

Original Article

# The demyelination and altered motor performance following electrolytic lesion in the ventrolateral white matter of spinal cord in male rats: benefit of post-injury administration of estradiol

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## Abstract

**Introduction:** Spinal cord injuries are accompanied with significant demyelination of axons and subsequent locomotor dysfunction. To identify the extent of damage following electrolytic lesion of ventrolateral white matter, essential area for initiation of locomotor activity, we assessed demyelination as well as alteration in motor performance. Moreover, the protective effect of estradiol as a candidate treatment for preservation of myelin and locomotor activity after injury was examined due to its anti-apoptotic and anti-inflammatory activities.

**Methods:** A unilateral electrolytic lesion positioned in the right ventrolateral funiculus (VLF) was applied following laminectomy at T8-T9. In the estradiol-treated injury group, animals received a pharmacological single dose of estradiol valerate (4 mg/kg) at 30min post injury. Locomotor function was assessed using rotarod and open field tasks during 4 weeks after injury.

**Results:** Obtained results showed significant demyelination at the site of injury and caudal areas following lesion as well as altered motor performance. Post-spinal cord injury administration of estradiol enhanced white matter maintenance at the site of lesion, restored the level of myelin basic protein (MBP), decreased TUNEL positive cells and improved functional recovery.

**Conclusion:** Taken together, these results indicate that demyelination after lesion in VLF may be a contributing factor to limited motor performance, and suggest that pharmacological doses of estradiol may have an early protective effect through sparing of white matter.

## Keywords:

Spinal cord injury;  
Estradiol;  
Demyelination

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## Introduction

Spinal cord injuries (SCI) cause sensory, motor and

autonomic dysfunctions and the resulting locomotor deficits severely impact the affected patients. Traumatic spinal cord injury induces a cascade of secondary deteriorating damages that contribute to

extensive tissue loss and functional impairment (McDonald and Sadowsky, 2002; Profyris et al., 2004). In different experimental animal models of SCI such as contusion (Blight, 1983; Lasiene et al., 2008), compression (Gledhill et al., 1973; Waxman, 1989) and hemisection, demyelination has been reported as a prominent feature of disease (Arvanian et al., 2009). In incomplete models, there is some extent of spared or preserved white matter (Bunge et al., 1997), prone to demyelination (Blight, 1983). Prolonged loss of oligodendrocytes leads to the demyelination of spare axons and functional deficits (McDonald and Sadowsky, 2002; Profyris et al., 2004). Degree of demyelination depends on the type and severity of injury, which is mostly studied on contusion injury as a clinically relevant model. However, the extent of demyelination after unilateral electrolytic lesion in ventrolateral spinal white matter remains elusive.

In the electrolytic lesion in ventrolateral white matter of spinal cord, a model established by Wang and Thompson (2008), damage to the white matter and subsequent changes in motor performance have not been well studied. Considering the importance of demyelination in SCI-induced locomotor dysfunction, further studies on the white matter damage in different models would be of great benefit to develop supplementary therapeutic agents for the maintenance of white matter. Therefore, we designed our experiment to better clarify these features. Due to the multifaceted and complex pathophysiology of SCI, successful treatment will require a multipotent curative pharmacological agent. Therapeutic agents tend to be moderately effective; however, there is still debate over this issue (Sribnick et al., 2010). Estradiol could be an appropriate candidate because of its anti-apoptotic and anti-inflammatory effects (Sribnick et al., 2005; Sribnick et al., 2010). These effects have been described to be protective for spared white matter, neuronal and glial cells (Sur et al., 2003; Biewenga et al., 2005), and contribute to enhanced motor function (Yune TY et al., 2004). Although various studies with different dosages and administration time points have revealed protective effects of estradiol after SCI (Hauben et al., 2002; Farooque et al., 2006; Webb et al., 2006; Kachadroka et al., 2010), it has not still been applied as a potential therapy. Further studies are required to find out appropriate therapeutic time window and dosages

as well as underlying protective mechanisms. The goal of this research was to evaluate the effects of a single high dose of estradiol on motor function and white matter sparing after a lesion of the right ventrolateral funiculus (VLF) which is a critical area for overall locomotion.

## Materials and methods

Adult male Sprague Dawley rats weighing 220-300 g were used. A total of 83 rats were used in this study including 30 rats for histological assessments and 53 rats for behavioral assessments. Animals were kept under 12 hour light/dark cycle and room temperature of  $21\pm 2^{\circ}\text{C}$ . All surgical procedures and experimental treatments were approved by ethical committee of Shahid Beheshti University of Medical Sciences.

### Spinal cord lesion and estradiol treatment

Unilateral electrolytic lesion was induced according to the method described by Wang and Thompson (2008) with some modifications. Briefly, rats were anesthetized by intraperitoneal (i.p.) injection of ketamine (60 mg/kg) and xylazine (10 mg/kg), the skin and muscle are incised to expose the vertebral column, and a laminectomy was performed at T8 or T9 vertebrae. In the electrolytic lesion group, the dura was opened by fine scissors. Then, a tungsten electrode (5 $\mu\text{m}$  tip, 1 M $\Omega$ ) was pointed to the VLF area, with stereotaxic coordinates (laterality to midline: 0.5–0.7 mm and depth: 1.6–1.9 mm). Lesion was made by a brief current pulse (300  $\mu\text{A}$ , 90 s) passed through the electrode. Treatment group received i.p. injection of a single dose of 4 mg/kg 17 $\beta$ -estradiol (Sribnick et al., 2005; Kachadroka et al., 2010) dissolved in sesame oil, 30 min after injury. Vehicle control group received only sesame oil. After surgery, rats received 5ml saline solution subcutaneously (S.C.) for rehydration. Penicillin was administered to prevent infection.

### Locomotion assessment

Motor coordination was assessed using the rotarod (Ugo Basile, Model 7750, Italy) (Dunham and Miya, 1957) for all experimental groups at 0, 7, 14 and 28 days post injury (dpi). The rats were pre-trained to stay on rotating rod and the endurance time on the revolving rod at 20 rpm was determined for a

maximum duration of 2 min. Each rat was given five trials before the actual reading was taken. The trained animals received lesion and estradiol treatment. A total of 3 trials was recorded to calculate the mean endurance time.

The open field test was designed to measure locomotor activity during 5min in all experimental groups prior to the surgery at 7, 14 and 28 dpi. Before the experiment, each animal was pre-trained to the test box for 5 days to get accustomed to the environment and walk through the center of open field. Each animal was placed individually from a specific corner of open field box and its behavior was recorded for 5 min. All activities were simultaneously recorded using a video camera mounted over the open field box and attached to a computer, where an image analyzer (EthoVision, version7, Noldus Information Technology, Netherlands) was used to track movements of rats in the arena at real time. The total distance moved during 5min was considered as measure of motor performance (Meredith and Kang, 2006).

### **Histological assessment**

The animals were anesthetized at 7, 14 and 28 dpi by i.p. injection of ketamine (60 mg/kg) and xylazine (10 mg/kg) and perfused transcardially with 150 ml PBS (pH 7.4), followed by 250ml of 4% paraformaldehyde (PFA). Segments of spinal cord, 5-6 mm, from lesion site toward caudal area were dissected and post-fixed overnight in PFA. Then, the tissue was embedded in paraffin and serial longitudinal sections of 5 $\mu$ m were used for immunohistological studies and TUNEL staining.

Toluidine blue stained resin sections were produced to examine the gross pathology of the lesion site. For resin processing, animals were perfused transcardially with 150ml PBS, then a fixative containing 4% PFA and 1% glutaraldehyde, in 0.1M phosphate buffer, (pH 7.4). The spinal cord sections at the lesion level were dissected and post-fixed in 4% glutaraldehyde for 72 hours. They were then rinsed in 0.1M phosphate buffer (pH 7.4) for 30 min, exposed to 1% OsO<sub>4</sub>, dehydrated in ascending graded alcohols, and finally embedded in resin. Transverse semi-thin (1 $\mu$ m) sections were cut with the ultramicrotome (LEICA, UCT) from each block, stained by toluidine blue (%1) for light microscopy (Kotter et al., 2006).

### **Myelin staining**

In order to assess the evidence for demyelination, the spinal cord paraffinated sections were stained by Luxol Fast Blue (LFB) (Sigma,USA) (Pistorio et al., 2006; Mozafari et al., 2011; Sherafat et al., 2012) which binds to the myelin, and counterstained with cresyl violet acetate (Sigma, USA). For LFB staining, paraffin sections were incubated in graded alcohols and placed in 0.1% LFB at 60°C, 3h, then differentiated in 0.05% LiCO<sub>3</sub> for 2min and 70% ethanol for 60-70 seconds, counterstained with cresyl violet acetate for 5min, and dehydrated in graded alcohol and xylene, and cover slipped. Five sections from the same area of the spinal cord of each animal were analyzed to quantify the extent of demyelination using the light microscope (Olympus BX51, Japan) and images were captured by camera (Olympus DP72). These images were then imported into the NIH Image J program and analysis of demyelinated areas was performed by manually outlining the determined areas including center of lesion area (1 mm in length and equal to 0.5-0.5 mm from the central part), 1 mm caudal to lesion (0.5-1.5 mm caudal) and 2 mm caudal (1.5-2.5 mm caudal) to the lesion center. For each section, the extent of demyelination was assessed as the percentage of total spinal cord area (Jasmin et al., 2000; Pomeroy et al., 2005).

### **Myelin basic protein immunohistochemistry**

In order to mark myelinating cells, immunohistochemical staining for myelin basic protein (MBP) (Mathis et al., 2001) was performed on paraffin sections.

Sections were deparaffinized and rehydrated. Antigen retrieval was performed by boiling the sections in citrate buffer (pH 6) for 5 min using microwave. Endogenous peroxidases were quenched with 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) in absolute methanol and the sections were blocked with 10% normal goat serum in PBS. Rabbit monoclonal primary antibody to MBP (Abcam7349, 1/100) was applied overnight at 4°C. The sections were washed and then incubated with a peroxidase labeled anti-rabbit secondary antibody (EnVision, Dako, K406189). The staining was developed using diaminobenzidine as chromogen, counterstained with hematoxylin, dehydrated using graded alcohols and xylene, and mounted with mounting medium (Entellan®, MERK).

Qualitative assessment of MBP immunoreactivity was performed by two blinded observers using a grading method. The grading score was considered from 0 to 3 with 0 for no staining and 1-3 for weak, intermediate and strong staining (Pang et al., 2010).

### TUNEL assay

We performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL reaction) on spinal cord sections from similar area in each experimental animal, according to the manufacturer's protocol (Roche Applied Science, Germany). In brief, the sections were deparaffinized in xylene, rehydrated and immersed in 3% H<sub>2</sub>O<sub>2</sub>. After rinsing with PBS, the sections were permeabilized using proteinase K solution at 37°C for 15 min. Incubation with TUNEL reaction mixture was performed for 60 min at 37°C after which the sections were incubated with 50 ml of converter-POD for 30 min at 37°C. After rinsing with PBS, the sections were incubated for 10 min at room temperature with 50ml of DAB solution. Counterstaining was carried out with 0.5% methyl green. For the positive controls, the sections were pre-incubated with 1- Unit of DNase1 in 10 mM PBS for 20 min. Negative controls were incubated only with buffer.

The number of TUNEL positive cells per microscopic field were counted in three to four sections per animal (Yune TY et al., 2004; Sribnick EA et al., 2006).

### Statistical analysis

All of the data are presented as Means  $\pm$  SEM and analyzed by two-way analysis of variance (ANOVA) with 2 factors of time and treatment followed by the Bonferroni post-hoc test.  $P \leq 0.05$  was considered as minimum significant difference of means.

## Results

### Behavioral assessment

Motor activity was investigated in all experimental groups. Animals with normal motor activity were selected for behavioral experiments. Consistent with results reported previously by Wang et al. 2008, we observed no paralysis of the animals after injury.

The results demonstrated a significant decrease in motor performance on rotating rod in injured animals versus control at day 7 ( $P < 0.01$ ). Estradiol significantly improved motor performance at day 14,

but not at day 28 ( $P < 0.05$ ) (Figure 1A).

Data from open field test indicated that the distance moved decreased in the injured animals but a significant difference was not observed between the injured and uninjured animals. Estradiol in injured animals significantly improved motor activity at 7 and 14 dpi, but not at day 28, in comparison with the lesion group ( $P \leq 0.001$ ) (Figure 1B).

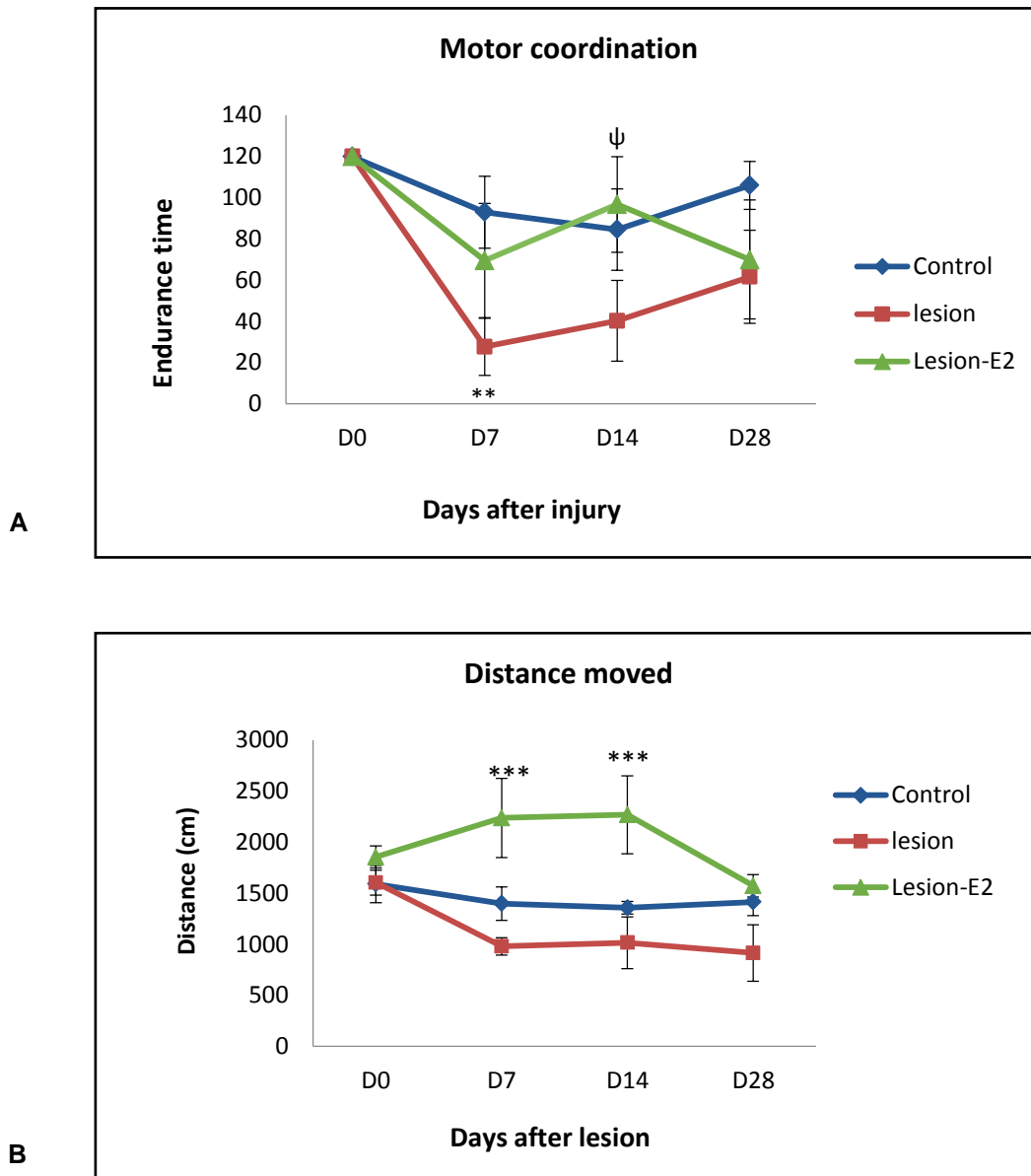
### Electrolytic lesion-induced demyelination and apoptotic cell death

Induction of electrolytic lesions in the VLF caused demyelination at 7, 14 and 28 dpi, shown as the disappearance of luxol fast blue-stained myelin. At all the time points, we observed significant demyelination for both estradiol-treated and untreated lesion groups as compared to the control at the lesion center as well as 1-2 mm caudal to the lesion (Figure 2 A, B and C). Estradiol reduced demyelination only at day 7 at the lesion center (Figure 2A).

To confirm the presence of demyelinated fibers in injured spinal cord, toluidine blue-stained resin sections were observed by light microscopy at the ventrolateral white matter where demyelination had occurred. In the normal spinal cord, axons are tightly packed with little extracellular space and with various diameters. In injured tissue, the extracellular space within VLF was expanded particularly in the vicinity of lesion site where the myelin sheaths were destroyed. Estradiol-treated animals had darker staining and limited demyelinated axons in toluidine blue stained sections (Figure 3B).

MBP, a specific protein component of myelin, immunostaining was performed in order to better represent myelin changes which was visually observed and scored on the sections. Lesion caused a decrease in MBP staining in all lesioned animals at the given time points. Estradiol increased MBP immunostaining intensity at each time point with significant effect at day 7 as compared with injured group ( $P < 0.001$ , figure 4A, B).

TUNEL staining was performed to measure apoptotic cells in the lesion center and caudal areas in the adjacent sections used for myelin staining and MBP immunohistochemistry. There was a significant increase in the number of TUNEL positive cells at the lesion site as well as 1-2 mm caudally in all lesion animals at all the time points at 7( $P < 0.001$ ), 14 ( $P < 0.001$  in 1mm,  $P < 0.01$  in 2mm) and 28 ( $P < 0.001$ )



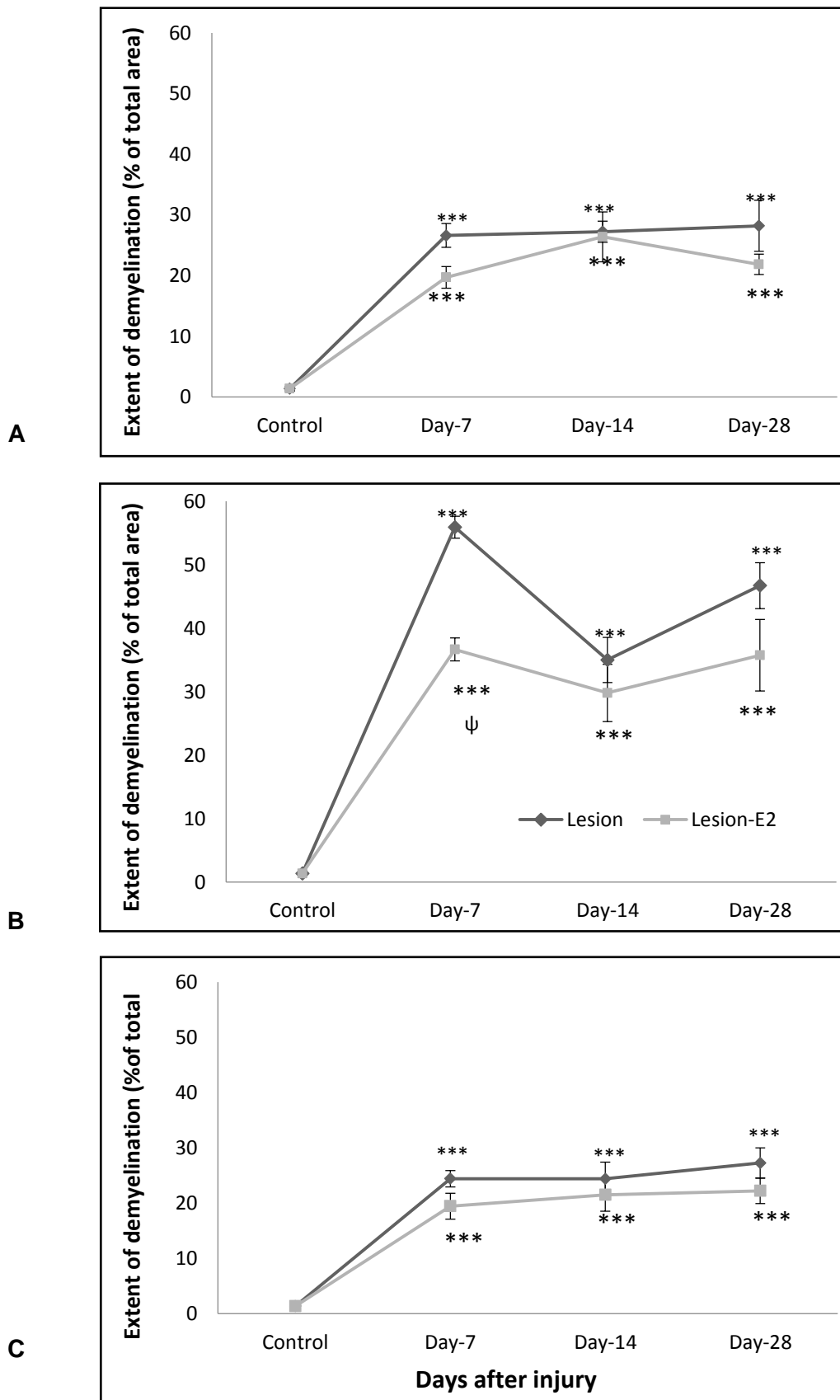
**Fig.1.** Assessment demyelination and the effect of post injury administration of estradiol on locomotor function. (A) Electrolytic lesion on right VLF decreased motor performance on rotating rod during experimental time points, significantly on day 7  $**P < 0.01$ . Estradiol-treated group had significant difference with lesion group on day 14  $\psi P < 0.05$ . (B) Distance moved in an open field. Profound difference between injured and estradiol-treated group on days 7 and 14 is indicated by  $***P < 0.001$ . The values are shown as Mean  $\pm$  SEM. (n=8), VLF: ventrolateral funiculus, E2: estradiol valerate 4 mg/kg, i.p., 30 min postinjury.

dpi (Figure 5 A, B, C and Figure 6 A, B, C). Treatment with estradiol reduced the number of apoptotic cells by about 50% at day 7 in the lesion center and 1 mm caudally (Fig 5 A and B).

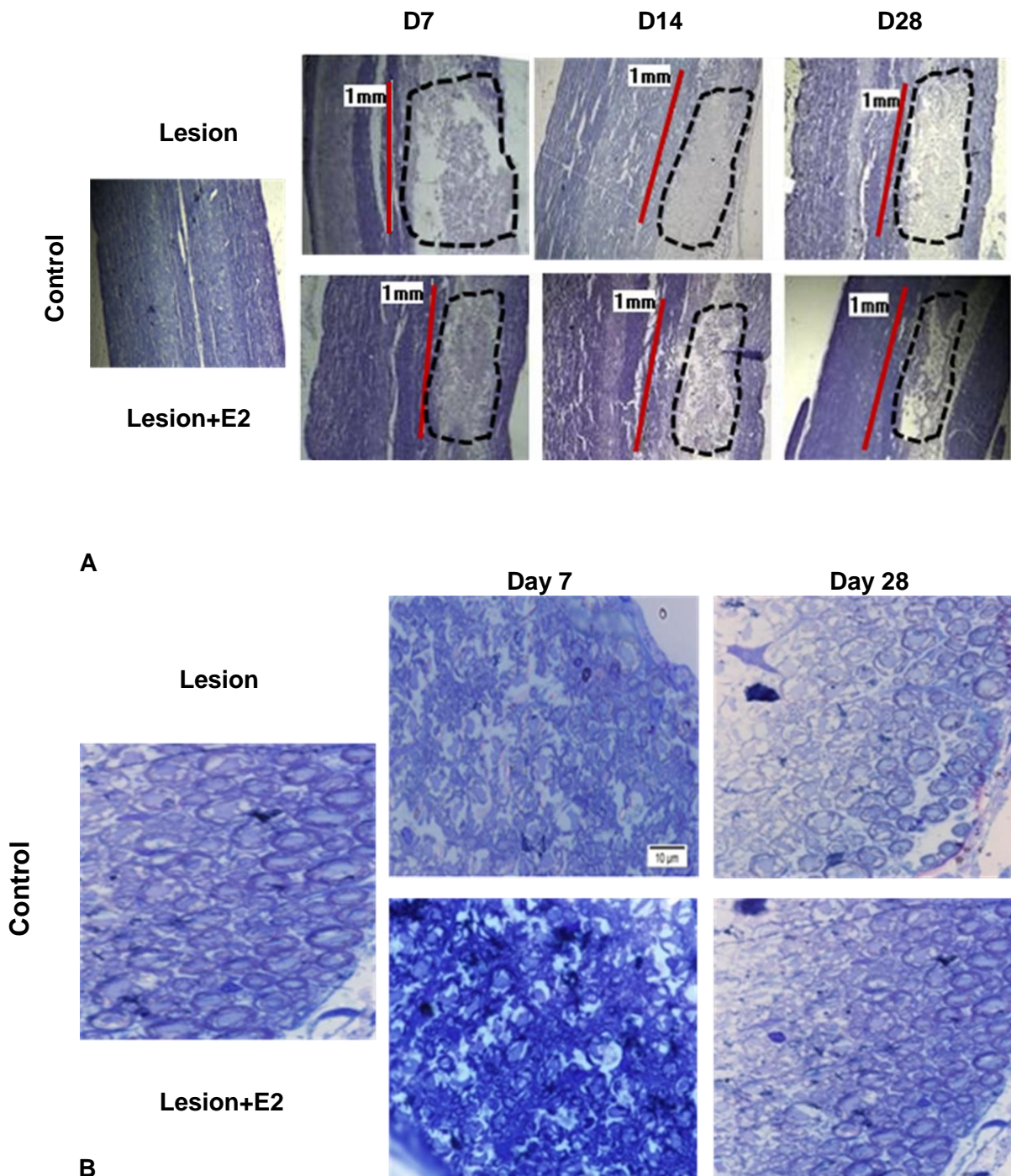
## Discussion

We designed this study to examine extent of myelin destruction after an electrolytic lesion model of SCI

entered on VLF and to assess the effect of estradiol on white matter in this area. The VLF contains interspersed descending pathways that are necessary to initiate locomotor function. Damage to these descending tracts may produce motor deficits due to extensive demyelination (Arvanian, V.L., et al. 2009). Supraspinal tracts interact with central pattern generators (CPG) to form final motor output. Supraspinal control of locomotor function through corticospinal motor pathways that reaches at the level



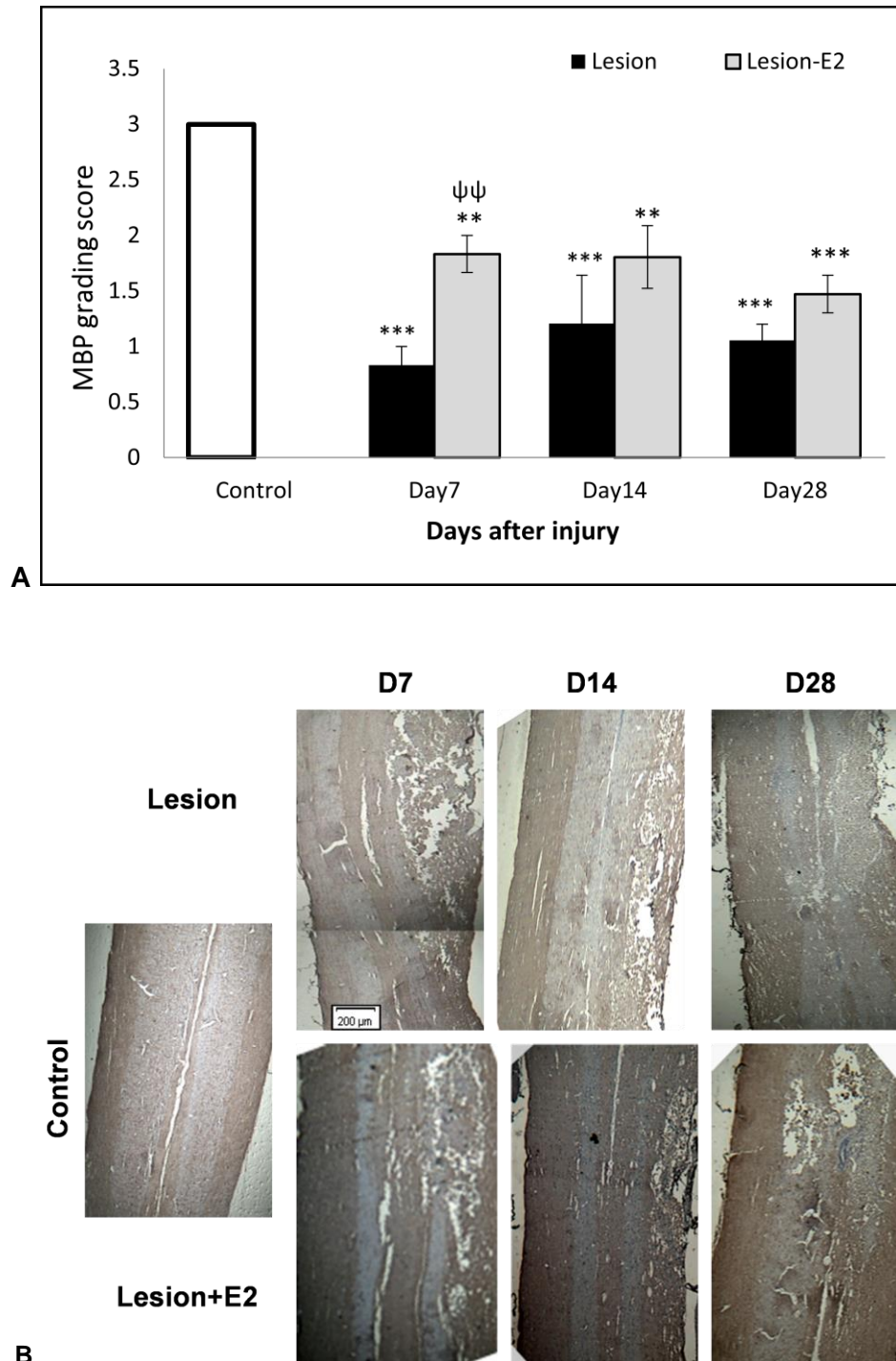
**Fig.2.** Quantification of the extent of demyelinated area following estradiol treatment of injured rats in the right VPL. Significant demyelinated area was observed on days 7, 14 and 28 at the lesion center (1 mm) (A) , 0.5-1.5 mm caudal (B) and 1.5-2.5 mm caudal (C) in both injured and estradiol-treated injured groups versus control group \*\*\*  $P < 0.001$ . Estradiol-treated animals represented less extent of demyelination. Significant reduction was observed at lesion center in E2-treated lesion group versus lesion group on day 7  $\psi P < 0.05$ . The data represented as Mean  $\pm$  SEM, (n=4-5), E2: estradiol valerate 4 mg/kg, i.p., 30 min postinjury.



**Fig.3.** The representative micrographs for the lesion center of both lesion and E2- treated lesion animals. (A) Longitudinal sections of lesion center for myelin stained with LFB. Discrete demyelination was shown at the lesion area in which damage was greatly noted as central cavity surrounding by abnormal myelin. Estradiol-treated lesion group demonstrated less extent of demyelination. Scale bar =200μm. (B) Toluidine blue –stained transverse resin sections on days 7 and 28. Electrolytic lesion resulted in demyelination in axons centered on right VLF. Tissue pathology and myelin deficiency were obvious in lesion group on days 7 and 28. After administration of estradiol, tissue damage and demyelinated axons apparently were limited and thin myelin was observed in the given time points. All histological sections are 1 μm toluidine blue resin sections, scale bar = 10μm.

of spinal cord activates central pattern generators. They have a regulatory function in preserving balance and coordination for locomotion. Injury to the spinal cord might affect general organization of spinal CPGs (Brown, 1971; Brown, 1974; Steeves JD, 1980; Houle

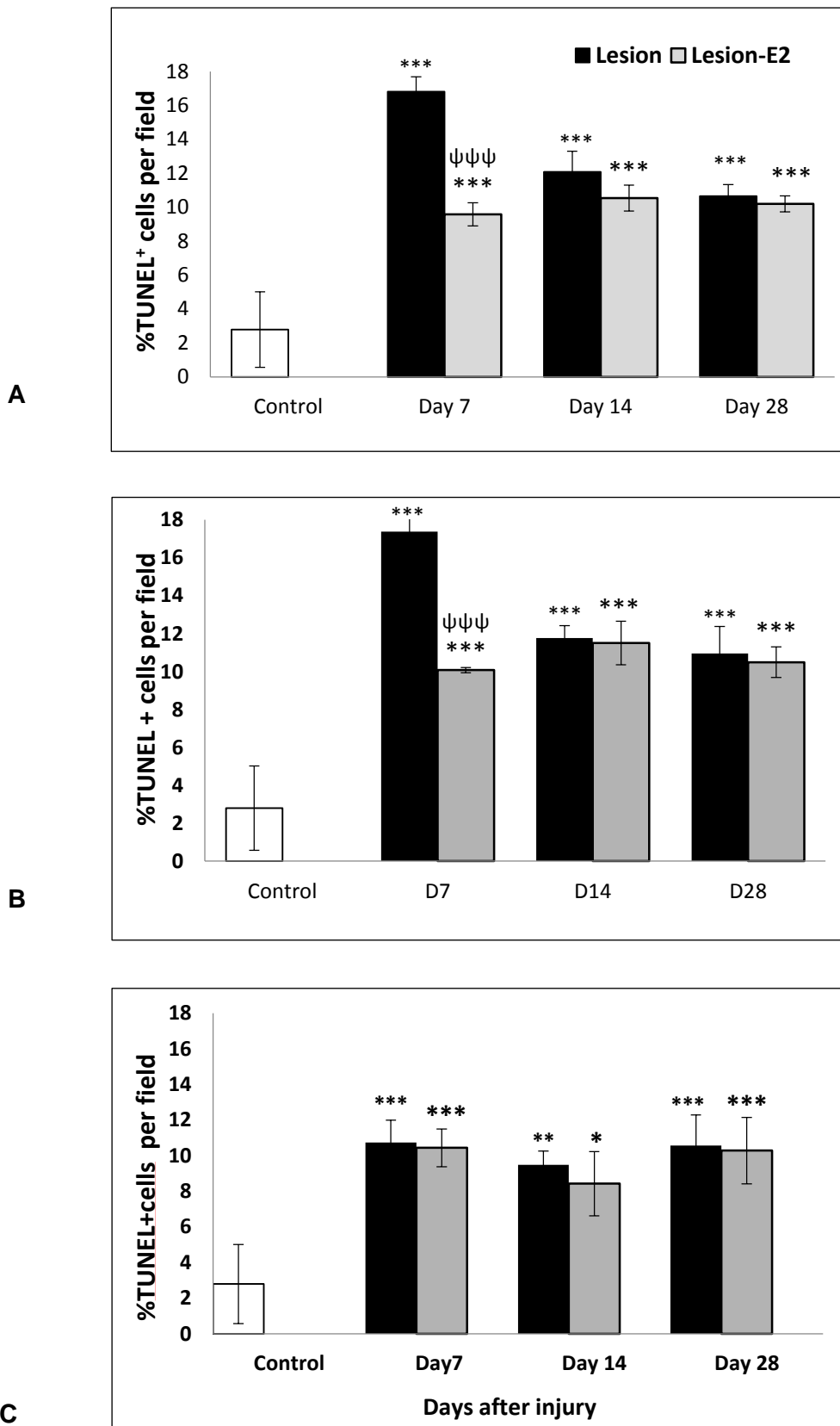
and Jin, 2001; Loy et al., 2002). Electrolytic lesion of VLF led to histological and behavioral changes. We did not observe a significant reduction in motor activity in open field task after injury, but endurance time on rotating rod significantly reduced on day 7.



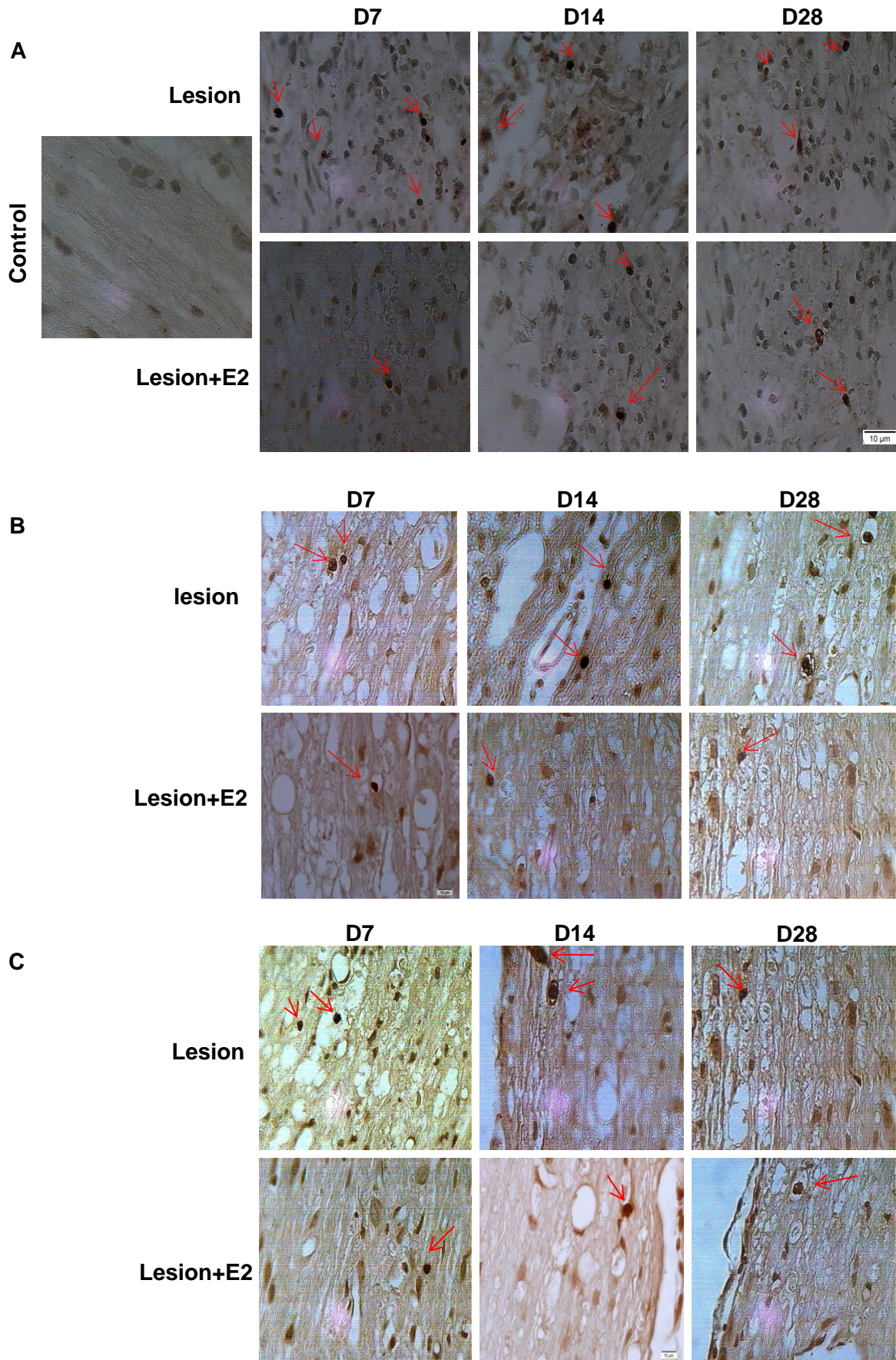
**Fig.4.** Evaluation of MBP protein expression after electrolytic lesion and effect of estradiol.

(A) electrolytic lesion caused a significant reduction in MBP expression on days 7,14 and 28 post lesion in comparison with control group \*\*\*  $P < 0.001$ . E2-treated lesion group represented significant difference with control group on days 7, 14 and 28 post lesion (\*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ ) and lesion group on day 7  $\Psi\Psi P < 0.01$ . The values are showed as Mean  $\pm$  SEM (n=5-6). (B) Representative micrographs are demonstrated of all groups at the given time points. Immunoreactivity in lesioned animals was faint. Scale bar =200 $\mu$ m.





**Fig.5.** Quantification number of the TUNEL- positive cells after electrolytic lesion in right VLF and effect of estradiol. TUNEL-positive cells were counted on 100x magnification. Both lesion group and E2-treated lesion group differed significantly from control group at lesion center (1mm)(A), 1mm caudal (0.5-1.5mm) (B) and 2mm caudal (1.5-2.5mm) to lesion center (C) at the given time point\*\* P< 0.01, \*\*\* P< 0.001, \*P<0.05. Estradiol treatment significantly reduced number of TUNEL-positive cells at the lesion center and also 1mm caudal on day 7 as compared to lesion group ψψψ P< 0.001.



**Fig.6.** Representative sections of TUNEL-positive cells (arrows) from each group at 7, 14 and 28 days post injury in lesion center (1mm)(A), 1mm caudal (0.5-1.5mm) (B) and 2mm caudal (1.5-2.5mm) to lesion center (C). Apoptotic cells were characterized by the compact chromatin and formation of irregular dense mass shape. The data are shown as Mean  $\pm$  SEM (n=5-6). Scale bar=10 $\mu$ m. E2: estradiol valerate 4 mg/kg, i.p., 30 min post-injury.

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Possible explanation for these results is that white matter damage in VLF may lead to changes in locomotor function (Cao et al., 2005). Damage to the descending pathways across the lesion area and disruption of their integrity, are in part responsible for this deficiency. It seemed that existing ventrolateral tracts had compensated functions after injury (Weidner et al., 2001; Webb and Muir, 2004) and integration of intraspinal circuit (Barriere et al., 2008) prevents paralysis after injury. Reduced motor function may be caused by damage to motor tracts due to extensive myelin destruction (Loy et al., 2002; Arvanian et al., 2009).

We quantified the amount of myelin destruction from lesion site to 2mm caudal, to characterize the extent of myelin damage and examine the effect of lesion on intraspinal circuits located caudally to lesion center. According to LFB-staining sections, demyelination was observed at the site of lesion, 1mm and 2mm caudally. The maximum demyelination was observed at day 7 corresponding to the time of maximum reduced motor activity on the rotarod. This is in accordance with the studies that have revealed demyelination after SCI occurring chiefly within 2-7 days, then persisting in a chronic manner (Norenberg et al., 2004; Totoiu and Keirstead, 2005). Toluidine blue-stained sections also further reflected demyelinated axons in ventrolateral white matter, corroborating the idea that demyelination limited recovery of neural function following injury (Hulsebosch, 2002; Totoiu and Keirstead, 2005). Demyelination is a consequence of damage or loss of oligodendrocytes (Emery et al., 1998) and activation of microglia (Krenz and Weaver, 2000; Hendriks et al., 2005) after SCI. In the previous study, we found microglia reactivity at lesion site (Naseri et al., 2012) which may contribute to demyelination.

The decreased level of MBP protein expression in damaged spinal cord can be the result of myelin loss, since MBP is expressed in myelinating cells, including mature and immature oligodendrocytes. Therefore, its expression can represent their presence or activity (Baumann and Pham-Dinh, 2001). Apoptotic cell death has been revealed following SCI in lesion site and in several millimeters away from injury site (Kato et al., 1996; Crowe et al., 1997; Li et al., 1999a; Beattie et al., 2000; Beattie et al., 2002). The high number of TUNEL positive cells in both the lesion center and the caudal at day 7

corresponded to the extent of demyelination and motor changes. Earlier study revealed that by first week, numbers of apoptotic cells increase steadily at the lesion site and the distal areas of white matter (Crowe et al., 1997). It can be assumed that apoptotic cell death may contribute to white matter loss. Myelinating cells in CNS are highly sensitive and are very susceptible to trauma (Ludwin, 1997). They are affected by secondary degenerative processes and commitment to death (Barres et al., 1992). Furthermore, apoptosis in CNS mainly involves oligodendrocytes (Liu et al., 1997; Shuman et al., 1997; Casha et al., 2001). It has been shown that after lesion in cervical corticospinal tracts, apoptosis in white matter penumbra is selective for oligodendrocytes while astrocytes, microglia and endothelial cells are resistant to damage (Li et al., 1999b). Inflammatory responses (Donnelly and Popovich, 2008) such as microglia activation (Beattie, 2004) may initiate death mechanisms as we observed microglia reactivity in the previous study (Naseri et al., 2012).

Demyelination in central nervous system is usually followed by endogenous remyelination due to proliferation, migration and differentiation of oligodendrocyte precursors and neural stem cells. Following the lesion, these endogenous cells within the lesion area are lost or inactivated, therefore the repair is thought to be mediated by cells migrating from the surrounding and it occurs in the rims of demyelinated area. To be able to discriminate between myelin repair in the rims and myelin protection by estradiol, we separately analyzed the extent of demyelination in center of lesion area (1 mm in length and equal to -0.5-0.5 mm from the central part), 1 mm caudal to lesion (0.5-1.5 mm caudal) and 2 mm caudal (1.5-2.5 mm caudal) to the lesion center.

The white matter preservation is an efficient therapeutic target, playing an essential role in functional recovery after SCI either by protection of myelin or by limiting demyelination (Young, 1988). Pharmaceutical treatments prevent and mitigate detrimental secondary injury pathways (Samantaray et al., 2010). The premise that estradiol has protective effect on myelin was mostly based on the in vitro evidence on the cell culture. Limited studies investigated estradiol effect in in vivo animal models. This study was designed to provide insight into

potential treatment of estradiol after electrolytic lesion in rat's spinal cord. Some studies indicated that estradiol has protective effect after SCI, capable of attenuating of secondary damage and locomotor dysfunction (Hauben et al., 2002; Farooque et al., 2006; Webb et al., 2006; Kachadroka et al., 2010). We detected significant effect of estradiol at the lesion center as compared with the rims of demyelinated area. It might be speculated that the effect of estradiol is mediated through protection and sparing the white matter rather than through enhancement of the repairing effect, however, further characterization of glial and neuronal response is worthwhile. Majority of animal models reported a beneficial effect of estradiol on attenuation of inflammation and apoptosis following SCI. We observed estradiol modulated pain sensitivity by inhibition of glia activity in VPL region. In fact, effect of estradiol in our study relied on type of injury and extent of tissue damage. It represented that dose and administration route of estradiol treatment has not had reparative potential. Another possible explanation is that estradiol couldn't induce differentiation and maturation of oligodendrocyte progenitor cells (Suyama et al., 2007), and just prevented pre-existing oligodendrocytes from apoptotic cell death and attenuated secondary damage. Estradiol-treated injured rats displayed significant improvement in motor performance in the first and second week, which may be associated with decrease of demyelination at the acute phase. This observation may support the idea that functional recovery is affected by preserving white matter (Basso, 2000). Although, we did not observe any improvement in functional recovery and maintenance of spared white matter at later phase at day 28, long-term experiments on functional recovery should be designed. It might be inferred therefore that estradiol concentration tends to drop after 28 days or amount of tissue damage might be increased, indicating pharmacological single dose estradiol treatment do not have a prolonged effect. Estradiol affected locomotion in injured animals in different ways. Motor activity in openfield exploratory task increased on 7 and 14 dpi, but motor coordination increased in first 2 weeks and then declined in lesioned group. This effect might be due to pain development after SCI. On the basis of our finding described in Naseri et al, 2013, allodynia appeared on day 14 after SCI and

continued to day 28, with underlying role of astrocytes activation in pain development. To maintain locomotion, some regions are involved including sensorimotor cortex, cerebellum and basal ganglia. Several areas in brainstem have important roles in initiation and sustaining of locomotion. Different supraspinal pathways regulate and control locomotion through effect on muscle activity and posture and support body weight. Cerebellum has an important role in locomotion and interlimb coordination through integration of information coming from peripheral motor apparatus (Majczyński H and Sławińska U, 2007). Cerebellum is a target of estradiol (Hedges et.al, 2012). One interpretation for observed results on rota rod after estradiol treatment might be due to influence of estradiol on cerebellum with this administration methods and single high dose of estradiol. We used high dose of estradiol at early stage, amount of estradiol appeared to drop with time. Therefore, we observed sharp decline during day's 14-28 dpi. It has been demonstrated that estradiol prevented the expanding damaged area during first week and increased locomotor function until 4 weeks post injury (Ritz and Hausmann, 2008). During the acute phase (first week), significant lower myelin loss and higher MBP staining at the lesion center found in estradiol-treated rats, suggested that estradiol potentiates myelin (Sim et al., 2000), and this is in line with several studies showing positive roles of estradiol on myelin and oligodendrocytes (Jung-Testas et al., 1992) and protection of myelin forming cells against cytotoxic insults (Takao et al., 2004). In our study high single dose of estradiol attenuated number of TUNEL positive cells in lesion site and 1mm caudal at day 7, in agreement with studies reported the effect of estradiol on spared white matter and improvement in hind limb locomotion (Kachadroka et al., 2010). Our findings reinforce accumulating evidence that estradiol makes the tissue to be conducive to repair and possibly prevents from spread of secondary injury. It seems that supportive effects of estradiol on activated microglia is responsible for limited demyelination and behavioral improvement, as we observed in earlier studies that estradiol treatment after injury reduced microglia reactivity at day 7 (Saghaei,et.al, 2013), however, it demands further characterization which has not been considered in our study; especially to elucidate the involvement of the subtypes of estrogen

receptors (ERalpha and ERbeta).

## Conclusion

In conclusion, electrolytic lesion in VLF of spinal cord, an area containing tracts with information about locomotion, primarily lead to white matter disruption and locomotor deficit. A single dose of 4mg/kg estradiol administration altered this insulting outcome and significantly prevented secondary damage. Our results can be looked at as conclusive evidence to support pharmacological enhancement of locomotion and white matter protection after SCI through estradiol treatment as a supplementary therapy. However, further research needs to confirm estradiol benefits and the associated molecular mechanism underlying white matter survival.

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## Conflict of interest

The authors declare that there are no competing and conflicts of interest.

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