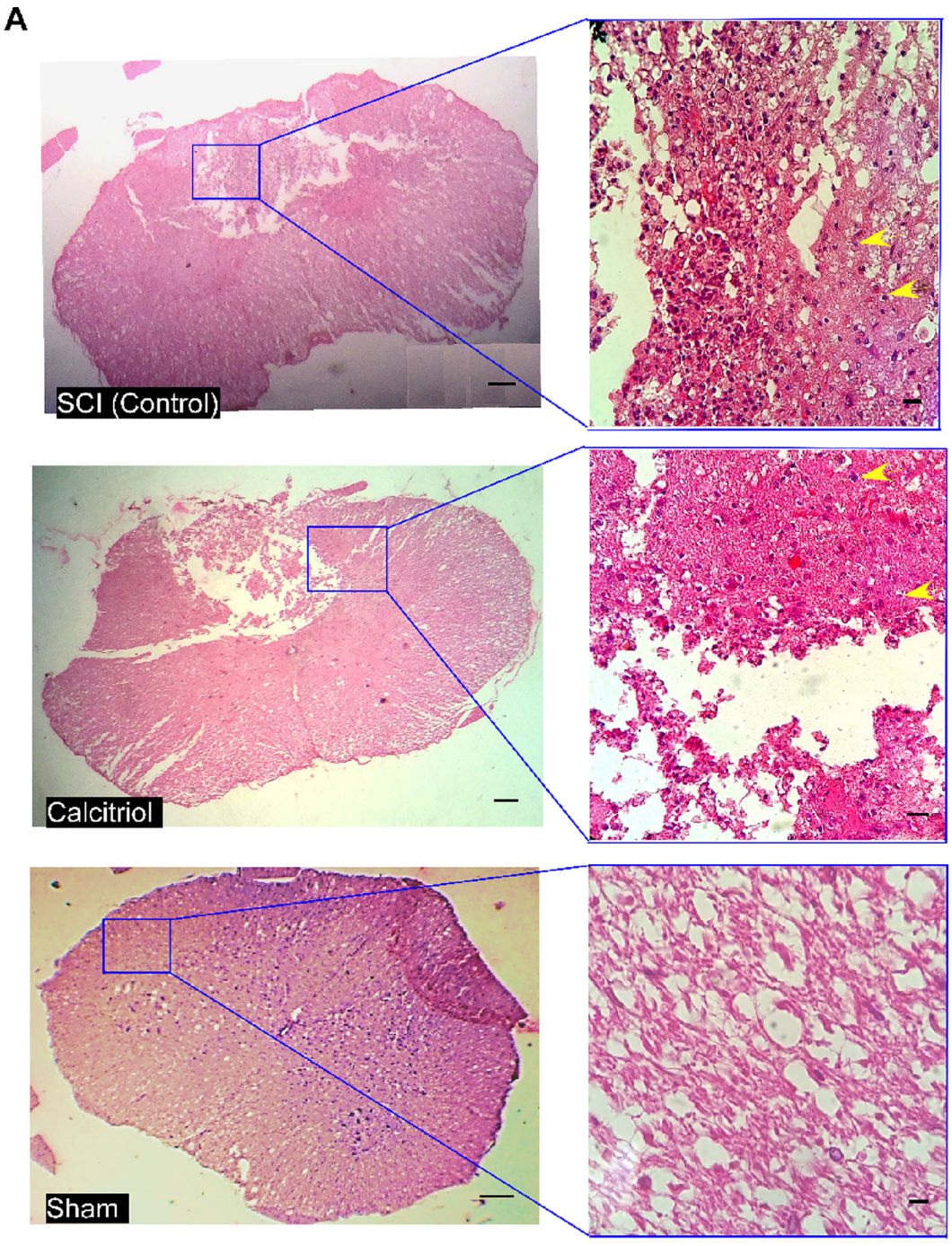


Fig. 7. No significant difference among experimental groups related to IL-10 levels in response to MBP stimulation measured in vitro by ELISA. A, B: Representative bar diagrams indicating the average of IL-10 levels produced by mononuclear cells isolated from spleen and lymph nodes one week after spinal cord injury and stimulated with MBP (50 μg/ml) in 96-well plates for 72 h. Data are expressed as the mean ± SEM of six rats for each group. Asterisk indicates significant differences (one-way ANOVA, Tukey's post hoc test).



(caption on next page)

Fig. 8. Calcitriol treatment reduced leukocyte infiltration into injury site after SCI. A: Representative images showing transverse section of spinal cord, leukocyte recruitment at lesion area of all groups evaluated in this study at 12 weeks after spinal cord injury by Hematoxylin and Eosin (H&E) staining. Yellow arrows illustrate infiltrated leukocytes. Scale bar represent 100 μm and 20 μm in left and right columns, respectively. B: Bar diagram shows the mean cell number per slide (n = 6 rat/group) of migrated leukocytes in experimental groups at 7 days after spinal cord injury. Data are expressed as mean ± SD. Asterisk indicates significant differences (independent samples *t*-test. $^{**}p < 0.01$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

increased. In agreement with our data, previous studies also showed that Calcitriol can inhibit the IFN-γ production by staphylococcal enterotoxin A -stimulated peripheral blood mononuclear cells (PBMC) in a dose-dependent manner [27]; However, the effects of Calcitriol on Th2 cells is more controversial with evidence that Calcitriol inhibits IL-4 transcriptionally as well as evidence that Calcitriol upregulates IL-4 in T lymphocytes [45,46]. Another report showed targeted disruption of the *IL-4* gene reduced slightly the protective function of Calcitriol in EAE [28]. Additionally, recent evidence suggests that impairment of IL-4 production in IL-4 null mice could worsen functional recovery after SCI [11]. Furthermore, It has been shown that IFN-γ-KO mice after contusive spinal cord injury have a significantly lower degree of impairment [10]. Adaptive immunity is biased toward the Th1 immune response after spinal cord injury [2], as a result, the preferential inhibition of Th1 immunity and Th2 induction by Calcitriol may prove to be neuroprotective after SCI [47].

Moreover, our findings demonstated that intraperitoneal Calcitriol administration in acute phase after SCI enhance motor function recovery with reduced IL-17 proinflammatory cytokine production and no significant effects on IL-10 as an anti-inflammatory cytokine. IL-17 is an important proinflammatory factor mainly produced by Th17 cells, which contributes to neuroinflammation and disease pathogenesis in most autoimmune diseases. It has been recently demonstrated that IL-17 deficiency could enhance significantly locomotor functional recovery after SCI [12]. Among rodent $CD4^+$ T lymphocytes subsets, IL-17-producing Th17 cells same as Th1 and Th2 lymphocytes express abundant calcitriol *receptor* (*VDR*) transcripts. Calcitriol inhibited IL-17 synthesis in a VDR-dependent fashion by a post-transcriptional mechanism In agreement with our data, previous studies also showed that the spleens of Calcitriol-treated mice in the EAE model had a reduced number of Th17 cells and lower IL-17 secretion than controls [23,44,48,49]. In contrast with our data, previous studies showed that Calcitriol treatment can induce $FoxP3^+$ Tregs in the lymph nodes, spleen, and spinal cord in EAE model and increase IL-10 secretion when $CD4^+$ cells are cultured under neutral conditions [48,50,51], while very low calcitriol receptor (*VDR*) transcript levels were detected in rodent $CD4^+Foxp3^+$ Treg cells produced in vitro and whether $CD4^+Foxp3^+$ Treg cells express the VDR is uncertain [43]. Additionally, it has been demonstrated that Calcitriol is not capable to ameliorate EAE when the VDR is absent in T cells [23]. Furthermore, in agreement with our finding, in animal model of Arthritis Calcitriol had a direct effect on T lymphocytes to inhibit Th17 differentiation, whereas had no effects on Tregs induction [52].

We observed spinal cord injury induced a robust inflammatory response and the *extravasation* of *leukocytes* into the *lesion site*, *whereas*, there was a tendency for Calcitriol-treated animals to have fewer leukocyte recruitment in the spinal cord at one-week post-SCI, which is in parallel with previous reports that Calcitriol treatment can reduce leukocyte recruitment to injured central nervous system and acute lung injury [53,54]. It has been demonstrated IL-17A can be crucial for chemokine induction and neutrophil influx during inflammatory response [55]. Furthermore, it has been shown that IL-17 treatment results in neutrophil infiltration in the spinal cord tissue at 14 days compared to untreated spinal cord injured rats [56]. Therefore, results obtained *from present study* revealed a possible mechanism by which leukocytes influx inhibition into injury site in acute phase after spinal cord injury might result from Calcitriol modulation of Th17 and IL-17A reduction. It has been shown Calcitriol could decrease oxidative stress in rats following 7 days after SCI, however, related mechanism has not been illustrated [21]. Immune response is critical in shaping the neuroinflammation which regulate oxidative stress and free radicals formation after SCI [57], additionally, our results revealed immunomodulatory effect of Calcitriol could be the major mechanism that dampening the damaging consequence of oxidative stress after SCI.

It should be noted that this study has not assessed whether Calcitriol treatment in combination with Methylprednisolone, the standard treatment for acute SCI, might have beneficial effect or not. However, for definitive conclusions, further studies need to be carried out in this regard with various combination therapy. Furthermore, Calcitriol analogue with enhanced immunomodulatory potency such as C 1,25-dihydroxyvitamin D3-3-bromoacetate could be evaluated after SCI, however, vitamin D analogs have been approved for treating psoriasis, osteoporosis, and secondary hyperparathyroidism [58,59]. Interesting results have been obtained from different studies on the role of Calcitriol receptor (VDR) in Calcitriol immunomodulatory effect, suggesting that change in this receptor expression after spinal cord injury may exacerbate recovery after SCI; According to evidence that induction and activation of this receptor in acute phase in traumatic brain injury (TBI) resulted in protective outcome [60], which needs to be considered in future studies related to spinal cord injury.

In conclusion, we demonstrated that treatment with Calcitriol in acute phase of spinal cord injury results in neuroprotective effects with suppressed activity of autoreactive lymphocytes against MBP. Overall, this study provides evidence suggesting that depending on cytokine axis, Calcitriol may affect inflammatory response targeting adoptive immune system, which contribute to protective effect of Calcitriol by administration in acute SCI.

Declaration of Competing Interest

None.

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