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Therapeutic Effects of Phosphatidylserine on Functional Recovery After Spinal Cord Injury

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Abstract

Spinal cord injury (SCI) initiates a cascade of secondary injury mechanisms, including inflammation, oxidative stress, and neuronal degeneration, which severely limit functional recovery. Phosphatidylserine (PS), a critical phospholipid in neuronal membranes, has shown potential neuroprotective and anti-inflammatory effects in central nervous system injuries. This study evaluated the therapeutic efficacy and molecular mechanisms of PS at varying doses (10, 20, and 40 mg/kg) in a rat SCI model over 28 days, a model widely accepted as a reliable representation of human SCI, compared with methylprednisolone (MP) and control groups.

Behavioral tests demonstrated dose-dependent improvements in locomotor function and a reduction in neuropathic pain in PS-treated rats, with the highest dose (40 mg/kg) producing effects comparable to those of MP. Histological analysis revealed that PS significantly decreased inflammatory infiltration, axonal degeneration, and cystic cavity formation, correlating with behavioral recovery. Molecular assays revealed that PS treatment significantly suppressed the pro-inflammatory cytokines TNF-α and IFN-γ, while restoring the levels of the anti-inflammatory cytokine IL-10. Furthermore, Western blot analysis showed that PS inhibited the phosphorylation of key signaling proteins in the MAPK (Mitogen-Activated Protein Kinase), ERK1/2 (Extracellular Signal-Regulated Kinase 1/2), and NF-κB (Nuclear Factor Kappa B) pathways, suggesting the attenuation of inflammatory signaling cascades.

These results underscore the potential of PS as a therapeutic adjunct after SCI. By modulating inflammatory responses and intracellular signaling, PS preserves spinal cord tissue integrity and enhances functional recovery. This finding paves the way for the future application of PS as a promising adjunct to current pharmacological treatments for SCI, instilling optimism in the audience.

1. Introduction

Spinal cord injury is a dramatic injury that results in devastating mortality and morbidity. The injury itself can be roughly explained in two phases: the primary phase and the secondary phase. The primary phase is attributed to the traumatic injury that occurs at the scene of the injury. In contrast, the secondary phase is attributed to the injury caused by inflammation, apoptosis, etc [1]. Despite considerable advances in neuroprotective research, spinal cord injury (SCI) remains an incurable condition with devastating consequences. The lack of effective therapeutic strategies is primarily attributed to the complexity of the injury's secondary phase, which triggers a cascade of molecular and cellular events, including oxidative stress, neuroinflammation, glial activation, and apoptotic cell death. These processes contribute to progressive tissue degeneration and long-term functional impairments [2, 3].

Several intracellular signaling pathways are activated following SCI, among which the MAPK/ERK1/2 and NF-κB/IκB-α pathways play critical roles [4]. The MAPK pathway, primarily through ERK1/2, regulates cell survival, differentiation, and inflammation [5]. While transient ERK1/2 activation may support repair processes, prolonged activation has been associated with enhanced inflammation, astrogliosis, and neuronal loss [6].

Concurrently, the NF-κB pathway acts as a master regulator of inflammation in the central nervous system. In its inactive state, NF-κB is retained in the cytoplasm by the IκB-α protein. Upon stimulation, the degradation of IκB-α enables NF-κB to enter the nucleus and initiate the transcription of pro-inflammatory genes. Hence, elevated IκB-α expression can reflect downregulation of NF-κB signaling, suggesting a shift toward an anti-inflammatory state [7, 8].

Phosphatidylserine (PS) is an essential phospholipid found in neuronal membranes, known for its anti-inflammatory, antioxidant, and neuroprotective properties [9, 10]. Prior studies have demonstrated that PS administration can suppress the production of pro-inflammatory cytokines and modulate immune responses in various models of CNS injury [11, 12, 13]. Reports indicate that PS has potential therapeutic effects in neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), major depressive disorder (MDD), and stroke [14].

Our study aims to explore further the potential of PS in SCI treatment, particularly its effects on MAPK/ERK1/2 and NF-κB/IκB-α signaling following spinal cord injury.

In the present study, we conducted a comprehensive investigation into the effects of PS administration at different doses in a rat model of contusive SCI. We assessed behavioral recovery, histological changes, cytokine levels, and the expression of key signaling proteins to provide a thorough understanding of the therapeutic potential and molecular mechanisms of PS in SCI.

Additionally, we compared the effects of STAT injection of phosphatidylserine with the administration of methylprednisolone over a seven-day period, a widely used drug for SCI management. This comparison is crucial for gaining a better understanding of the pharmacological anti-inflammatory and neuroprotective effects of phosphatidylserine, as well as providing a clearer perspective on its potential clinical applications.

2. Materials and Methods

2.1.Animals

This study was conducted on 56 male Sprague–Dawley rats (200–250 g, n = 8 per group) obtained from the Experimental Medicine Research Center at Tehran University of Medical Sciences. The animals were settled under standard laboratory conditions (23 ± 2°C, 50 ± 5% humidity, and with a 12-hour light/dark cycle) and had unlimited access to food and water. Additionally, animals were kept in the lab for about a week before the study began to acclimate them to the environment.

2.2.Drugs

Phosphatidylserine and Buprenorphine were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Ketamine and Xylazine were obtained from Alfasan (Woerden, The Netherlands), and Methylprednisolone (MP) was supplied by Pfizer (New York, NY, USA). Phosphatidylserine was dissolved

in olive oil and administered via intraperitoneal injection immediately after the SCI surgery with a dose of 1 ml/kg as a single dose.

2.3.Induction of Spinal Cord Injury (SCI)

Rats experienced a deep anesthetization with intraperitoneal ketamine (87.7 mg/kg) and xylazine (12.3 mg/kg). A T9 laminectomy was done to expose the spine, followed by a severe-to-moderate contusion injury induced by an aneurysm clip (110 g, 60 sec). This is a widely accepted model that highlights secondary damage caused by post-traumatic inflammation and apoptosis. After injury, the muscles and skin were sutured, and the animals were kept in individual private cages for post-operative monitoring. Manual bladder compression was performed twice daily until spontaneous urination resumed, and by this simple maneuver, urinary retention and its complications were prevented to a reasonable extent [15,16].(as shown in Figure 1)

On day 0, spinal cord injury was induced using a specified method, immediately followed by a single intraperitoneal injection of Phosphatidylserine. Behavioral assessments were conducted on predetermined days to evaluate motor function recovery. Magnetic resonance imaging (MRI) was performed on day 7 to assess structural changes in the spinal cord. Finally, tissue samples were collected on day 28 for histological examination and molecular analyses.

2.4.Post-Surgical Care

To prevent infection and manage post-operative pain, the following treatments were administered:

• Cefazolin (25 mg/kg, IP) – A broad-spectrum antibiotic, administered once, 15 minutes before the surgery, followed by daily injection for 3 days after the operation [17].

• Buprenorphine (0.05 mg/kg, SC) – An analgesic opioid drug, administered twice a day for 3 days [18].

Additionally, the animals were closely monitored throughout the survey to ensure they were generally healthy and not experiencing a water deficit or any other significant health issues.

2.5.Experimental Groups

Rats were randomly assigned to six experimental groups (n = 8 per group):

1. Control (SCI + Saline) – Received normal saline (0.9%) intraperitoneally.

2. Sham (Laminectomy Only) – Underwent laminectomy without spinal cord injury.

3. Olive oil Control (SCI + Vehicle) – Received Olive oil intraperitoneally to monitor the potential vehicle effects.

4. Phosphatidyl-Serine 10 mg/kg – Administered intraperitoneally immediately after the SCI.

5. Phosphatidyl-Serine 20 mg/kg – Administered intraperitoneally immediately after the SCI.

6. Phosphatidyl-Serine 40 mg/kg – Administered intraperitoneally immediately after the SCI.

7. Methylprednisolone (MP) as positive control group – administered 30 mg/kg intraperitoneally (IP) immediately after the SCI and for 7 days [19].

Phosphatidyl-Serine was dissolved in Olive oil and injected immediately after the surgery. Additionally, the Olive oil control group received the same volume of solvents as the other treatment groups to detect the efficacy of Olive oil in SCI rehabilitation precisely.

2.6.MRI Acquisition

Magnetic resonance imaging (MRI) was conducted on day 7 after the operation to evaluate structural changes in the spinal cord. The scan was performed on three rats from three groups: one from an uninjured rat (Sham), one from the SCI control group, and one from those treated with Phosphatidylserine at a dosage of 40 mg/kg.

Animals were deeply anesthetized with the peritoneal administration of ketamine (87.7 mg/kg) and xylazine (12.3 mg/kg) before the scan.

An MRI scan was performed using a clinical human MRI scanner (Siemens Avanto 1.5 Tesla, Siemens Healthcare, Erlangen, Germany) equipped with a knee coil, which enables high-resolution imaging of the spinal cord at the injured site. We used a T2-weighted protocol with a sagittal view to evaluate edema, lesion extent, and tissue integrity. Subspecial imaging parameters, such as repetition time (TR), slice thickness, echo time (TE), and echo train length (ETL), were adjusted to optimize visualization of the rats' spinal cords. Also, all imaging procedures and the results were verified by an expert Radiologist.

These rats used for the MRI scan were not a part of the study at the beginning and were only subjected to the surgery for imaging purposes, and after the scan, the animals were euthanized. Their data have not been used elsewhere in this study to prevent any confounding effects due to anesthesia and the imaging process.

2.7.Behavioral Assessments

2.7.1.BBB Locomotor Scoring

Locomotor recovery was assessed using the Basso, Beattie, and Bresnahan (BBB) locomotor scale, a widely accepted scoring system for measuring functional recovery after spinal cord injury [18]. This scoring system evaluates the animals based on the hind limb movement, weight support, joint coordination, and stepping ability. The scoring process was conducted by two independent, blinded researchers using BBB scores on locomotor performance. The rats were placed in an open-field arena (90 × 90 × 20 cm) for 10 minutes. The scoring system varies from 0 (that means complete paralysis) to 21 (which represents normal locomotion):

• Scores 0–7: interpreted as Minimal movement with slight joint flexion, and no weight support.

• Scores 8–13: Some weight support with uncoordinated stepping but no consistent limb coordination.

• Scores 14–21: Interpreted as Near-normal movement with coordination of limbs and remarkable gait patterns.

BBB scoring was performed on days 0, 3, 5, 7, 14, 21, and 28 post-injury, providing a comprehensive spectrum of locomotor status before and after surgery.

In general, a score below 10 is considered severe functional impairment, while a score above 15 is interpreted as significant recovery [20].

2.7.2.Neuropathic pain assessment

Tail-Flick Test (Thermal Nociception)

The tail-flick test was used to evaluate both thermal pain sensitivity and spinal reflex function. The test was performed on days 0, 7, 14, 21, and 28 after the surgery.

In this test, the rats' tails were exposed to a specific infrared light source. Then, the tail withdrawal time after the exposure was recorded. While a prolonged withdrawal time would be interpreted as reduced pain sensitivity because of the SCI injury, a shorter withdrawal time would be regarded as exaggerated pain sensitivity or thermal hyperalgesia. Each rat was tested three times with a 5-minute interval to minimize variability and obtain more reliable data [21].

2.8. Euthanasia and Tissue Collection

Twenty-eight days after spinal cord injury, and upon completion of all behavioral assessments, animals were deeply anesthetized by intraperitoneal injection of ketamine (87.7 mg/kg) and xylazine (12.3 mg/kg). Euthanasia was subsequently carried out using an overdose of the same anesthetic agents (ketamine 200 mg/kg and xylazine 20 mg/kg, intraperitoneally), in accordance with institutional ethical guidelines. Death was confirmed by the complete cessation of respiration and heartbeat.

Following euthanasia, the spinal cord was carefully exposed and dissected. From each experimental group, four animals were allocated for histopathological analysis, and tissue samples were immediately fixed in 10% neutral-buffered formalin. The remaining samples were rapidly collected, snap-frozen, and stored at −80 °C for subsequent molecular analyses, including ELISA and Western blot.

2.9.Histopathological scoring

Histopathological analysis was conducted on the spinal cord specimen collected from animals at day 28 post-operation. The specimen of each animal was fixed in 4% paraformaldehyde in a special tube for 72 hours before undergoing the process of stabilization and staining, which an expert laboratory technician

conducted. After the paraffin embedding process, the tissues were cut into 5-micrometer-thick vertical sections. The sections were then deparaffinized at 70 °C for approximately 20 minutes. After that, the tissues were floated in Xylene for 3 minutes, just before they were rehydrated using a serial technique with ethanol. Finally, the specimens were stained with Hematoxylin (Sigma-Aldrich, USA) for 15 minutes. After the washing process, eosin staining was performed for 2 minutes. The slides were then placed in 90%, 96%, and 100% ethanol for 2 minutes each in that order. Eventually, they were cleared in Xylen just before being mounted with mounting medium. (Merck, Germany)

The specimens were carefully examined by a blinded professional pathology professor using a professional laboratory microscope (Zeiss, Germany) under great magnification (400) each specimen was evaluated rostrally and caudally and three fields spaced 100 micrometers apart from each other were assessed for each specimen also to have better histological evaluation we used a special semi-quantitative scoring in which three parameters were examined: (1) interstitial hemorrhage (2) inflammatory cells infiltration and (3) neuronal vacuolation each of these three were scored from 0 to 3 while zero was interpreted as none and three was interpreted as severe so on and so, the maximum score for each specimen would be 9 [22].

2.10.Molecular Analysis

2.10.1.ELISA (Enzyme-Linked Immunosorbent Assay)

In this study, the key inflammatory cytokines were evaluated. These cytokines include IL-10, INF-γ, and TNF-α. Initially, frozen tissue specimens from the spinal cord injury site of each rat were homogenized thoroughly using a mechanical homogenizer. The mixture was then centrifuged at 4000 rpm for approximately 15 minutes at 4 °C, and the supernatant was used for analysis.

Cytokine levels were quantified using commercially available ELISA kits, following the manufacturers' protocols:

• IL-10 Cat No: R1000, R&D, USA)

• INF-γ (Cat No:DIF50C,R&D,USA)

• TNF-α (Cat No: RTA00, R&D,USA)

Absorbance was measured at 450 nm using a microplate reader. For each cytokine, a standard curve was plotted from known concentrations, and sample concentrations were interpolated using the curve's linear regression equation. The measured cytokine concentrations were statistically analyzed to compare inflammatory responses among different experimental groups [23].

2.10.2.Western blot analysis

Western blot analysis was performed by another expert laboratory technician, following standardized protocols with a few modifications tailored to this method. Frozen spinal cord tissue samples were

homogenized in RIPA lysis buffer supplemented with protease and phosphatase inhibitor cocktails. The mixtures were then centrifuged at 14,000 rpm for 20 minutes at 4°C to collect the clear supernatant. Total protein concentration was measured using the Bradford Protein Assay Kit (DB0017, DNAbioTech, Iran) following the manufacturer's instructions.

Equal amounts of protein (20 µg per sample) were mixed with an equal volume of 2X Laemmli sample buffer, boiled at 95°C for 5 minutes, and separated on 10% SDS-PAGE gels. The proteins were transferred onto 0.2 µm PVDF membranes (Cat No: 162-0177, Bio-Rad Laboratories, CA, USA) using a semi-dry transfer system.

Membranes were blocked with 5% BSA (Cat No: A-7888, Sigma Aldrich, MO, USA) in TBST (Tris-buffered saline with 0.1% Tween-20) for 1 hour at room temperature and then incubated overnight at 4°C with the following primary antibodies:

• p38 MAPK (1:1000, Cat No: ab308333, Abcam)

• Phospho-p38 MAPK (p-p38)(Phospho-Tyr182) (1:1000, Cat No: a50384, antibodies)

• pNF-kB (p65 phospho S536)(1:1000, Cat No: ab76302, Abcam)

• IKB alpha (phospho S36)(1:1000, Cat No: ab133462, Abcam)

• ERK1/2 (1:5000, Cat No: ab76299, Abcam)

• β-actin (1:2500, Cat No: ab8227, Abcam) as an internal loading control

After three washes with TBST, membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (1:10,000, Cat No: ab6721, Abcam) for 1 hour at room temperature.

Protein bands were visualized using an Enhanced Chemiluminescence (ECL) detection system and imaged using a chemiluminescent imaging system. Densitometric analysis was performed using ImageJ software (NIH, USA). Protein expression levels were normalized to β-actin, and relative expression levels were calculated by dividing the area under the curve (AUC) of each target protein by that of the corresponding β-actin band. The results were then statistically compared among experimental groups [24].

2.11.Statistical Analysis

All data were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using Stata software version 17 (StataCorp LLC, College Station, TX, USA). The normality of data distribution was assessed using the Shapiro–Wilk test. Depending on the study design, one-way or two-way ANOVA was applied, followed by Tukey's post hoc test for multiple group comparisons. For behavioral assessments conducted over time (e.g., BBB and tail-flick tests), repeated-measures ANOVA was used. A p-value less than 0.05 was considered statistically significant.

Graphs were generated using GraphPad Prism version 10 (GraphPad Software, San Diego, CA, USA).

2.12.Statement of ethics

All experimental procedures were formally reviewed and approved by the Ethics Committee of Tehran University of Medical Sciences (Approval Code: IR.TUMS.MEDICINE.REC.1404.351). All animal experiments were conducted in accordance with the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals and relevant institutional regulations.

All efforts were made to minimize animal suffering and to reduce the number of animals used, while maintaining the scientific integrity of the study. The study was also conducted in accordance with the guidelines of the International Association for the Study of Pain [25].

3. Results

3.1.MRI Findings

MRI analysis on day 7 post-injury demonstrated distinct pathological features in each group. In the SCI control group (Figure2-A), T2-weighted images showed a pronounced hyperintense signal at the lesion epicenter, extending longitudinally along the spinal cord, consistent with severe edema and parenchymal disruption. The margins of the lesion were poorly defined, indicating diffuse tissue damage and secondary degeneration. In the phosphatidylserine-treated group (Figure 2-B), the hyperintense area was markedly smaller, with more precise lesion boundaries and partial preservation of the surrounding white and gray matter. These changes suggest that phosphatidylserine reduced vasogenic edema, limited secondary injury progression, and preserved spinal cord microarchitecture. The sham-operated group (Figure 2-C), which underwent laminectomy without SCI induction, exhibited homogeneous signal intensity and intact cord morphology, with no evidence of edema or structural abnormalities. Collectively, these MRI findings indicate that phosphatidylserine exerts a protective effect on spinal cord integrity following traumatic injury.

3.2. Behavioral Outcomes Following SCI and Phosphatidylserine Treatment

3.2.1. Phosphatidylserine-Mediated Modulation of Body Weight Following SCI

Monitoring of body weight over 28 days after spinal cord injury (SCI) revealed significant differences among the groups. In the sham-operated group, body weight increased steadily, maintaining the normal growth trend. In contrast, the SCI control group and the olive oil group showed a gradual and significant decrease in body weight, which persisted until day 28 (****$p < 0.0001$). Treatment with phosphatidylserine (PS) significantly improved weight maintenance in a dose-dependent manner. The 40 mg/kg PS group exhibited the most significant weight preservation, with values significantly higher than those of the SCI control group from day 14 onward (****$p < 0.0001$). The 20 mg/kg PS group also demonstrated a gradual and significant weight gain compared to the SCI control group (***$p < 0.001$). In contrast, the 10 mg/kg PS group showed a milder improvement, although it still exhibited a slower rate

of weight loss than the SCI control (**p < 0.01). Moreover, the methylprednisolone (+Control) group displayed a significant weight gain comparable to that of the high-dose PS group (****p < 0.0001). These findings suggest that PS, particularly at higher doses, can effectively help maintain general health status and prevent weight loss following SCI. (as shown in Figure 3)

3.2.2. Locomotor Recovery Following Phosphatidylserine Treatment

Locomotor function assessment using the BBB scoring method showed that on day 1 after injury, all SCI groups exhibited very low scores, indicating complete hindlimb paralysis. In the sham group, scores remained at the maximum value of 21 throughout the study period.

Over the 28-day follow-up, all treatment groups demonstrated gradual improvement, the extent of which depended on the type and dose of treatment. In the SCI control and olive oil groups, locomotor recovery was minimal, and BBB scores did not exceed approximately five by day 28.

In contrast, phosphatidylserine treatment markedly improved locomotor function, with the 40 mg/kg group achieving a score above 15 on day 28, showing a statistically significant difference compared to the SCI group (***p < 0.001). The 10 mg/kg and 20 mg/kg doses also produced moderate recovery that was significantly better than that of the SCI group.

The methylprednisolone (+ control) group showed rapid and pronounced functional improvement, maintaining higher scores than the SCI and olive oil groups at all time points. These findings indicate that phosphatidylserine, particularly at higher doses, significantly enhances locomotor recovery after spinal cord injury. (as shown in Figure 4)

3.2.3. Phosphatidyl serine decreases the hyperalgesia induced by the SCI

(Improved sensory function assessed by Tail Flick Test)

The tail flick test was conducted on days 0, 7, 14, 21, and 28 to assess the latency after thermal stimulation, which can be interpreted as thermal nociceptive thresholds. In this method, the animals experience two phases of thermal nociception, as shown in Figure 6. In the first phase, the latency of the tail flick increases significantly in the SCI control group compared to the sham group, indicating that the animals are experiencing a slowed-down signaling process in the spinal cord. In contrast, during the second phase, the animals experience a shorter latency time, indicating that hyperalgesia begins mildly in the second week and continues to increase throughout the remainder of the study. Comparing the SCI control group with the treatment groups suggests a smaller range of thermal threshold in the treatment groups. The threshold range becomes narrower as the dosage increases, indicating a dose-dependent effect. Consequently, in the group treated with Phosphatidylserine at 40 mg/kg, the animals exhibited a near-normal threshold of thermal nociception.(as shown in Figure 5)

3.3. Histopathological Assessment

Histopathological assessment of spinal cord sections at day 28 post-injury revealed that the SCI group had the highest pathology score (mean ≈ 3.0), indicating severe structural disruption characterized by extensive inflammatory cell infiltration, neuronal degeneration, axonal vacuolation, and the formation of cystic cavities. The olive oil–treated group also exhibited high scores (mean ≈ 2.8) with a similar pattern of damage. Notably, the hemorrhage score was zero across all experimental groups.

Phosphatidylserine (PS) treatment produced a dose-dependent reduction in pathological damage. Rats receiving PS at 10 mg/kg showed a moderate but significant improvement compared to the SCI group ($p < 0.01$). In contrast, PS at 20 mg/kg produced a more pronounced reduction in pathology scores. Remarkably, PS at 40 mg/kg resulted in a marked decrease in pathological scores (mean ≈ 1.5), reflecting better preservation of tissue structure and reduced inflammatory cell infiltration ($p < 0.01$ vs. SCI). The methylprednisolone-treated group exhibited the lowest pathology scores (mean ≈ 1.0), indicating near-complete preservation of spinal cord morphology ($p < 0.0001$ vs. SCI).(as shown in Figure 6)

Overall, these findings demonstrate that phosphatidylserine exerts a dose-dependent neuroprotective effect, significantly mitigating secondary injury following spinal cord trauma, with the highest dose (40 mg/kg) approaching the protective efficacy of methylprednisolone.(as shown in Figure 7)

Representative H&E-stained sections from different experimental groups are shown, illustrating the degree of tissue preservation or damage. The SCI group exhibits marked structural disruption, including axonal vacuolation, neuronal degeneration, cystic cavity formation, and dense infiltration of inflammatory cells. Olive oil–treated animals show a similar pattern of injury. Phosphatidylserine (PS) treatment yields a dose-dependent improvement in tissue morphology, with a dose of 40 mg/kg demonstrating substantial preservation of typical architecture. Methylprednisolone-treated rats display near-normal histological features. The hemorrhage score was zero in all groups.

Quantitative histopathological scores are presented as mean ± SD. Statistical significance: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

3.4. Molecular Analysis: Impact of Phosphatidylserine on Post-Injury Inflammation and Signaling Pathways

To elucidate the molecular mechanisms underlying the neuroprotective effects of phosphatidylserine (PS), we examined the expression of key pro-inflammatory cytokines alongside the activation status of major intracellular signaling pathways in spinal cord tissues on day 28 post-injury. The analysis encompassed all PS treatment doses (10, 20, and 40 mg/kg) and was compared with the SCI, sham, and methylprednisolone (MP) groups. This approach enabled a detailed evaluation of how PS modulates inflammatory responses and intracellular signaling events following spinal cord injury.

3.4.1. ELISA Analysis of Pro- and Anti-inflammatory Cytokines

The levels of TNF-α, IFN-γ, and IL-10 in spinal cord tissue homogenates were quantified using ELISA to assess the inflammatory status following SCI and subsequent treatments.

As shown in Figure (8-A), the SCI group exhibited a marked and significant elevation in TNF-α levels (52.13 ± 3.12 pg/mg protein) compared to the sham group (22.47 ± 2.68 pg/mg protein, *p < 0.0001). Administration of phosphatidylserine (PS, 40 mg/kg) significantly reduced TNF-α concentrations to 34.86 ± 2.91 pg/mg protein compared to the SCI group (*p < 0.001). Similarly, methylprednisolone (MP) treatment reduced TNF-α levels to 32.95 ± 3.07 pg/mg protein (**p < 0.001 vs. SCI). There were no statistically significant differences between the PS and MP groups in TNF-α reduction (p > 0.05).

For IFN-γ (Figure 8-B), SCI induction resulted in a significant increase (95.74 ± 6.38 pg/mg protein) compared to sham-operated rats (41.35 ± 4.87 pg/mg protein, **p < 0.001). PS treatment markedly reduced IFN-γ levels to 54.27 ± 4.15 pg/mg protein (p < 0.01 vs. SCI), and MP treatment similarly decreased IFN-γ concentrations to 57.21 ± 4.42 pg/mg protein (*p < 0.01 vs. SCI). Again, no significant differences were detected between PS and MP groups (p > 0.05).

In contrast, IL-10 levels (Figure 8-C) were profoundly reduced in the SCI group (72.15 ± 5.18 pg/mg protein) compared to the sham group (305.74 ± 10.29 pg/mg protein, **p < 0.001). Treatment with PS significantly increased IL-10 levels to 231.84 ± 9.65 pg/mg protein (*p < 0.01 vs. SCI), and MP administration elevated IL-10 concentrations to 198.63 ± 8.74 pg/mg protein (*p < 0.05 vs. SCI). The increase in IL-10 was more pronounced in the PS group compared to the MP group, although the difference was not statistically significant (p > 0.05).

These findings suggest that phosphatidylserine, like methylprednisolone, exhibits potent anti-inflammatory effects in SCI by suppressing pro-inflammatory cytokines (TNF-α and IFN-γ) and restoring the anti-inflammatory cytokine IL-10 to normal levels.

3.4.2. Western Blot Analysis of Signaling Pathways

To evaluate the effects of phosphatidylserine (PS) on inflammation-related signaling pathways after spinal cord injury (SCI), the expression and phosphorylation levels of key proteins, including p38 MAPK, ERK1/2, NF-κB, and IκB, were assessed in spinal cord tissue samples at day 28 post-injury using Western blot analysis. Samples were obtained from four experimental groups: Sham, SCI, PS (40 mg/kg), and methylprednisolone (MP, positive control).

Phosphorylation of MAPK (P-MAPK/MAPK)

In the SCI group, the ratio of P-MAPK to MAPK was significantly elevated compared to the Sham group (p < 0.001), indicating activation of the MAPK pathway in response to SCI (Figure 9-A).

Treatment with PS markedly reduced this increase (p < 0.01), with levels comparable to those in the MP group.

Phosphorylation of ERK1/2 (P-ERK1/2/β-actin)

Levels of phosphorylated ERK1/2 were significantly higher in the SCI group than in the Sham group ($p < 0.0001$) (Figure 9-B). Administration of PS significantly attenuated ERK1/2 phosphorylation ($p < 0.01$), with no statistical difference from the MP group, suggesting a modulatory effect of PS on the ERK1/2 pathway.

IκBα (IκBα/β-actin)

SCI led to a significant decrease in IκBα protein levels ($p < 0.01$) (Figure 9C), indicating the degradation of IκBα and subsequent activation of the NF-κB pathway. PS treatment effectively prevented this decrease ($p < 0.05$), restoring IκBα levels close to those observed in the MP group.

Phosphorylation of NF-κB (P-NF-κB/β-actin)

The SCI group exhibited markedly elevated NF-κB phosphorylation compared to the Sham group ($p < 0.001$) (Figure 9-D). PS administration significantly reduced this phosphorylation ($p < 0.01$), reaching levels similar to those in the MP-treated animals.

Representative Western blot images (Figure 9-E) clearly illustrate the differences in phosphorylated protein expression across groups. The SCI group displayed intense bands for P-MAPK, P-ERK1/2, P-IκB, and P-NF-κB, while treatment with PS or MP markedly reduced band intensities.

These findings suggest that PS may exert its neuroprotective effects, at least in part, by inhibiting the activation of MAPK, ERK1/2, and NF-κB signaling pathways following SCI. Such inhibition could contribute to the attenuation of post-injury inflammation and improvement of neurological outcomes. Offering potential new avenues for treating SCI.

4. Discussion

The present study provides novel evidence that phosphatidylserine (PS) exerts potent neuroprotective effects in a rat model of spinal cord injury (SCI), promoting functional recovery, attenuating neuroinflammation, and modulating key intracellular signaling pathways. These findings expand upon previous reports of PS's anti-inflammatory and antioxidant activities in the central nervous system (CNS) and highlight its therapeutic potential for traumatic SCI [26, 27].

The behavioral improvements observed in the present study, particularly the dose-dependent enhancement of locomotor recovery and reduction of hyperalgesia, suggest that PS confers benefits beyond mere structural preservation. This aligns with earlier work showing that PS supplementation improves synaptic function and neuronal survival in various models of CNS injury and neurodegeneration [28]. In SCI, secondary injury processes, including inflammation, oxidative stress, and apoptosis, play a decisive role in determining long-term outcomes [29, 30]. By targeting these mechanisms, PS appears to not only limit lesion expansion but also promote an environment conducive to neural repair.

Our histopathological findings further corroborate this neuroprotective profile, with PS treatment—especially at 40 mg/kg—reducing inflammatory infiltration, axonal degeneration, and cystic cavity formation. These structural improvements are consistent with the observed behavioral recovery and indicate that PS mitigates the destructive cascade of secondary injury. The histological benefits were comparable, and in some parameters nearly equivalent, to those achieved with methylprednisolone (MP), the current pharmacological standard for SCI management. Given the limitations and side effects of MP, the potential of PS as a safer alternative or adjunct therapy is a beacon of hope in the field of SCI management.

At the molecular level, phosphatidylserine (PS) significantly reduced the expression of pro-inflammatory cytokines (TNF-α, IFN-γ) and restored the anti-inflammatory cytokine IL-10 to physiological levels. Such modulation of the inflammatory milieu likely plays a crucial role in preserving neural tissue. Significantly, PS inhibited the activation of MAPK and ERK1/2 pathways, which are known to be persistently upregulated after SCI and contribute to astrogliosis, inflammation, and neuronal loss. Concurrently, PS increased the level of IκBα and decreased the phosphorylation of NF-κB p65, indicating suppression of the NF-κB signaling pathway and inhibition of the transcriptional program driving inflammatory gene expression. This dual regulation of the MAPK/ERK and NF-κB pathways has been implicated in neuroprotection in other CNS injury models; however, the present study is among the first to demonstrate such effects for PS in SCI [31, 32, 33, 34, 35].

The observed dose-dependent effects further support the pharmacological relevance of PS. The 40 mg/kg dose consistently outperformed lower doses in behavioral, histological, and molecular assays, highlighting the importance of dose optimization in translating these findings to clinical practice.

One of the strengths of this study lies in its comprehensive approach, integrating behavioral, imaging, histological, and molecular assessments. This multidimensional analysis strengthens the conclusion that PS confers genuine neuroprotection rather than nonspecific systemic benefits. The thoroughness of our approach should instill confidence in the robustness of our findings. Nevertheless, several limitations should be acknowledged. First, the study focused on acute and subacute outcomes (up to 28 days post-injury), and long-term effects on chronic SCI remain to be determined. Second, while the modulation of MAPK/ERK and NF-κB pathways suggests a mechanistic basis for PS's effects, causality was not directly confirmed through pathway-specific inhibitors or genetic approaches. Finally, pharmacokinetic and safety profiling in SCI-specific contexts will be necessary before advancing to human trials.

5. Conclusion

This study demonstrates that phosphatidylserine (PS) confers significant neuroprotective effects in a rat model of spinal cord injury, enhancing locomotor recovery, reducing neuropathic pain, and preserving spinal cord tissue integrity. The attenuation of pro-inflammatory cytokines accompanied the therapeutic benefits of PS, the restoration of anti-inflammatory IL-10, and the inhibition of MAPK/ERK1/2 and NF-κB signaling pathways,

suggesting a multi-targeted mechanism of action. Notably, high-dose PS (40 mg/kg) achieved Outcomes comparable to methylprednisolone, the current pharmacological standard, but potentially with a more favorable safety profile. These findings position PS as a promising candidate for the treatment of SCI. However, the need for further studies to focus on long-term safety evaluation, optimization of dosing regimens, and well-designed human clinical trials is urgent and cannot be overstated. This research is crucial for confirming the therapeutic potential of PS and facilitating its translation into clinical practice.

Declarations

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Author Contributions: Ali Darabniya spearheaded the study, performed the spinal cord injury surgeries, designed the scientific framework, carried out all experiments, analyzed the data, conducted the literature review, and prepared the initial draft of the manuscript.

Ahmad Reza Dehpour supervised the overall study design and conceptual development, oversaw the research process, and contributed to the critical revision of the manuscript.

Reza Fazeli and Sara Shahriari played key roles in conducting experiments and collecting data. Both also contributed to writing sections of the manuscript and provided valuable feedback on the draft.

Seyed Mohammad Tavangar conducted histopathological evaluations, contributed to the pathology section, and assisted in reviewing and refining the manuscript with constructive suggestions.

Alireza Parto Azar originated the study concept, conducted the literature review, thoroughly reviewed the manuscript, and provided insightful comments to enhance its quality.

All authors read and approved the final version of the manuscript.

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Consent to Participate: Not applicable. No human participants were involved in this study.

Consent for Publication: Not applicable.

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Figures

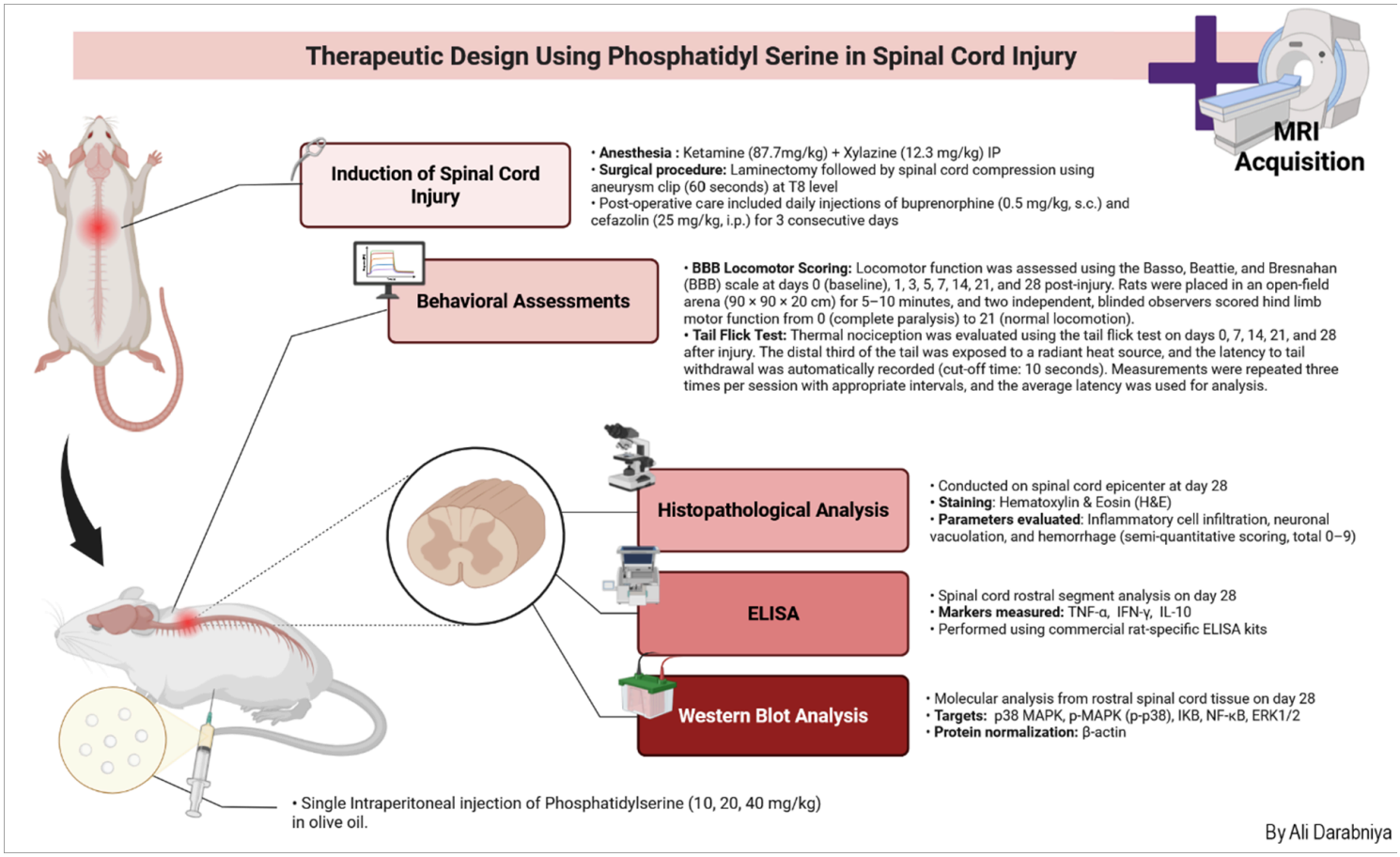


Figure 1

Schematic representation of the experimental design.

On day 0, spinal cord injury was induced using a specified method, immediately followed by a single intraperitoneal injection of Phosphatidylserine. Behavioral assessments were conducted on predetermined days to evaluate motor function recovery. Magnetic resonance imaging (MRI) was performed on day 7 to assess structural changes in the spinal cord. Finally, tissue samples were collected on day 28 for histological examination and molecular analyses.

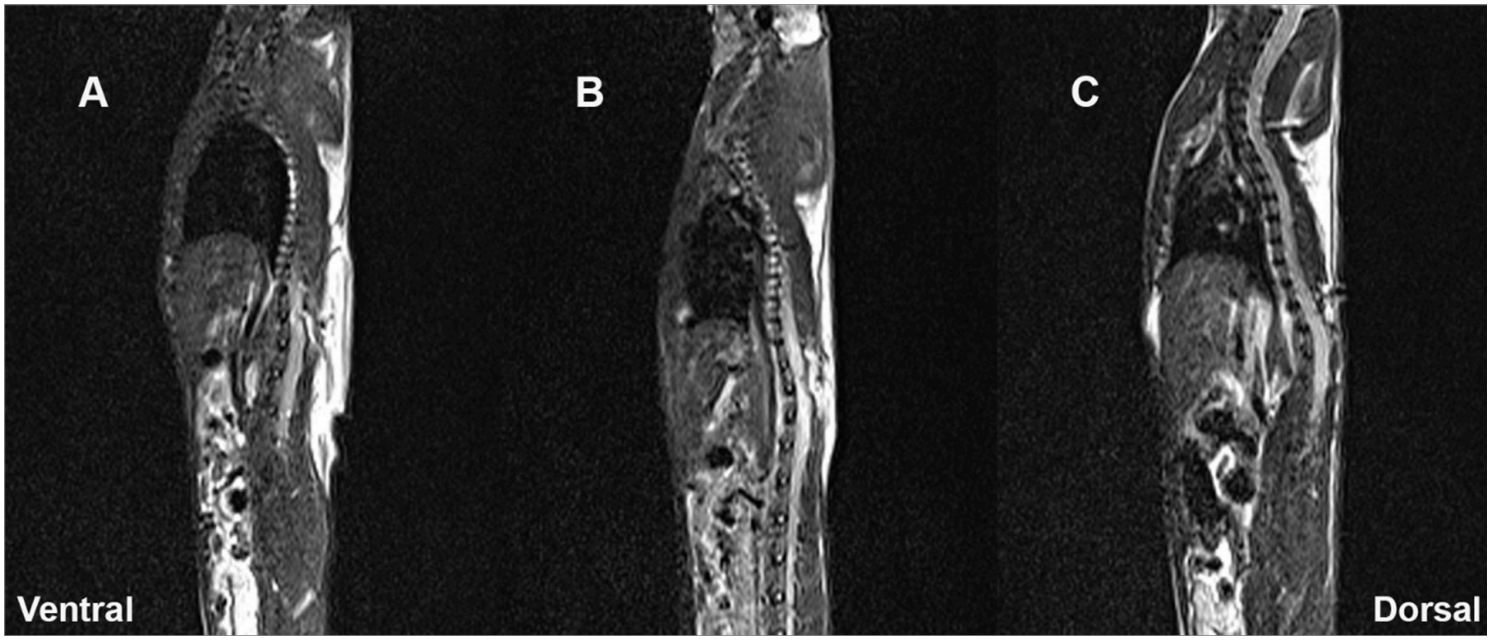


Figure 2

Sagittal T2-weighted MRI images of the spinal cord in different groups.

(A) Spinal cord injury (SCI) without treatment: a large hyperintense area and marked disruption of spinal cord architecture, consistent with edema and tissue damage.

(B) Phosphatidylserine-treated group: reduced size and intensity of the hyperintense area with better preservation of spinal cord continuity at the injury site.

(C) Sham surgery group (laminectomy without SCI): intact spinal cord with normal morphology and uniform signal intensity.

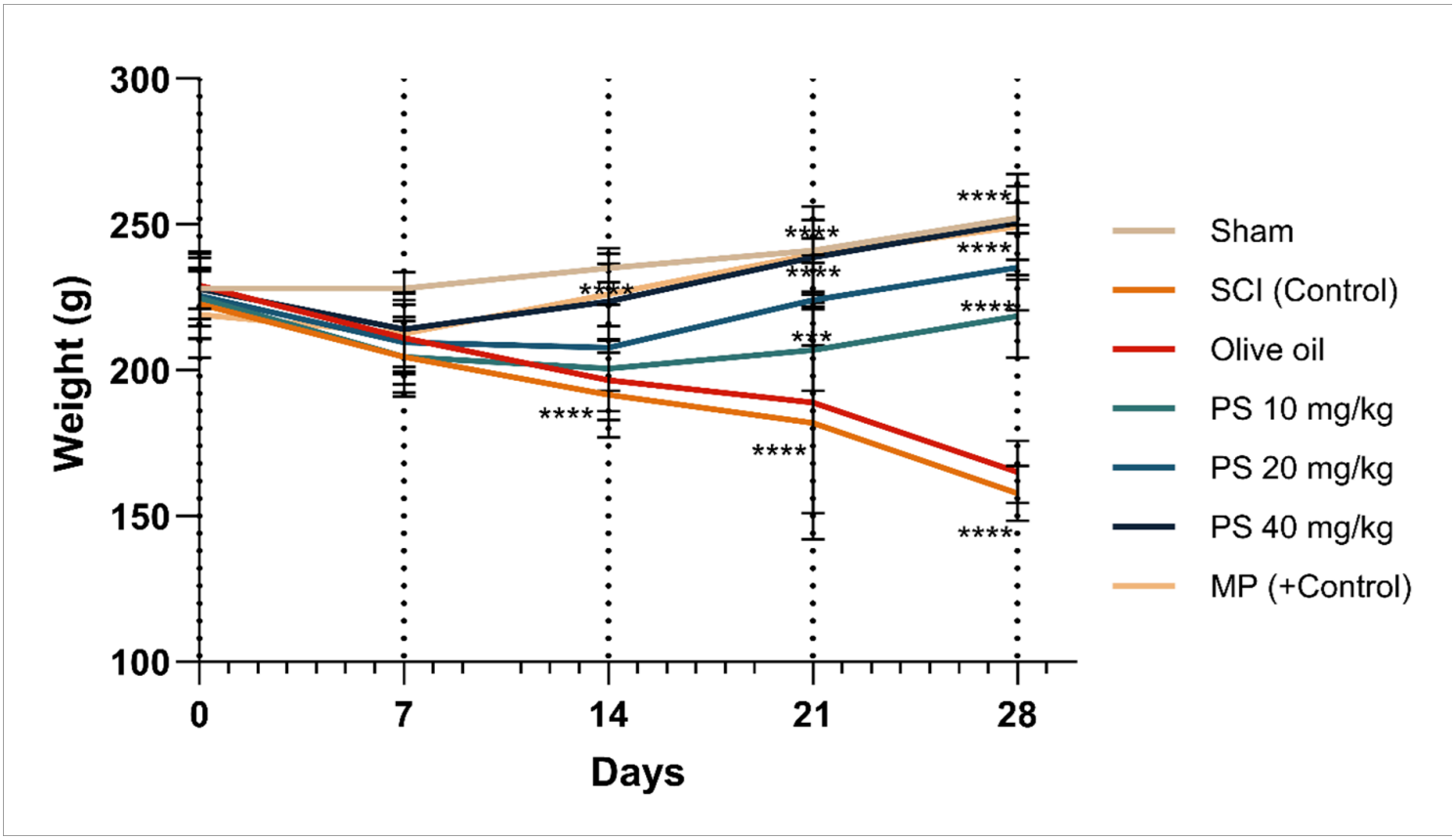


Figure 3

Changes in body weight over 28 days following spinal cord injury (SCI) in different experimental groups. The SCI control and olive oil groups showed a progressive decline in body weight, whereas treatment with phosphatidylserine (PS) at doses of 10, 20, and 40 mg/kg attenuated weight loss in a dose-dependent manner. The highest preservation of body weight was observed in the 40 mg/kg PS group, comparable to the methylprednisolone-treated group. The sham-operated group (laminectomy only) exhibited a continuous increase in body weight throughout the study period. Data are presented as mean ± SEM. $p < 0.05$ compared with SCI control.

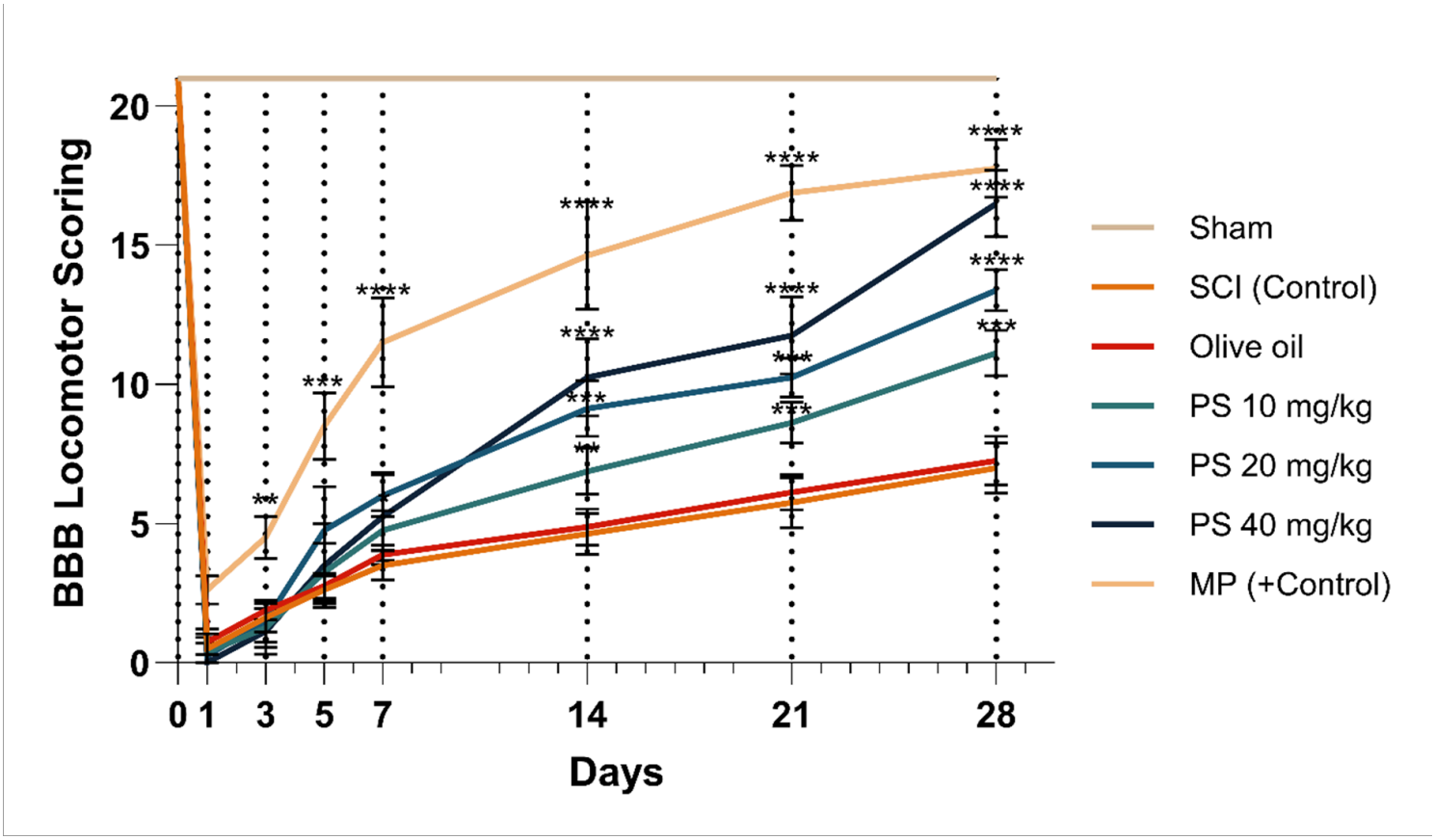


Figure 4

Effect of phosphatidylserine on locomotor function recovery after spinal cord injury in rats. Locomotor performance was evaluated using the Basso, Beattie, and Bresnahan (BBB) scoring system over 28 days. The sham group maintained the maximum BBB score of 21 throughout the study. Rats treated with phosphatidylserine at 40 mg/kg achieved scores above 15 by day 28, indicating substantial recovery compared to the untreated SCI control group. Data are presented as mean ± SD. Data are mean ± SEM. Significance vs. SCI group: **p < 0.01, ***p < 0.001, ****p < 0.0001.

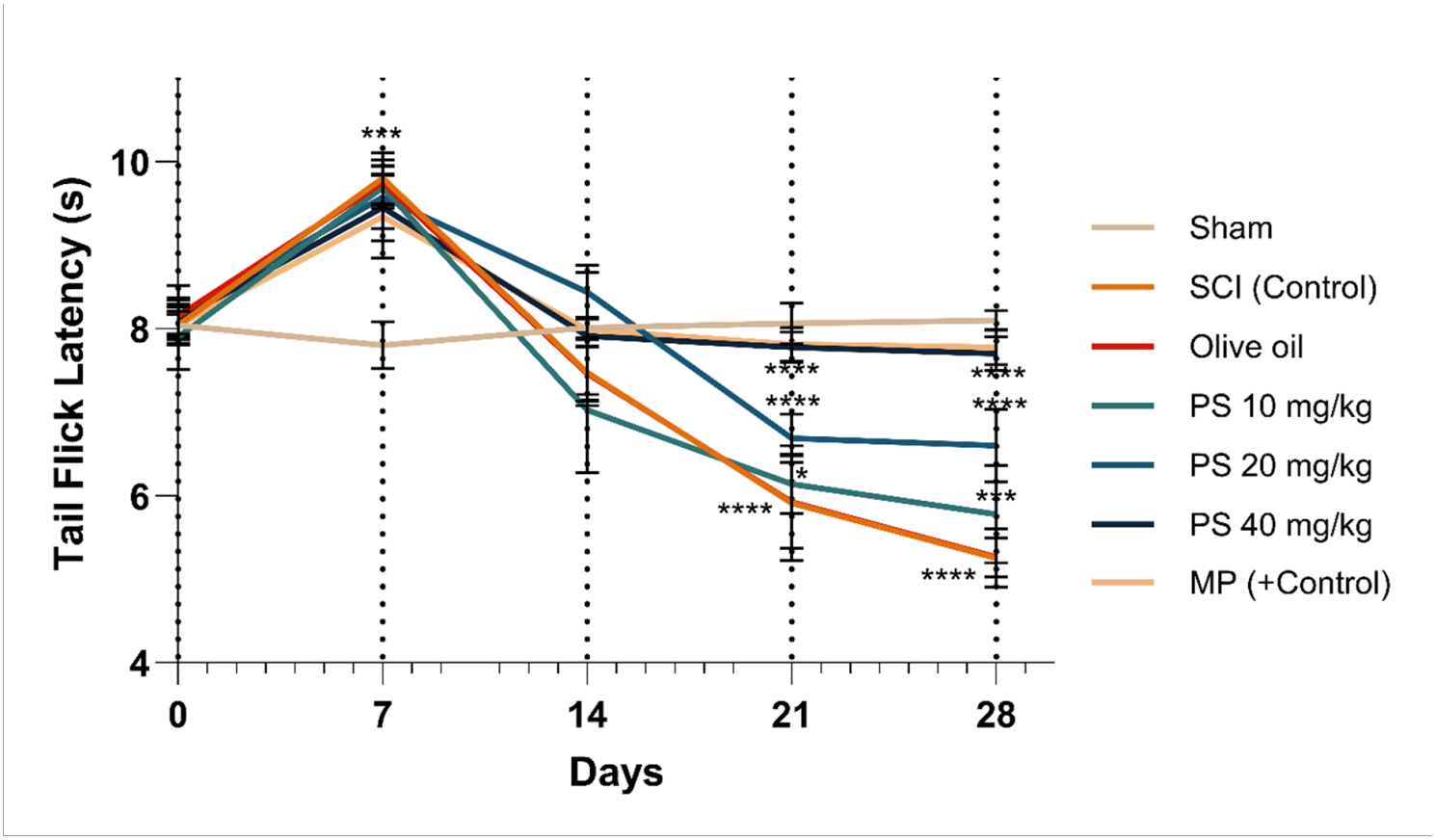


Figure 5

Tail flick latency, measured in seconds, shows that the SCI control group has developed greater hyperalgesia compared to phospatidyl-serine-treated animals and the sham group. Data are mean ± SEM. ***p < 0.001, ****p < 0.0001 vs. SCI.

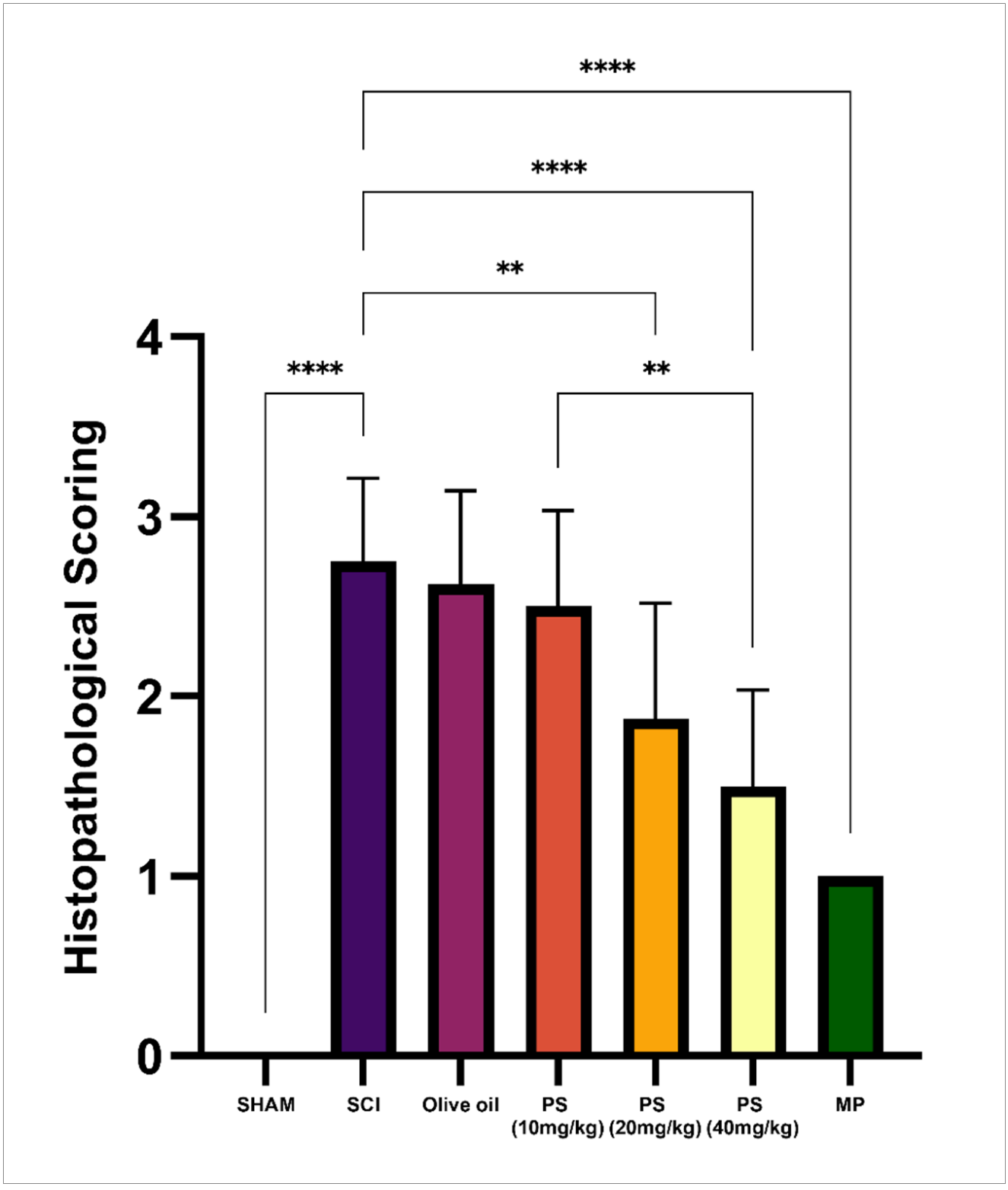


Figure 6

Histopathological assessment of spinal cord tissue at day 28 post-injury.

Representative H&E-stained sections from different experimental groups are shown, illustrating the degree of tissue preservation or damage. The SCI group exhibits marked structural disruption, including axonal vacuolation, neuronal degeneration, cystic cavity formation, and dense infiltration of inflammatory

cells. Olive oil–treated animals show a similar pattern of injury. Phosphatidylserine (PS) treatment yields a dose-dependent improvement in tissue morphology, with a dose of 40 mg/kg demonstrating substantial preservation of typical architecture. Methylprednisolone-treated rats display near-normal histological features. The hemorrhage score was zero in all groups.

Quantitative histopathological scores are presented as mean ± SD. Statistical significance: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001

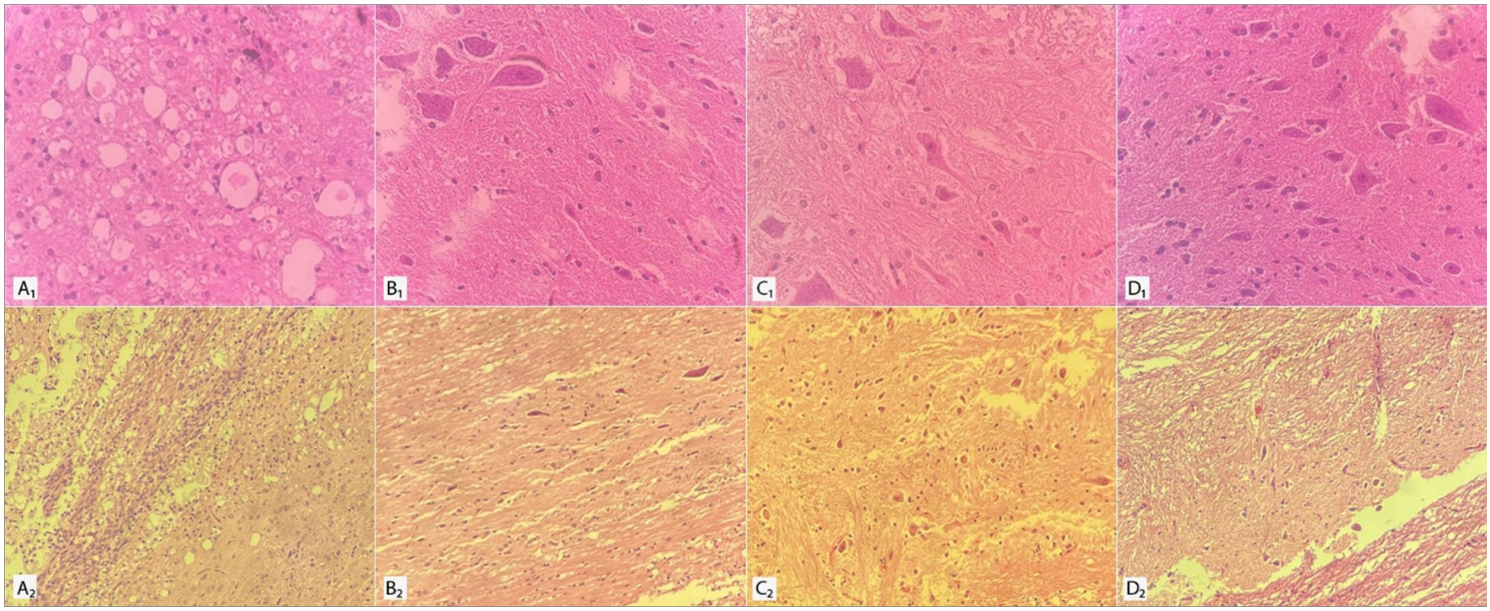


Figure 7

Histopathological evaluation of spinal cord tissue following SCI and different treatments (H&E staining).

(A_1, A_2) Untreated SCI control group showing severe tissue disruption, extensive neuronal vacuolation, inflammatory cell infiltration, and loss of normal architecture (A_1: ×400, A_2: ×100).

(B_1, B_2) Sham group demonstrating preserved spinal cord structure with intact neuronal morphology and absence of significant inflammation (B_1: ×400, B_2: ×100).

(C_1, C_2) The phosphatidylserine-treated group (40 mg/kg) exhibited improved histological preservation, reduced vacuolation, and decreased inflammatory infiltration, indicating neuroprotective effects (C_1: ×400, C_2: ×100).

(D_1, D_2) The methylprednisolone-treated group showed near-normal tissue morphology with minimal neuronal damage and inflammation, reflecting strong protective efficacy comparable to high-dose phosphatidylserine (D_1: ×400, D_2: ×100).

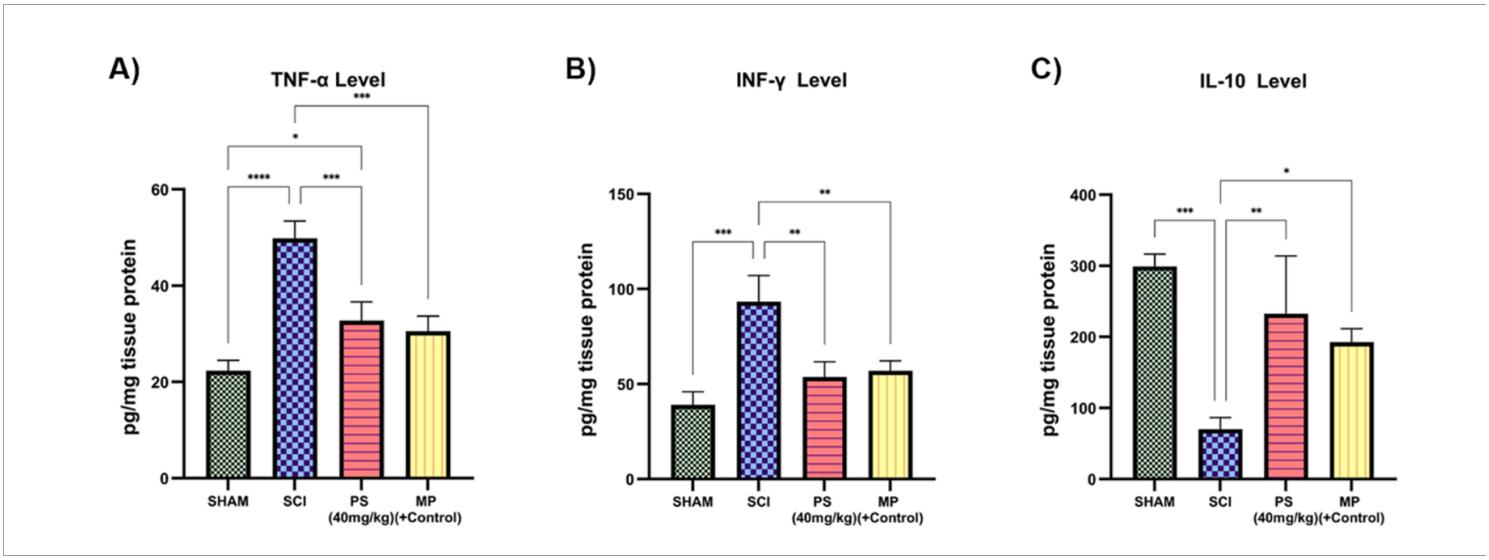


Figure 8

Effect of phosphatidylserine (PS) and methylprednisolone (MP) on TNF-α (A), IFN-γ (B), and IL-10 (C) levels in spinal cord tissue homogenates following spinal cord injury (SCI), as determined by ELISA. Data are presented as the mean ± SD. ****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05. Phosphatidylserine markedly reduced pro-inflammatory cytokines (TNF-α and IFN-γ) and restored the anti-inflammatory cytokine IL-10 to normal levels, with effects comparable to those of methylprednisolone.

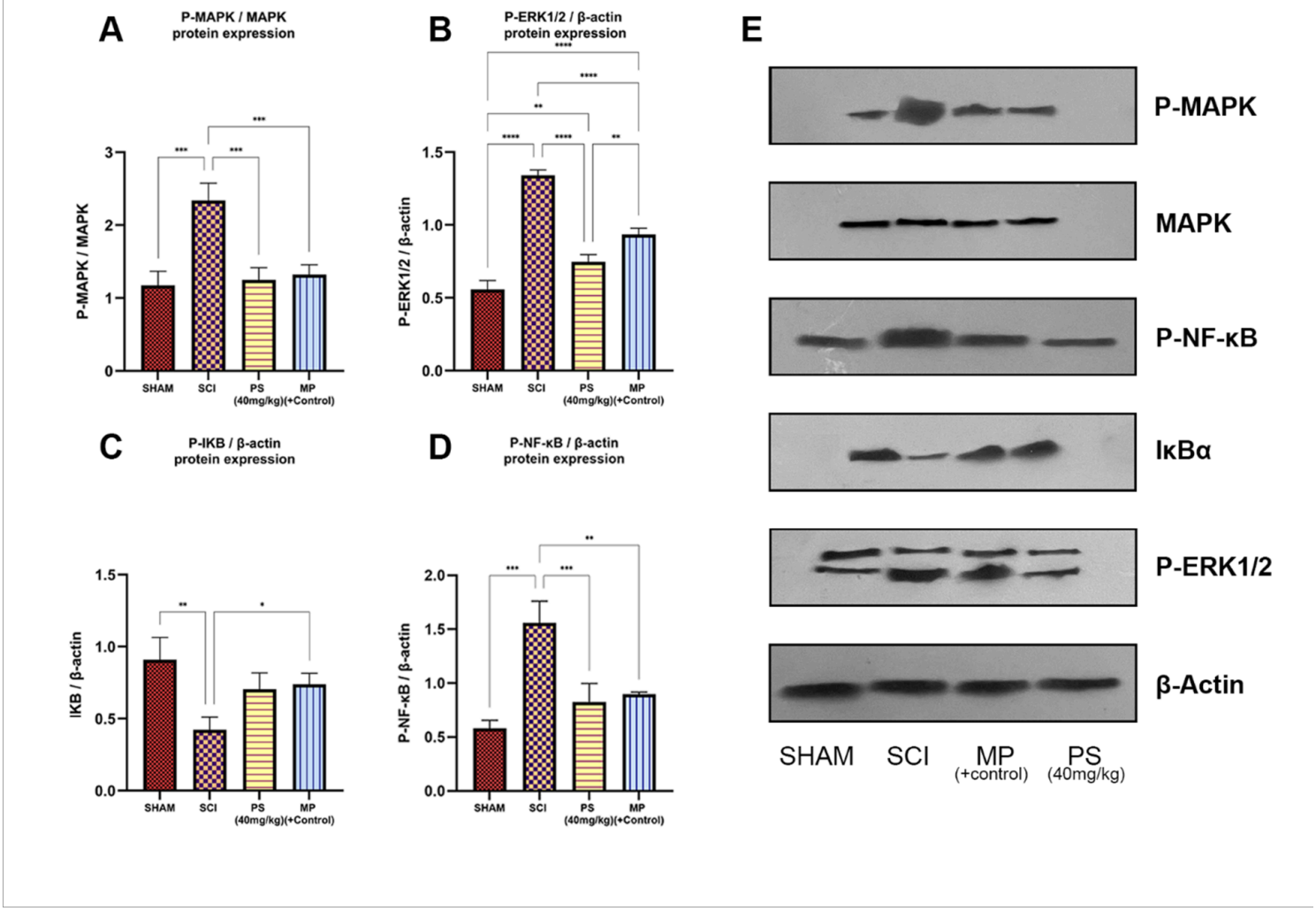


Figure 9

Effects of phosphatidylserine (PS) treatment on MAPK, ERK1/2, and NF-κB signaling pathways in spinal cord tissue at 28 days post-injury.

(A) Ratio of phosphorylated MAPK (P-MAPK) to total MAPK.

(B) Levels of phosphorylated ERK1/2 normalized to β-actin.

(C) IκBα levels normalized to β-actin.

(D) Phosphorylated NF-κB levels normalized to β-actin.

(E) Representative Western blot bands for P-MAPK, MAPK, P-ERK1/2, IκBα, P-NF-κB, and β-actin.

Data are presented as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test.

****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05.

Full-length uncropped blots are provided in the Supplementary Information. Blots for different antibodies were obtained from independent gels.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- MAPK1P1.jpg
- MAPK1P2.jpg
- PMAPKP1.jpg
- PMAPKP2.jpg
- pNFKBP1.jpg
- pNFKBP2.jpg
- pERKP1.jpg
- pERKP2.jpg
- pIKBalphap1.jpg
- pIKBalphap2.jpg
- BactinP1.jpg
- BactinP2.jpg