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Spinal Cord Injury Affects Gene Expression of Transmembrane Proteins in Tissue and Release of Extracellular Vesicle in Blood: In Silico and *In Vivo* Analysis

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Abstract

Objective: Spinal cord injury (SCI) can disrupt membrane transmission by affecting transmembrane channels or neurotransmitter release. This study aimed to explore gene expression changes of transmembrane proteins underlying SCI through bioinformatics approaches and confirming in SCI model in rats.

Materials and Methods: In this experimental study, the differentially expressed genes (DEGs) in acute and subacute SCI were obtained based on microarray data downloaded from the gene expression omnibus (GEO). Transmembrane proteins of DEGs were recognized by using the UniProt annotation and transmembrane helices prediction (TMHMM) methods. The model of SCI was established through a weight-dropping procedure in rats. To confirm the SCI model, hematoxylin and eosin (H&E) staining was performed. Total mRNA was extracted from spinal cord tissues, and the RNA expression profile of some of the significantly changed genes in the previous part that has been confirmed by real-time polymerase chain reaction (PCR). Blood was collected from rats before sacrificing. Extracellular vesicles (EVs) were isolated by high-speed centrifugation from plasma. For the assessment of protein expression, western blotting was used.

Results: Based on bioinformatics analysis, we candidated a set of membrane proteins in SCI's acute and sub-acute phases, and confirmed significant upregulation in *Grm1, Nrg1, CD63, Enpp3*, and *Cxcr4* between the acute and control groups and downregulation in *Enpp3* between acute and subacute groups at the RNA level. Considering *CD63* as an EV marker, we examined the protein expression of *CD9* and *CD63* in the plasma-derived EVs, and *CD9* has significant expression between acute and control groups. We also demonstrate no significant *CD63* and *Cxcr4* expressions between groups.

Conclusion: Our results provide new insight into the relationship between candidate transmembrane protein expression and different stages of SCI using in-silico approaches. Also, results show the release of EVs in blood in each group after SCI helping enlarge strategies to enhance recovery following SCI.

Keywords: Differentially Expressed Genes, Extracellular Vesicles, Membrane Protein, Signaling Pathways, Spinal Cord Injury

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Introduction

Spinal cord injury (SCI) is one of the most important causes of sensory and motor disorders (1). The side effects may vary widely, from pain to loss of movement (2). Microscopic events following tissue injury, including inflammation, apoptosis, necrosis, and glial scar formation, are more important consequences of SCI (3). The acute phase, which begins when an injury occurs, causes a rupture of capillary networks and damage to the blood-spinal barrier, causing bleeding, activation of microglia, and the influx of inflammatory agents into the lesion site (4). In the subacute phase, astrocytes, along with extracellular matrix proteins, begin to form glial scars (5). Activation of astrocytes helps to restore ion homeostasis reducing edema and inflammation (6). Based on many studies, most recovery occurs before entering the chronic stage, especially at the subacute phase that is the optimal time to perform treatments such as cell transplantation (7). Therefore, a comparison of these phases is important.

About 30% of the entire human genome encodes membrane proteins (8). Approximately two out of third of the drug targets are membrane proteins (60-70%) (9). Membrane proteins in the nervous system, specially transmembrane proteins which are integral membrane proteins, play an important role in the development (10), generation, and transmission of electrical messages (11). Damage to the cell surface proteins frequently occurs following damage to neural cells, which can cause problems with synthesis and transmission between neurons (12). They can play an important role in inflammation that are upregulated by sensing changes in the nervous system microenvironment (13). Therefore, investigation of changes in the gene expression of transmembrane proteins after SCI is important.

Extracellular vesicles (EVs) released from almost all cell types. The possibility of isolating them from different biofluids, makes EVs valuable biomarkers to be analysed for the diagnosis or prognosis of various conditions (14). Recent studies show that EVs participate in the progression of spinal cord secondary injury by transporting parent cell-specific signaling cargoes that change the function of recipient cells within the central nervous system (15). Also, EV-mediated functions are altered in association with many pathological features of neurotrauma (16). In contrast, EVs have emerged as alternatives to cell-based therapies due to their potential for improved safety and therapeutic efficacy across diverse regenerative applications (17). Additionally, due to EVs' diagnosis, therapeutic and cell-targeting potential, they have emerged as a suitable candidate for cell-free therapies in SCI (16).

Since finding key proteins in different stages is important for controlling molecular events following SCI, we hypothesized that the expression of some genes may differ in acute and subacute phases and there will be some common genes in the two phases. Indeed, due to the importance of transmembrane proteins, we aimed to study them with a bioinformatics approach and using available datasets. In this study, we investigated changes in the expression of transmembrane protein genes before and after injury and also introduced altered signaling pathways following injury. Also, after confirming the SCI model, the selected transmembrane proteins were examined at the RNA and protein levels. Due to the results, plasma-derived EVs playing an important role in molecular events after SCI, were isolated and characterized in different groups and compared together.

Materials and Methods

Microarray data

In this experimental study, the expression profiles of GSE464 (GPL6247; Affymetrix, Inc., Santa Clara, CA, USA), GSE45006, GSE46988, and GSE2599 based on the Affymetrix Rat Gene 1.0 ST Array were obtained from the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/; accessed August 2021) (Fig.S1, See Supplementary Online Information at www.celljournal.org) (18). Data included noninjured spinal cord control samples and contusion spinal cord at 7-, 14-, 28-, and 35-days post-lesion.

In the acute phase, the expression profiles of GSE464, GSE45006, and GSE46988 were selected. Each dataset included a control group and a SCI group (7 days after the injury, which is in the acute phase) to compare gene expression.

To compare gene expression in the subacute phase, the expression profiles of GSE464, GSE45006, and GSE2599 were selected. Each dataset has a control group and groups of spinal cord injuries (14, 28, and 35 days after injury that are in the subacute phase interval) (Table S1, See Supplementary Online Information at www.celljournal.org).

Differentially expressed genes analysis

For genes with different expression enrichment analysis, comparisons between 7-, 14-, 28-, and 35-day post-SCI groups vs. control groups were performed using the GEO2R tool. Then, the genes with a threshold of adjusted $P \leq 0.05$ were screened out as DEGs. Furthermore, genes with log2FC (fold-change) ≥ 1.5 or ≤ -1.5 were categorized as up and down-regulated expressed, respectively. Venn diagrams were plotted for three datasets in each phase, and the overlaps of DEGs in at least two datasets were obtained.

Gene Ontology and signaling pathway enrichment analyses

Pathway enrichment analysis for the identified

up and down-expressed genes was performed by the Enrichr tool (19) considering KEGG (2019), Reactome (2016), Wikipathway (2019) libraries, and Biological Process for Gene Ontology analysis. All pathways in different libraries with an adjusted $P \leq 0.5$ were selected and merged. Then, to draw the interaction network of the gene ontology, we used the ShinyGO v0.75 database ($P<0.5$) for shared expressed genes in both stages demonstrating a hierarchical clustering tree that summarizes the correlation between significant signaling pathways and network plots displays relationships between enriched pathways (20).

Membrane protein annotation

For membrane protein annotation, we utilized our previously published approach (21, 22). Firstly, we downloaded all the rat's membrane proteins from UniProt (23) (release 2021_03) and investigated the overlap of DEGs and plasma membrane annotated proteins. To determine whether these reported proteins were integral membrane containing transmembrane (TM) domains, we predicted the TM helices using TMHMM (v 2.0) (24) and also SignalP (v 5.0) (25) confirmation for proteins with a single TM. Therefore, proteins with single or fewer helices and also signal proteins are excluded for further analysis.

Experimental animals and spinal cord injury model establishment

Twenty adult male Wistar Rats (220-270 g) were used in this study. All animals were housed double per cage in a room under a controlled condition, (ad libitum food and water, 22 ± 2°C, 12 hours dark/light cycle). All animals were randomly divided into five groups; intact groups, acute laminectomy (sham) group, acute injury group, subacute laminectomy (sham) group, and subacute injury group (Table S2, See Supplementary Online Information at www.celljournal.org). Animals were anesthetized by intraperitoneal injection of Ketamine (80 mg/kg) and Xylazine (20 mg/kg). To expose the spinal cord at the T9-T10 level, we performed a laminectomy with a dental drill. A 10 g weight dropped on the exposed spinal cord from 25 mm height via an NYU-impactor. After spinal cord compression, the muscles and skin were sutured. Animals were kept on a hot stage in 37°C for recovery. After SCI surgery, dextrose saline (10 ml) for 5 days and Enrofloxacin (5 mg/Kg) for 7 days was injected into each animal. Manual bladder discharge was performed twice daily until every animal regained full bladder control (Fig.S2, See Supplementary Online Information at www.celljournal.org).

To confirm the contusion SCI model, hematoxylin and eosin (H&E) staining was performed on sagittal and transverse sections of the spinal cord 7 days after the surgery.

Ethics statement

All of the procedures conducted on animals were approved by The Institutional Animal Care Using Committee (IACUC) at The Royan Institute, Iran. All animal experiments were performed by international guidelines that approved by the Royan Ethics Committee (IR.ACECR.ROYAN.REC.1400.122). All efforts were made to decrease the suffering of the animals used and reduce the number of animals used in this research.

Behavioral assessment

The Basso, Beattie, and Bresnahan (BBB) (26) score is a scale of 0 (complete hind limb paralysis) to 21 (normal motor activity) that rat models in injury groups were placed in a surrounded field and observed for 3 minutes which can confirm spinal cord injury. First, each rat's bladder was emptied to avoid affecting its behavior. Then BBB Scale locomotor tests of all rats were blindly performed after SCI in 3-, 7-, and 14-days post-injury. Finally, the average integer value of each rat was recorded.

Histological analysis

Animals were sacrificed and perfused transcardially with 4% paraformaldehyde (pH=7.4). The spinal cords of the upper and lower 10 mm entered on the injured site with bone that were collected and kept in 4% paraformaldehyde for 10 days and fixed in formalin for 24 hours with bone. The tissue was dehydrated in gradient alcohol, then cleared in xylene, and embedded in paraffin. Subsequently, the embedded tissues were cut into 5-µm thick slices. The sagittal section contained the full length of the longitudinal axis of the tissues, passing through the center of the spinal cord, and the transverse section was located at the site of the injury. Next, these sections were stained with H&E.

Real-time polymerase chain reaction

To isolate RNA from each injured spinal cord which was kept in RNA later at -80°C after sacrificing the animal, Trizol (Kiagene/Kiazist) was used. cDNA was synthesized via Royan Biotech kit, according to the company's guidelines. It was used as a template in real-time polymerase chain reaction (RT-PCR) analyses and quantifying the RNA levels of *Grm1*, *Nrg1*, *Scn1*, *Kcna1*, *Cxcr4*, *CD63*, and *Enpp3* genes. mRNA expression levels were normalized against the reference gene *GAPDH* and measured using the 45-ΔCT method, which is the result of subtracting ΔCT from the total number of cycles (which is 45 in this study). The final volume was 10 µl which included cDNA (2 µl, 12.5 ng/µl), forward and reverse primers (5 pmol/µl), SYBER Green (2.5 µl), and DEPC treated

water up to the final volume. The concentration of RNA for each cDNA reaction is 2000 ng. Three biological replicates and two independent technical replicates were used in all experiments.

Extracellular vesicles isolation and characterization

EVs were isolated from plasma using differential centrifugation. Since we are going to focus on the membrane proteins, we isolated EVs by high-speed centrifugation at 20,000 g for 60 minutes. Isolated EVs were stored at -80°C.

Western blotting

Protein expression of EVs was measured using western blot for CD9, CD63 (EVs markers), and Cxcr4 proteins. The protein concentration of each group was determined by BCA assay kits. Samples (10 µg) were separated by 10% SDS-PAGE electrophoresis (Fig. S3, See Supplementary Online Information at www.celljournal.org). The probed proteins were transferred to Bio-RAD polyvinylidene fluoride membranes using an electroblotting transfer system (Bio-Rad) 25V for 150 minutes at room temperature (RT). Then polyvinylidene difluoride membranes (PVDF) membranes were blocked overnight at 4°C buffered with 2% bovine serum albumin (BSA) in Tris-saline plus 0.1% Tween for 1 hour and incubated with primary antibodies [anti-CD9 (Sc13118, Santacruz; 1:1000), and anti-CD63 (Sc-5275, Santacruz; 1:200) and anti-Cxcr4 (sc-6190, Santacruz; 1:200)]. The membranes were washed three times with Tris buffered saline with tween 20 (TBST) and then incubated with anti-goat secondary antibodies (diluted at 1:2000) at room temperature for 1 hour. At the end, the membranes were rinsed three TBST, and incubated with Super Signal West Femto Substrate (ThermoFisher Scientific) and bands visualized by using the Alliance Q9 Advanced Chemiluminescence Imager gel documentation system. According to the SDS-PAGE gel (Fig.S3, See Supplementary Online Information at www.celljournal.org) image, the band intensities were normalized to the loading control and quantified using ImageJ software version 1.46 (National Institute of Health, USA).

Statistical analysis

All results were analysed using GraphPad Prism v.9 software (GraphPad, USA). The qRT-PCR and western blot data analysed through one-way ANOVA and Tukey post-test and the data was presented by means ± SD. $P<0.05$ were considered significant.

Results

Data pre-processing and differentially expressed genes screening in spinal cord injury

DEGs between the SCI and control samples at both stages were screened. In both phases, genes that were common to at least two datasets are presented in Figure S4 (See Supplementary Online Information at www.celljournal.org). After removing duplicate genes, in total, 3382 and 745 genes with a threshold of adjusted $P\leq0.05$ as DEGs have been reproducibly reported in acute and subacute datasets respectively.

After choosing genes with log2FC (fold-change) ≥1.5 or ≤-1.5, 389 upregulated and 141 downregulated DEGs in the acute phase, and 143 upregulated and 62 downregulated DEGs in the subacute phase were identified. At both stages, there was a greater number of upregulated DEGs than those downregulated DEGs.

Pathway enrichment analysis

The biological process and hierarchical clusters between the shared expressed genes in both phases were detected. In hierarchal clustering trees, bigger dots indicate more significant P values. Analysis of genes commonly upregulated between acute and subacute phases by ShinyGO identified biological processes with the most significant p-value, including the immune and defense responses, and biological processes involved in interspecies interaction between organisms. Since darker nodes in ShinyGO network plots are shown as more significantly enriched gene sets, these processes are related together, and other pathways such as regulation of immune system process, inflammatory response, response to external biotic stimulus, cell activation, cytokine production, and leukocyte activation. Also, a similar analysis revealed that the commonly downregulated genes in both phases were enriched in biological processes associated with anterograde trans-synaptic signaling, chemical synaptic transmission, synaptic signaling, and cell-cell signaling (Fig.1). The hierarchal clustering tree represents related gene ontology (GO) terms grouped together based on the number of shared genes. They are related together and some pathways, including ion transport, behavior, modulation of chemical synaptic transmission, and regulation of trans-synaptic signaling. The relationship between enriched pathways is shown and two nodes with 20% or more shared genes are connected.

Moreover, we have checked our DEGs in signaling pathways with enrichment-adjusted $P\leq0.05$ via Wikipathway, KEGG, Biological process, and Reactome databases. Some of the enriched signaling pathways that were found by at least two databases with greater input sizes that were immune response and apoptosis pathways in upregulated pathways, and also membrane transmission in downregulated pathways (Table 1).

Table 1: Enriched signaling pathways

Phase		Pathway	Term	Input/references size	Adjusted P value	Database
Acute	UP	Immune response	Regulation of immune response	14/179	1.15E-05	Biological process
			Macrophage activation involved in the immune response	5/13	3.03E-05	Biological process
			Cytokine Signaling in Immune System Homo Sapiens R-HSA-1280215	28/620	8.57E-07	Reactome
			Inflammatory response pathway WP458	4/30	0.00353	Wikipathway
		Apoptosis	Regulation of glial cell apoptotic process	2/7	0.026198	Biological process
			Apoptosis WP1254	6/81	0.003715	Wikipathway
			Apoptosis	9/141	8.45E-04	KEGG
Sub-acute	UP	Immune response	Cytokine-mediated signaling pathway	38/621	5.83E-19	Biological process
			Positive regulation of the production of molecular mediator of immune response	3/38	0.021754	Biological process
			Neutrophil activation involved in immune response	28/485	5.02E-13	Biological process
			Inflammatory response pathway WP458	3/30	3/30	Wikipathway
Acute	DOWN	Membrane transmission	Ion channel transport Homo sapiens R-HSA-983712	10/203	1.86E-05	Reactome
			GABA receptor activation Homo sapiens R-HSA-977443	6/55	2.73E-05	Reactome
			GABAergic synapse	10/90	3.54E-08	KEGG
			Calcium signaling pathway	10/189	7.70E-06	KEGG
			Neurotransmitter secretion (GO:0007269)	7/44	2.45E-06	Biological process
			Sodium ion transport (GO:0006814)	5/90	0.011817	Biological process
			Potassium ion transmembrane transport (GO:0071805)	11/139	5.95E-07	Biological process
Sub-acute		Membrane transmission	Glutamate receptor signaling pathway	5/37	8.03E-06	Biological process
			Sodium ion transmembrane transport	3/87	0.026798	Biological process
			GABAergic synapse	7/90	4.53E-07	KEGG
			Potassium ion transmembrane transport	5/139	0.002162	Biological process
			Calcium signaling pathway	8/189	0.04148	

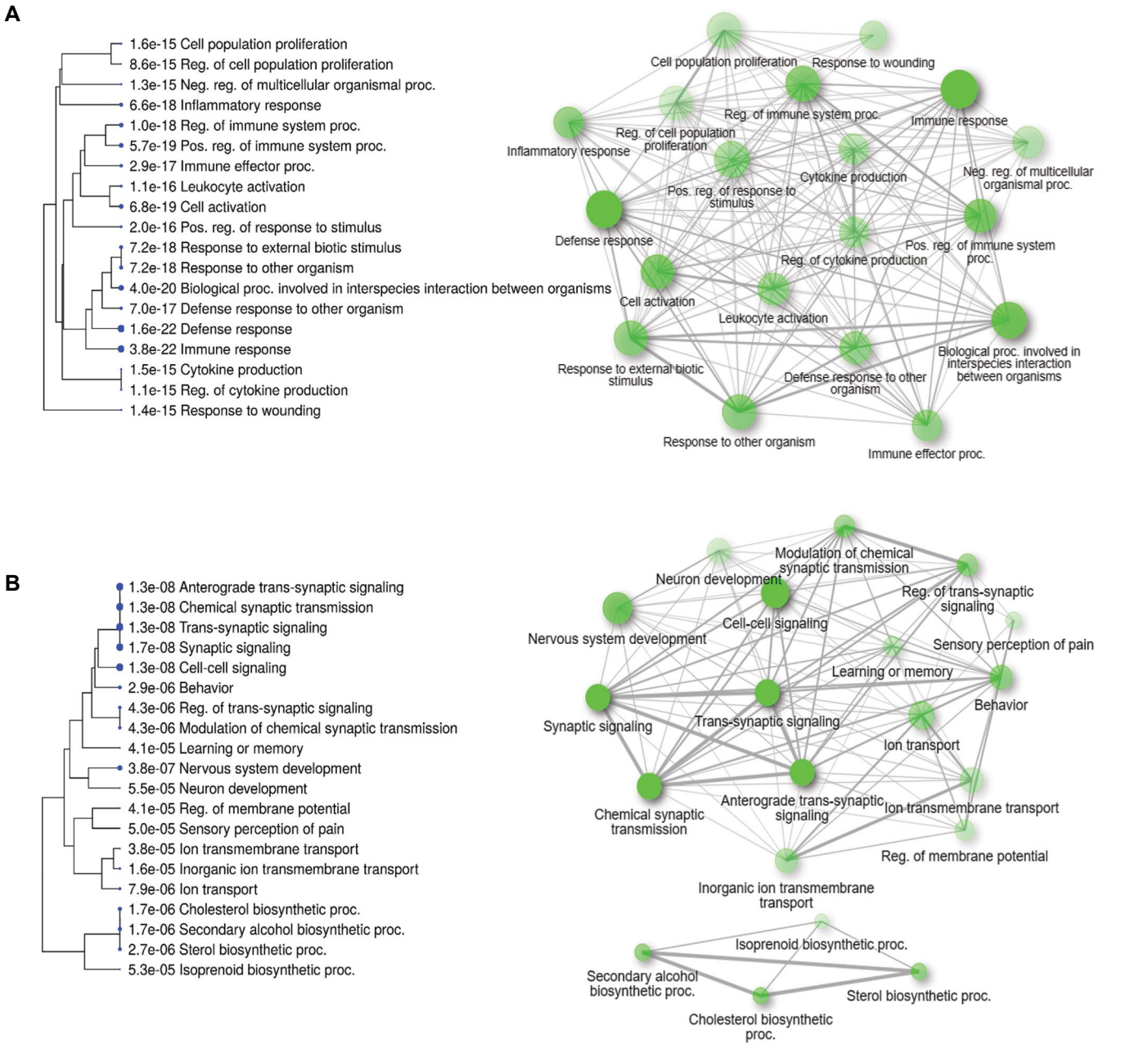

Fig.1: The biological process and hierarchical clusters between the shared expressed genes in acute and subacute phases. **A.** Upregulated and **B.** Downregulated genes in both acute and subacute phases. The intensity of color and the circles' size in networks indicate the significance of the biological process. The hierarchal clustering tree shows related GO terms grouped based on the number of shared genes (FDR P≤0.05). Bigger dots indicate more significant P values. To draw the interaction network of the gene ontology we used the ShinyGO v0.75 database. FDR; False discovery rate and GO; Gene ontology.

Analysis of differentially expressed membrane and transmembrane proteins

DEGs encoding membrane proteins in rats that were up- or down-regulated at each stage of injury were detected. In this study, proteins with more than one helix are considered transmembrane proteins. Transmembrane proteins which are upregulated or downregulated in both stages of SCI are separated. *Tlr4*, *Cd36*, *Olr1*, *Enpp3*, *Cd63*, *Ptprc*, *Cd53*, *Adcy4*, and *Cxcr4* are upregulated, and *Kcna1*, *Scn1a*, *Grm1*, *Cnr1*, and *Nrg1* are downregulated in both phases. The rest of them are specific to one of the two phases of SCI (Fig.2).

Spinal cord injury model establishment and behavioral test

To confirm contusion SCI model H&E staining was performed in sagittal and transverse sections of the spinal cord sections 7 days after the surgery, H&E demonstrated tissue damage as well as neuronal degeneration and cell elimination around the lesion site (Fig.3).

To confirm the SCI model and to show the rate of recovery after injury, the BBB test was performed on 1, 7, and 14 days after injury, showing the motor score immediately after surgery, and at each phase, respectively

(Table S3, See Supplementary Online Information at www.celljournal.org). Up to one week after the injury, the animal had scores below five, which confirms the SCI model. The BBB score of all animals was determined to be 21 before the SCI surgery. This score shifted to zero after surgery and remained the same for at least 3 days.

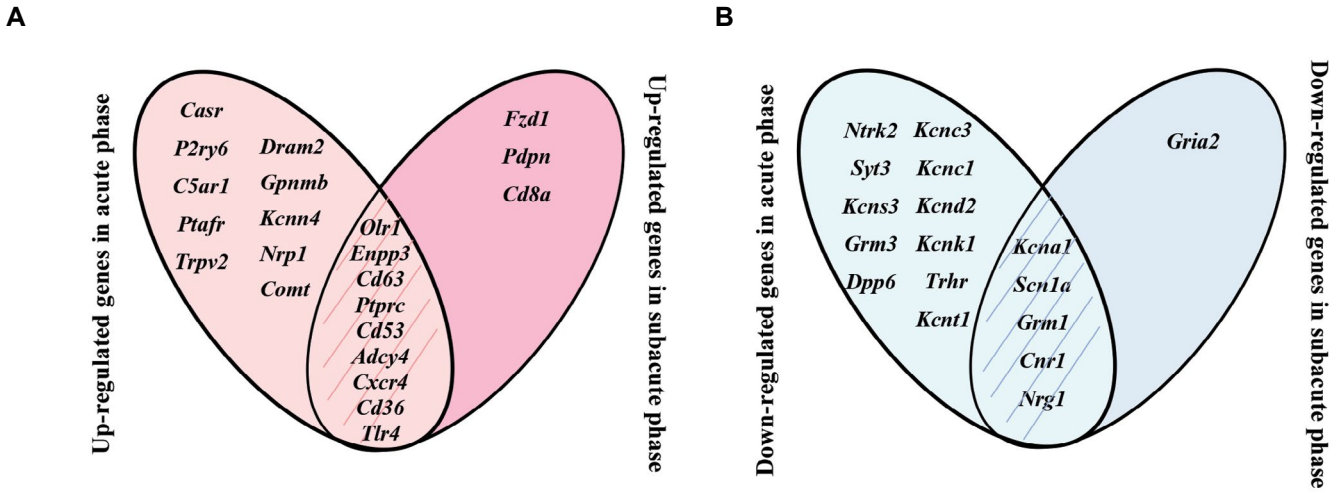

Fig.2: Upregulated and downregulated transmembrane proteins in both phases. **A.** Upregulated and **B.** Downregulated transmembrane proteins in the acute and subacute phases of injury. They also show common and specific proteins in two phases of SCI. SCI; Spinal cord injury.

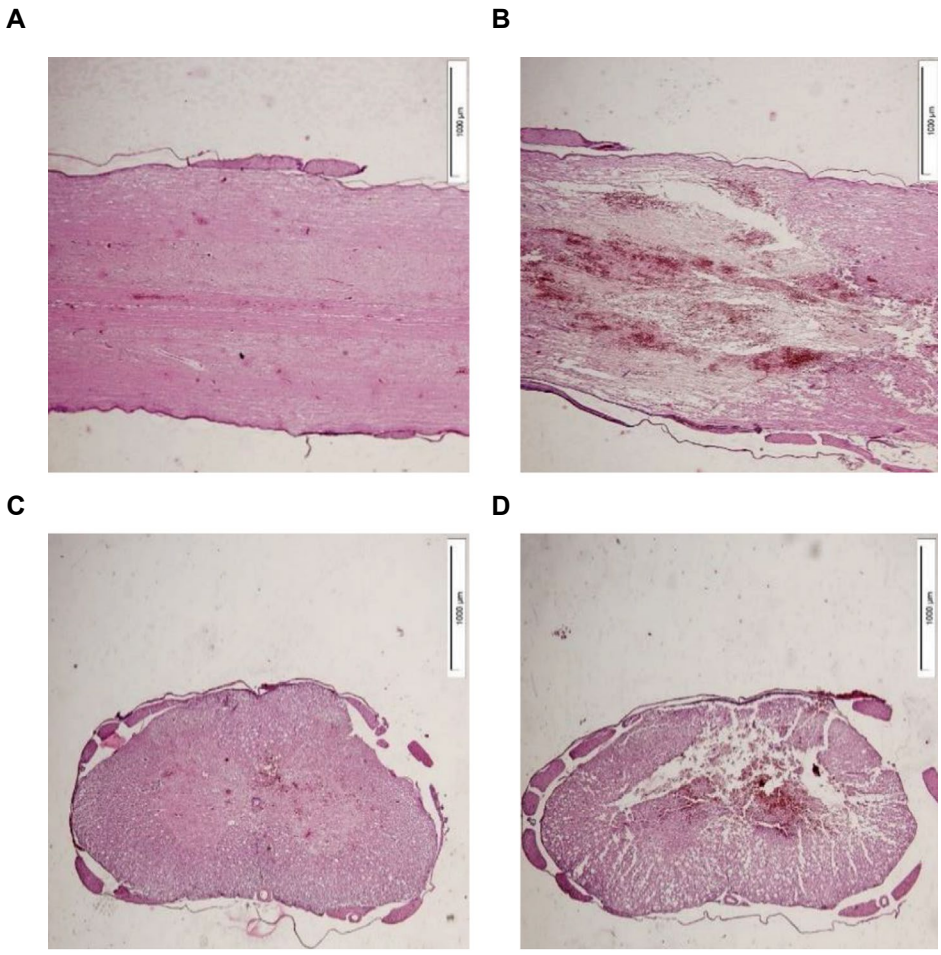

Fig.3: Spinal cord injury (SCI) model confirmation. H&E staining 1 week after SCI. **A.** H&E staining of a sagittal section of intact and **B.** injured spinal cord. The lesion site is clearly demonstrating a severe injury. **C.** Shows a transverse section of approximately the 9th thoracic level of the vertebrae which is intact. **D.** A transverse section of about the 10th thoracic vertebrae which is in the lesion site.

Gene expression at the RNA level

We examined some genes among common ones in both phases to confirm them at the RNA level. The selected genes are *Enpp3*, *Cd63*, and *Cxcr4* as upregulated genes and *Kcna1*, *Scn1a*, *Grm1*, and *Nrg1* as downregulated. In this section, the primers designed in Table S4 (See Supplementary Online Information at www.celljournal.org) are used. The melting curve of the two strands of cDNA is also shown in Figure S5 (See Supplementary Online Information at www.celljournal.org).

The genes which were upregulated in the bioinformatics part of the study were compared in the intact, sham acute, sham subacute, acute, and subacute groups. In the comparison of the sham acute groups with the acute groups, there was a considerable expression of *Cd63*. Additionally, there was a significant difference in *Cxcr4* expression between acute and intact groups as well as acute and sham groups. There was a noticeable difference in the expression of the *Enpp3* gene between the acute and subacute groups as well as between the acute and sham groups.

Also, the genes that were downregulated in the bioinformatics section of the study were compared in the intact, sham acute, sham subacute acute, and subacute groups. There was a significant expression in this comparison between the acute group and the sham group in *Grm1*. In the acute and subacute groups, *Kcna1* and *Scn1a* genes did not have any notable expression. Additionally, there was a significant expression difference between the acute and sham groups in *Nrg1*. To compare groups, a One-way ANOVA and a Tukey post-test were applied (Fig.4).

Western blot analysis

Among all the identified genes, CD63 (EV's marker) and Cxcr4 were selected to test their protein expression in plasma-derived EV. Western blots of plasma-derived EV have shown the expression of CD63, CD9, and Cxcr4 in intact, sham acute, sham subacute, acute, and subacute groups. CD63 has not shown any significant expressions between groups. While there was a significant difference in CD9 expression between sham acute and intact groups as well as acute and sham acute. Also, there was no significant difference in Cxcr4 expression between groups (Fig.5).

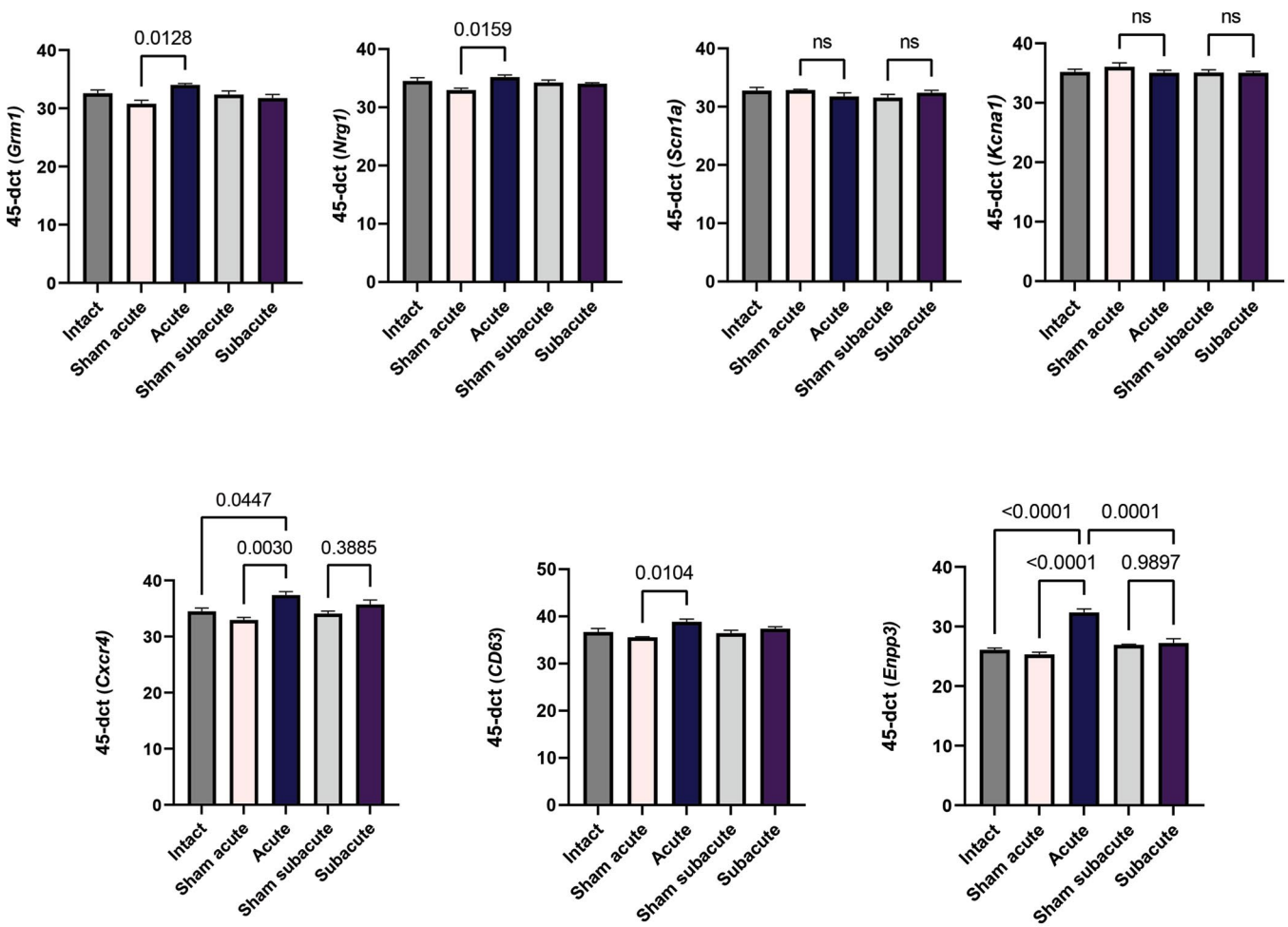

Fig.4: Quantitative RT-PCR analysis of selected genes in the spinal cord of intact, laminectomy, and injury groups in acute and subacute phases. One-way ANOVA with Tukey posttest was performed and data has been presented by means ± SD. ns; Non-significant and RT-PCR; Real time polymerase chain reaction.

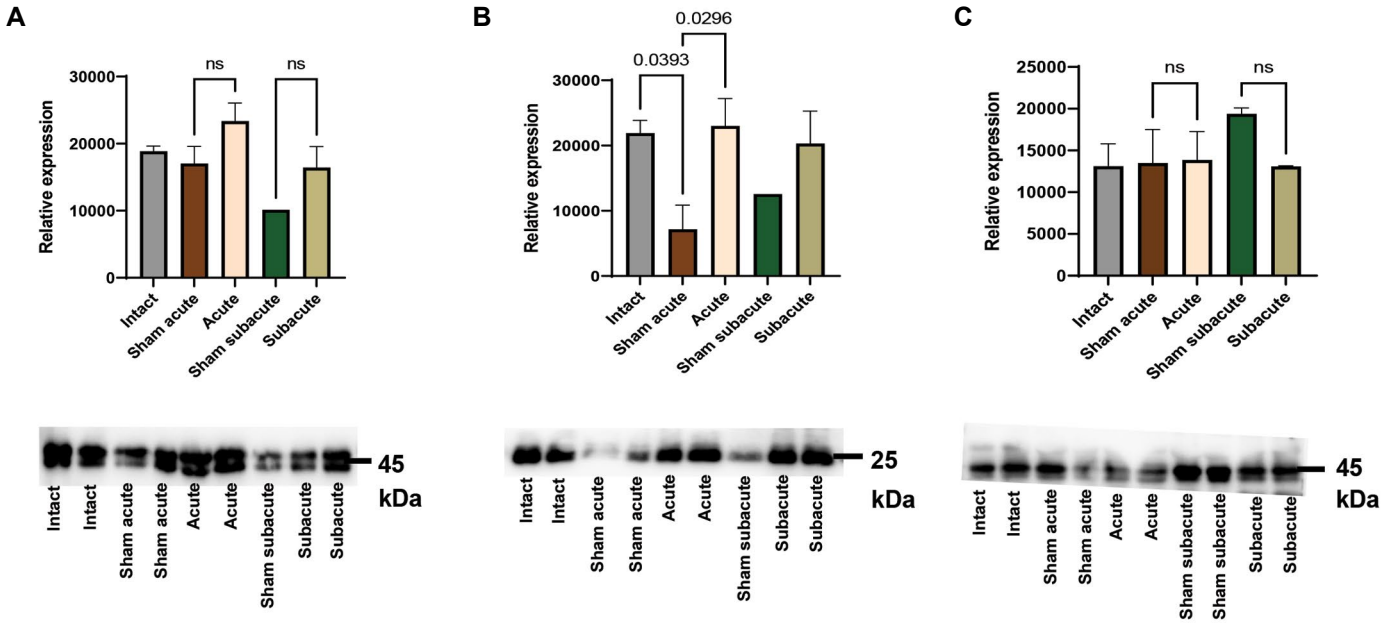

Fig.5: Western blots of plasma derived-EV show the expression of CD63, CD9, and Cxcr4 in intact, laminectomy and injury groups. EVs were characterized by expressions of **A.** CD63 and **B.** CD9. **C.** Also, Cxcr4 protein levels were measured in all groups. 10 µg of protein was loaded per lane. One-way ANOVA with Tukey posttest was performed and data has been presented by means ± SD. ns; Non-significant.

Discussion

SCI is a fatal nerve injury that has irreversible effects on the sensory, motor, and nervous systems that is no complete treatment for it (27). It is important to have a comprehensive understanding of the conditions of injuries and to provide treatment strategies.

In this study, a total of 3382 DEG in the acute phase and 745 DEG in the subacute phase were found. Based on biological process enrichment, the up-regulated genes in both phases were related to the immune, defense, and inflammatory response and also cell activation and cytokine production. Also, the down-regulated genes were related to synaptic transmission, cell-cell signaling, and nervous system development. Similar results were obtained from Enrichr analysis of pathways, the most up-regulated pathways were related to the immune response such as inflammation and cytokine signaling, and apoptosis. Also, the down-regulated pathways were related to membrane transmission such as ion channel transport and neurotransmitter receptors. In 2015, research on a different dataset demonstrated that the enriched KEGG pathways of the downregulated genes were predominantly associated with pathways of neurological diseases. While the upregulated genes were enriched in immune response-associated pathways (28).

Due to the important role of transmembrane receptors in neurons, in the next step we defined analyses to find transmembrane proteins and obtained a short list of them that finally confirmed some of the genes among the selected genes at the RNA level. These genes included a subunit of the sodium and potassium channels and the glutamate receptor, as well as the growth factor Neuregulin genes that were downregulated in the bioinformatics section. Additionally, the immunogenic genes *Enpp3*, *Cxcr4*, and *Cd63* were selected from the genes that were up-regulated in the previous section. Following RT-PCR for tissues, we showed significant upregulation in *Grm1*, *Nrg1*, *CD63*, *Enpp3*, and *Cxcr4* between the acute and control groups and downregulation in *Enpp3* between acute and subacute groups.

Due to the important role of EVs in cell communication, stimulation or suppression of the immune system and tissue injury, it has recently been considered as a therapeutic method. Also, considering the ability of EVs to activate or suppress many signaling pathways, in this study, we investigated the expression changes of EVs markers (29, 30). Considering *CD63* as an EV marker, to characterize EVs we examined the protein expression of *CD9* and *CD63* plasma derived-EV (31). *CD9* has significant expression between acute and control groups. We also demonstrate no significant *CD63* and *Cxcr4* expressions between groups.

According to studies on chronic pain after spinal cord injury, it has been proposed that a significant increase in *CXCL12* expression occurs in activated astrocytes. Also, the expression of the *Cxcr4* receptor increases on the surface of macroglia cells, and the connection between this ligand and the receptor causes an increase in pro-inflammatory cytokines, which will eventually lead to chronic pain (32). Moreover, studies have shown that *Enpp3* has a decreasing effect on ATP concentration in most tissues and leads to the suppression of basophil cells and mast cells. And these changes will eventually lead to the control of severe inflammation (33).

According to the bioinformatic studies in this project, we expected that the factors related to immune response and inflammation would increase in expression, and we observed this increase in both *Enpp3* and *Cxcr4* genes in the acute phase of injury.

As we mentioned, to prevent the infection and death of the animal, we used the antibiotic enrofloxacin, which in some studies has shown the mechanism of action of this drug orally leading to anti-inflammatory effects in some tissues (34). It seems that the daily use of antibiotics and serum, as well as the daily emptying of the animal's bladder, has led to the prevention of extensive inflammation and infection in the subacute phase, and it may also affect the expression of *Cd63*, which is one of the important proteins on the surface of EVs and involved in immunity and causes the adhesion of leukocytes to endothelial cells and has a role in signaling angiogenesis (35).

As we have shown, no significant difference was observed in sodium (*Scn1a*) and potassium (*Kcna1*) genes selected in the injury phases compared to the control group. Due to the important role of potassium channels and also the difference in their different isoforms, the study on the expression of these channels in neuronal damage has also been done on human data sets. Some articles reported significant changes in the *Kcna1* gene after injury. In a study, these changes were seen in the chronic phase in the mouse model. Also, they have shown the expression of another subunit of the sodium channel (*Scn8a*) was changed (36).

In our study, the *Grm1* did not significantly change between the injury groups and the control groups, but this expression was significant between the acute and subacute phases. We suppose that using ketamine to anesthetize the animal is the reason for this result, because this drug directly affects glutamate receptors and inhibits them (37).

The *Nrg1* gene, which encodes Neuregulin-1, leads to myelination of some neurons and spontaneous healing of the injury after SCI (38). It has been shown in studies that the expression of this gene decreases after injury, and severe secondary injury is formed after it (39). We guess that in our study, this change was not seen in the acute phase due to the absence of severe secondary damage in a short time after the injury occurred, and in the sub-acute phase, this lack of significance is due to the use of drugs that controlled the severity of the damage.

Generally, the possible reasons for the lack of significance in some of our genes in this study can depend on various factors. For example, the type of damage selected in bioinformatics studies was medium damage, which in this type of damage is considered to be 12-25 mm weight distance in the articles. In this research, we chose the maximum distance, 25 mm, and as seen in the histological examination, the intensity of the damage was high and scattered. Therefore, the type of injury may have influenced the study. Moreover, there are various types of subunits and isoforms in ion channels and neurotransmitter receptors. The subunits of ion channels have different roles, including regulatory, or functional, and some of them may be active in different conditions. Also, some of these isoforms do not change when cell conditions change.

According to the mentioned cases, it can be suggested that the anesthetic drug and antibiotic used should undergo a more detailed study or be changed. Secondly, for the bioinformatics part of the work, it is possible to create more filters by changing analyses such as the amount of logFC and reaching a shorter gene list. Thirdly, in this study, we used real-time PCR for spinal cord tissue samples and the Western blotting technique for blood samples. This can be one of the explanations for the difference in expression in genes such as *Cd63*. It is suggested to investigate protein level expression in the candidate genes in tissue samples.

In the subacute phase, due to the activity of the immune system after injury, the spinal cord can undergo a slight spontaneous recovery. For example, the inflammation in the spinal cord decreases in this phase (40). Therefore, we expect that the expression of genes has undergone more changes in the acute phase, and the bioinformatics results regarding the number of genes in these two phases can support this theory.

Conclusion

Our results provide novel insight into the relationship between transmembrane protein expression and SCI by bioinformatics approaches followed up at RNA level in animal models. Also, results indicate that SCI affects EV release in the blood at different times, which can help enlarge strategies to enhance recovery following SCI.

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Authors' Contributions

Y.M.; Conceptualization, Methodology, Software, Formal Analysis, Writing- Original Draft, and Visualization. N.E., Y.M., A.I.; Investigation. N.E., Y.M., F.Sh.; Writing- Review and Editing. F.Sh., S.K.; Supervision, Project administration, and Funding acquisition. All authors read and approved the final manuscript.

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