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Abscisic Acid Regulates Immune-inflammatory Responses to Induce Neuroprotection in Spinal Cord Injury: Insights from Gene Expression and Network Analysis

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ABSTRACT

Spinal cord injuries (SCI) lead to complex primary and secondary damage that disrupts neural function. Current treatments are often insufficient and unable to fully repair spinal cord injuries, highlighting the urgent need for new medicines and innovative therapies.

This study aimed to evaluate the therapeutic potential of abscisic acid (ABA) in SCI by examining its effects on immune-inflammatory genes' expression in rats. This phytohormone possesses anti-inflammatory and neuroprotective properties, rendering it a potential agent for reducing secondary damage following spinal cord injury. Additionally, we performed protein-protein interaction (PPI), pathway enrichment, functional annotation, and gene ontology (GO) analyses to gain a comprehensive understanding of the functions of the affected genes.

Based on the results, SCI led to changes in the expression of immune/inflammation-related genes in rats. However, the administration of ABA alleviated the effects. ABA downregulated proinflammatory genes (*IL-6, IL-1β, MCP, TLR2, TLR4*) and neural signaling components (*NMDA*, *AMPA*, *NK1R*), while upregulating adrenergic receptors (*ADRA1A*, *ADRB1*) and a gamma-aminobutyric acid receptor (*AGBRA2*). PPI analysis identified *FOS*, *IL-1β*, *IL-6, MMP9*, and *TLR4* as crucial nodes in the network, exhibiting the highest degree of interaction. Functional analyses revealed potential impacts on cellular responses, metabolic processes, and synapse-associated extracellular matrix components. Notably, these genes were enriched in inflammatory signaling pathways according to KEGG analysis.

These findings suggest that ABA has a significant modulatory effect on gene expression following SCI, particularly in reducing inflammation and immune responses, thereby highlighting its potential as a novel therapeutic agent for SCI.

Keywords: Inflammatory response; Plant hormones; Regulation of gene expression; Spinal cord injuries

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INTRODUCTION

The phrase spinal cord injury (SCI) pertains to harm to the spinal cord caused by trauma (such as falls and road traffic accidents) or non-traumatic reasons like tumors, degenerative and vascular conditions, infections, toxins, or birth defects. The World Health Organization (WHO) reported that worldwide calculations indicate that about 15.4 million individuals were living with SCI in 2021 (https://www.who.int/news-room/fact-sheets/detail/spinal-cord-injury, available on 16 April 2024). These injuries often result in significant and long-lasting physical, emotional, and economic burdens for those affected and their families. Current treatment options are limited, highlighting the urgent need for innovative therapies and improved medical interventions to enhance recovery and quality of life for SCI patients.

The pathophysiology of SCI is complicated and can be categorized into 2 main types: primary and secondary injuries. The primary phase occurs immediately after the injury. It involves direct mechanical damage to the spinal cord tissue due to trauma, such as fractures, dislocations, or compression. This phase leads to disruption of blood flow, damage to the blood-spinal cord barrier, cell death (including neurons, glial cells, and endothelial cells), and disruption of neural fiber tracts in the spinal cord. The secondary phase of SCI is triggered by the primary injury and involves complex pathological processes that last for several weeks. This phase includes chemical and mechanical damage to spinal tissues, leading to neuronal excitotoxicity due to high calcium accumulation, increased reactive oxygen species, and elevated glutamate levels. These changes damage nucleic acids, proteins, and phospholipids, resulting in neurological dysfunction. Clinically, secondary injury manifests as increased cell permeability, apoptotic signaling, ischemia, vascular damage, edema, excitotoxicity, ionic deregulation, inflammation, lipid peroxidation, free radical formation, demyelination, Wallerian degeneration, fibroglial scar, and cyst formation. Also, blood vessel disruption causes hemorrhage and the invasion of immune cells like monocytes, neutrophils, T and B lymphocytes, and macrophages, which release inflammatory cytokines TNF-α within 6 to 12 hours post-injury. These processes worsen tissue damage and hinder neuroplasticity.[1–3]

The current clinical treatment for SCI includes early surgical decompression, spinal cord perfusion augmentation, corticosteroids, anti-inflammatory therapy, and neurological rehabilitation. However, these approaches have limited clinical benefit, and there are currently no effective strategies to repair SCI, leading to long-term dysfunction and lifelong disability for patients.[4] Methylprednisolone (MP) is a corticosteroid proposed to inhibit the inflammatory cascades contributing to secondary spinal cord damage, but its clinical utility remains controversial.[5,6] Additionally, the prolonged use of MP has been linked to adverse effects such as gastrointestinal bleeding, increased risk of infection, hyperglycemia, and delayed wound healing.[7,8] As a result, there is an urgent need to explore alternative therapeutic agents that can provide better efficacy, safety, and long-term outcomes for treating spinal cord injuries.

In recent decades, phytochemical compounds have demonstrated considerable pharmaceutical potential across various biomedical domains.[9–11] Abscisic acid (ABA) is a plant-derived compound classified as a sesquiterpenoid, with the chemical formula $C_{15}H_{20}O_4$.[12] As a plant hormone, ABA is essential for regulating a variety of physiological and defensive processes in plants.[13,14] Moreover, recent studies have uncovered that abscisic acid possesses a wide range of pharmacological activities. It has been found to have anti-inflammatory, antioxidant, anti-atherosclerosis, anti-depressant, pro-cognitive, anti-anxiety, and anti-cancer effects.[15] Additionally, the antinociceptive properties of this phytohormone have been highlighted in recent research.[16,17] Furthermore, a recent study revealed that ABA contributed to an increase in the response latency to nociceptive thermal stimuli and enhanced locomotor function in rats with SCI. However, the full extent of ABA's beneficial effects in the context of SCI remains incompletely investigated. While ABA shows promise as a therapeutic agent, its exact mechanism of action remains elusive. Identifying the specific genes and proteins modulated by ABA in SCI patients is essential to unraveling its protective mechanisms.

This study aimed to investigate the effect of ABA administration on the expression of 18 key genes involved in various aspects of SCI, including immune and inflammatory response, neurotransmitter receptors, transcription factors and signaling molecules, enzymes and matrix remodeling, pain modulation, and neuropeptides. To understand the potential mechanism of ABA-mediated neuroprotection in SCI, we further

explored the interactions of these genes through protein-protein interaction analysis using the STRING database, Gene Ontology (GO) term enrichment analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment studies.

MATERIALS AND METHODS

Animals

36 male Wistar rats, weighing between 220 and 250 grams, were sourced from the Laboratory Animal Maintenance and Breeding Center of Kerman University of Medical Sciences. The rats were then acclimated to the new environment for a week at the animal house of Shahid Bahonar University of Kerman before beginning the experiments. During this period and throughout the study, the rats had ad libitum access to food and water and were housed in a controlled environment with a 12-hour light/dark cycle at a temperature of 22°C± 2°C. Subsequently, the rats were randomly assigned to their respective treatment groups (n=6) for further assessments. It is important to note that all experimental protocols were ethically reviewed and approved by the Ethics Committee of Shahid Bahonar University of Kerman (Approval No. IR.KMU.REC.1399.096) in compliance with the ARRIVE guidelines.[18]

Drugs and Animal Treatments

The (±)-cis-trans-abscisic acid was purchased from Sigma-Aldrich (USA) and dissolved in dimethyl sulfoxide (DMSO; Merck, Germany). A stock solution of ABA was prepared in DMSO and then diluted with artificial cerebrospinal fluid (aCSF) to a final intended concentration for administration. The final vehicle consisted of a 2:1 (v/v) mixture of aCSF and DMSO. Methylprednisolone sodium succinate (MP) was obtained from Exir Pharmaceutical Co. (Iran).

Rats were randomly assigned to six groups (n=6):

- Control: Rats received no surgery or treatment.
- SCI: Rats underwent SCI induction without subsequent treatment.
- SCI+Vehicle: Rats received an intrathecal injection of 5 µL of ABA vehicle (2:1 aCSF:DMSO) immediately following SCI induction.
- SCI+ABA10: Rats received an intrathecal injection of 5 µL of ABA (10 µg/rat) immediately following SCI induction.
- SCI+ABA15: Rats received an intrathecal injection of 5 µL of ABA (15 µg/rat) immediately following SCI induction.
- SCI+MP: Rats received an intraperitoneal injection of 0.5 mL MP (30 mg/kg) immediately following SCI induction.

Spinal Cord Injury Induction

Ketamine and xylazine were administered at doses of 60 mg/kg and 10 mg/kg intraperitoneally for anesthesia induction in the animals. Subsequently, the rats were secured in a stereotaxic apparatus for the surgical procedure. Following skin preparation and disinfection using a 7.5% povidone-iodine solution, the fascia and paravertebral muscles were delicately dissected to expose the lamina. A dorsal laminectomy was performed at the T9-T10 level without disrupting the dura mater to reveal the spinal cord.[19] Spinal cord injury was induced using the weight-drop contusion model, where a 10-gram weight was dropped from a height of 25 mm onto the exposed spinal cord.[20] The injections were then administered as per the experimental design. Finally, the muscle and fascia layers were sutured using absorbable sutures, and the animals were placed in a recovery cage equipped with a circulating heating pad.

The animals were anesthetized and euthanized 3 hours following spinal cord injury and injections. In preparation for the molecular analysis, intracardiac perfusion was carried out using phosphate buffer solution (pH 7.35), and the collected tissue samples were then preserved in a −80°C freezer.

Evaluation of Gene Expression (Real-time PCR)

After separating the injured site of the spinal cord tissue of the studied groups, the RNA extraction steps were performed by the manual protocol of the Cinna Gen Co. by the RNX-Plus reagent (Tehran, Iran), followed by dissolving in 30 µL of DEPC-treated water. Subsequently, cDNA was synthesized using the Easy cDNA Synthesis Kit (Pars Tous, Iran) following the manufacturer's instructions. In order to investigate gene expression, a Rotro gene detection system and RealQ Plus 2× Master Mix were utilized. The real-time PCR protocol was set as follows: initial denaturation at 95ºC for 10 minutes, followed by 40 cycles for real-time PCR, including denaturation phase: 20 seconds at 95ºC, annealing phase: 30 seconds at 58–61ºC, and elongation phase: 30 seconds at 72ºC. Primer sequences, RT-PCR product length, as well as NCBI accession numbers are shown in Table 1. All samples were evaluated in duplicate, and the means were used in subsequent

analyses. The linearity and efficiency of PCR amplification were evaluated using standard curves generated by increasing the amount of cDNA. The relative mRNA levels were calculated by the expression $2^{-\Delta\Delta CT}$.

Bioinformatic Analysis

Protein-protein Interaction (PPI) Network Analysis

To further explore the functional relationships among the 18 identified genes, we constructed a protein-protein interaction (PPI) network using the STRING database (https://string-db.org). STRING integrates known and predicted protein-protein interactions, allowing us to infer functional relationships between proteins [21]. STRING was used to identify interactions among the 18 genes, focusing on interactions supported by active sources, including text mining, experiments, databases, and co-expression. To ensure relevance to our study, the analysis was limited to "Rattus norvegicus" (rat) and used an interaction score threshold of >0.4. This threshold reflects a high confidence level in the predicted interactions.

The resulting PPI network was visualized using Cytoscape software version 3.10.1. In the network, nodes represent individual proteins, and edges represent their interactions.

Pathway Enrichment Analysis

To identify enriched pathways associated with the upregulated genes, we performed WikiPathways enrichment analysis using Enrichr (https://maayanlab.cloud/Enrichr/).[22] Enrichr is a web-based tool that facilitates the evaluation of annotations using extensive gene-set libraries, allowing us to assess the over-representation of specific pathways within our gene set.

Significant pathways were identified using an adjusted *p*-value threshold of <0.05. A pathway scatter plot was generated using the ggplot2 package in R software version 4.3.1. This plot visualizes the degree of enrichment for each pathway, where the y-axis represents the number of genes annotated in a pathway, and the "Rich Factor" (x-axis) is the ratio of this number to the total number of genes annotated in that pathway. A higher Rich Factor indicates a greater degree of enrichment. This analysis identified the top 23 enriched pathways based on their adjusted *p*-values.

Statistical Analysis

GraphPad Prism version 9 was used to analyze molecular datasets. To assess the differences between the study groups, the one-way analysis of variance with Tukey's post hoc test was employed. All data were presented as mean ± SEM, and statistically significant values were considered for $p<0.05$.

RESULTS

Gene Expression Assessment (RT-PCR)

Based on the results, the expression of all 18 tested genes considerably changed following SCI. However, treatment with MP significantly regulated the expression of the genes. ABA administration was also successful in regulating the expression of these genes, although it was slightly less effective than MP. Notably, the vehicle treatment had no significant effect on none of these genes' expression. The following details the effects of MP and ABA treatments on the expression of the 18 tested genes.

Cytokines and Inflammatory Response

Based on the findings, SCI resulted in a notable upregulation in the expression of genes associated with cytokines and inflammatory responses, including *IL-6, IL-1b, MCP, TLR2*, and *TLR4* (Figure 1). Specifically, the expression of *TLR2* doubled, while the others exhibited an increase of over five times compared to the control group. On the other hand, the expression of these genes in the PM-treated rats markedly decreased and was close to that of the control group. Likewise, ABA administration was successful in reducing the expression of these genes. Notably, the administration of 15 µg of ABA proved to be considerably more effective than 10 µg, reducing these genes' expression by approximately 60% compared to the SCI group

Table 1. Primer sequences, RT-PCR fragment lengths, and NCBI accession numbers

Genes	Full names	Primer sequence	Size of PCR product	NCBI accession number
NMDA/GRIN1	Glutamate ionotropic receptor NMDA type subunit 1 (Grin1)	F: CTCATCTCTAGCCAGGTCTACG R: GTCAGAGTAGATGGACATTCGGG	147	NM_001287423.1
Ampa/GRIA1	Glutamate ionotropic receptor AMPA type subunit 1 (Gria1)	F: GGACAACTCAAGCGT CCAGA R: CCACACAGTAGCCCTCATAGC	125	NM_031608.2
COX-2/PTGS2	Prostaglandin-endoperoxide synthase 2 (Ptgs2)	F: CTCAGCCATGCA R: GGGTGGGCTTCAGCAGTAAT	172	NM_017232.4
TLR4	toll-like receptor 4 (Tlr4)	F: CGGAAAGTTATTGTGGTGGTGT R: GGACAATGAAGATGATGCCAGA	173	NM-021578.2
TLR2	toll-like receptor 2 (Tlr2)	F: GGGTTCTGACATTGGAGTCC R: CAGTGTCCTGTAAGGATTTCC	182	XM_008761102/1
MMP9	matrix metallopeptidase 9 (Mmp9)	F: CATCACCTATTGGATCCAAAGC R: TGGATGACAATGTCTGCTTCG	147	NM-031055.2
MMP3	matrix metallopeptidase 3 (Mmp3)	F: GCTCATCCTACCCATTGCAT R: GCTTGTGCATCAGCTCCATA	219	XM_039080743.1
MCP/Ccl2	C-C motif chemokine ligand 2 (Ccl2)	F: TGTCTCAGCCAGATGCAGTT R: AGCCGACTCATTGGGATCAT	79	NM_031530.1
GABA-R	Gamma-aminobutyric acid type A receptor subunit alpha 2 (Gabra2)	F:GCTTCTTGTTTGGTTCTGGAGTAG R: AAGAGAAAGGCTCCGTCATG	135	NM_001135779.3
α1-adrenergic/ADRA1A	Adrenoceptor alpha 1A (Adra1a)	F: GTGTGCTTGTTTCTGTCTTG R: TATCGGGTAGGTTTCTTCCA	126	NM_012739.3
β1-adrenergic/ADRB1	adrenoceptor beta 1 (Adrb1)	F: CATCATGGGTGTGTTCACGCTCTG R: GCGTAGCCCAGCCAGTTGAAGAA	120	NM_012701.2
C-fos/FOS	Fos proto-oncogene, AP-1 transcription factor subunit (Fos)	F: GCCTTCACCCTGCCTCTTC R: GCTCCATGTTGCTAATGTTCTTGA	79	NM_022197.2
C-jun/JUN	Jun proto-oncogene, AP-1 transcription factor subunit (Jun)	F: GCTGAGTGTCTGTATGCTGGG R: GGACTTGTGGGTTGCTGGG	119	NM_021835.3
IL-6	Interleukin 6	F: CTGGTCTTCTGGAGTTCCGT R: TGGTCCTTAGCCACTCCTTCT	219	NM_012589.2
IL-1β	Interleukin 1 beta (Il-1β)	F: AAGACACGGGTTCCATGGTGAAGT R: TGGTACATCAGCACCTCTCAAGCA	97	NM_031512.2
NF-κB	Nuclear factor kappa B subunit 1 (Nfkb1)	F: AGAGCAACCGAAACAGAGAGG R: ATATGCCGTCCTCACAGTGC	227	NM_001276711.1
NK-R	Tachykinin receptor 1 (Tacr1)	F: CTTCTTCTTCCTGCCCTACATC R: TAATCACCTGCACTGATGAAGGG	195	NM_012667.3
Substance P/TAC1	Tachykinin, precursor 1 (Tac1)	F: CTGTTTGCAGAGGAAATCGGTG R:TCTCTGAAGAAGATGCTCAAAGGG	114	XM_063285566.1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: GTCTTCACCACCACGGAGAAGGC R: ATGCCAGTGAGCTTCCCGTTCAGC	392	NM_017008

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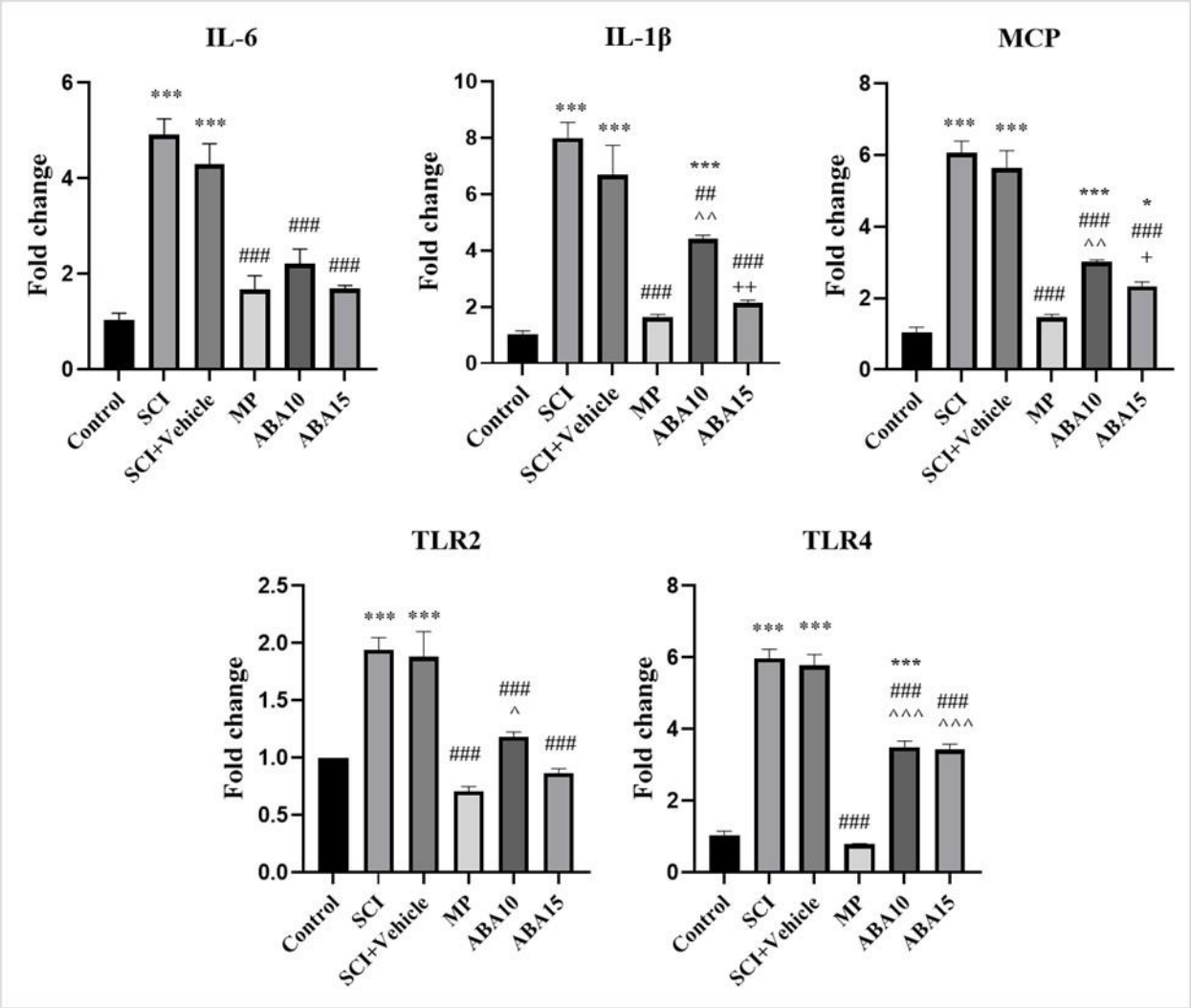

Figure 1. The expression level of genes associated with cytokines and inflammatory response in rats with spinal cord injury (SCI), in comparison to SCI rats who received methylprednisolone (MP) and abscisic acid (ABA 10 µg/rat and 15 µg/rat). * significant differences compared to the control group (*$p \leq 0.05$, ***$p \leq 0.0.001$); # significant differences compared to the SCI and SCI+Vehicle groups (##$p \leq 0.0.01$, ###$p \leq 0.0.001$); ^ significant differences compared to the MP group (^$p \leq 0.05$, ^^$p \leq 0.0.01$, ^^^$p \leq 0.0.001$); + significant differences compared to the ABA10 group (+$p \leq 0.05$, ++$p \leq 0.0.01$)

Neurotransmitter Receptors

Based on the results, SCI notably influenced the expression of neurotransmitter receptor genes, such as *NMDA, AMPA, AGBRA2, ADRA1A, ADRB1*, and *NK1R* (Figure 2). In the SCI group, *NMDA* and *AMPA* expression increased by 3-fold, while NK1R indicated a 7-fold increase compared to the control group. Conversely, the expression of *ADRA1A, ADRB1*, and *AGBRA2* decreased by 75%, 25%, and 25%, respectively, compared to the control group. PM treatment effectively restored the expression levels to those found in the control group. Furthermore, ABA administration successfully regulated the expression of these genes. ABA significantly reduced the expression of *NMDA, AMPA*, and *NK1R*, while increasing the levels of *ADRA1A*, *ADRB1*, and *AGBRA2* compared to the SCI group. Interestingly, there were no significant differences in the effectiveness of 10 µg/rat versus 15 µg/rat of ABA, except for AMPA.

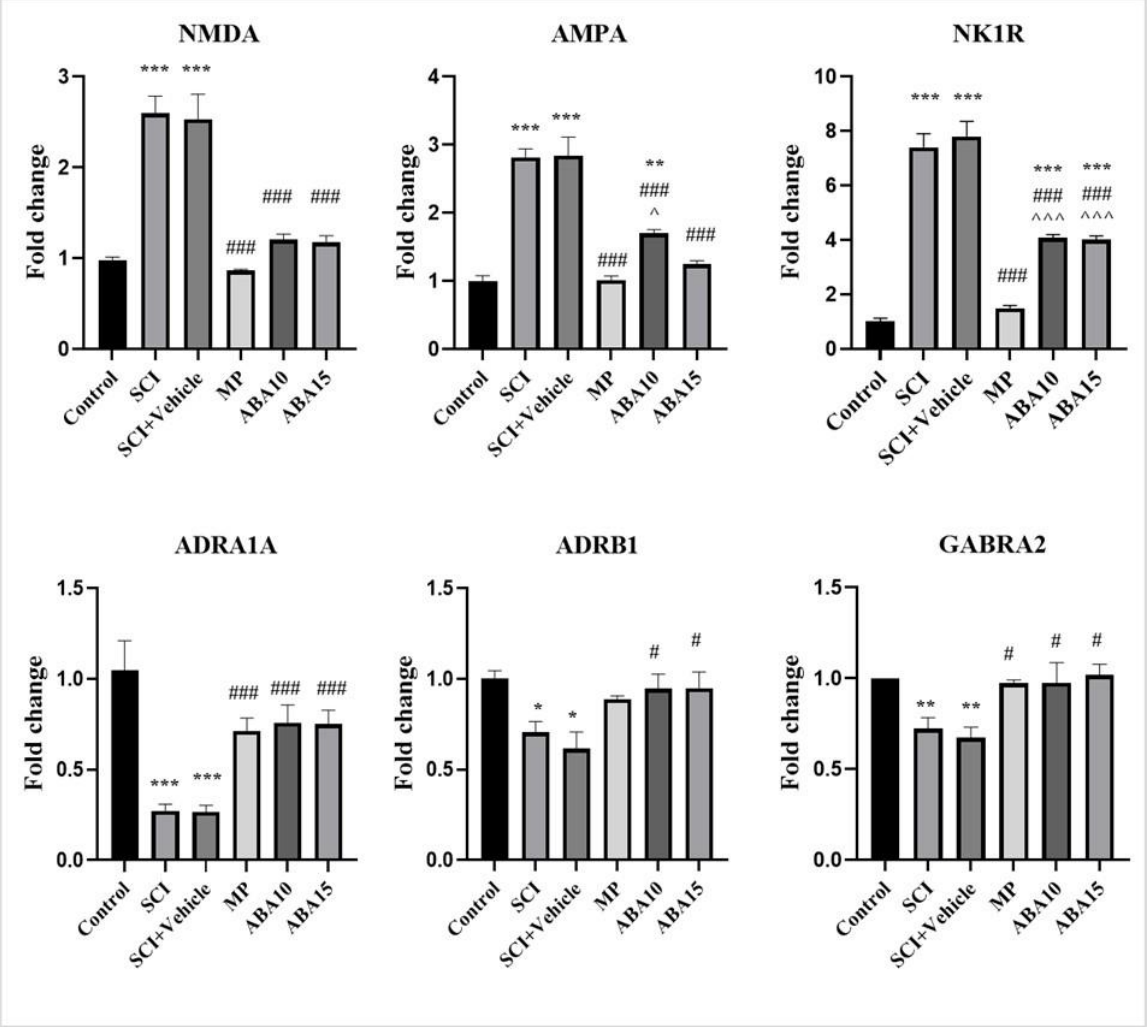

Figure 2. The expression level of neurotransmitter receptor genes in rats with spinal cord injury (SCI), in comparison to SCI rats who received methylprednisolone (MP) and abscisic acid (ABA, 10 µg and 15 µg). * significant differences compared to the control group (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$); # significant differences compared to the SCI and SCI+Vehicle groups (#$p \leq 0.05$, ###$p \leq 0.001$); ^ significant differences compared to the MP group (^$p \leq 0.05$, ^^^$p \leq 0.001$)

Transcription Factors and Signaling Molecules

Based on the findings, spinal cord injury (SCI) led to a significant increase in the expression of genes related to transcription factors and signaling molecules, such as *FOS, NF-κB*, and *JUN* (Figure 3a). Specifically, the expression of these genes increased by 6-fold, 5-fold, and more than 3-fold compared to the control group. Expression of the genes notably decreased in rats treated with PM and was similar to the control group. Additionally, ABA administration successfully reduced the expression of these genes, showing similar effectiveness to MP. Importantly, the administration of 15 µg of ABA was notably more effective than 10 µg. This concentration reduced the expression of *FOS, NF-κB*, and *JUN* by approximately 3-fold, 2.5-fold, and 2-fold, respectively, compared to the SCI group.

Enzymes and Matrix Remodeling

Following SCI, there was a notable increase in the expression of enzymes and matrix remodeling genes (Figure 3b). Specifically, the expression of *PTGS2, MMP3*, and *MMP9* genes increased by 3-fold, 9-fold, and 5-fold, respectively. On the other hand, treatment with MP led to a significant reduction in the expression of these genes, aligning their levels with those of the control group. Similarly, ABA treatment at both 10 µg and 15 µg concentrations proved effective in decreasing the expression of these genes. Both concentrations

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resulted in a 2-fold reduction in *PTGS2* expression, a 4-fold reduction in *MMP3* expression, and a 5-fold reduction in *MMP9* expression compared to the SCI group.

Pain Modulation and Neuropeptides

Our findings demonstrated that SCI led to a significant increase in TAC1 gene levels by more than 3-fold compared to the control group. Conversely, treatment with MP was able to decrease this gene expression by approximately 50%. ABA treatment at both concentrations also resulted in a reduction of this gene expression. However, the effectiveness of 15 µg of ABA was notably superior to that of 10 µg, achieving a 50% reduction in the expression of this gene (Figure 4).

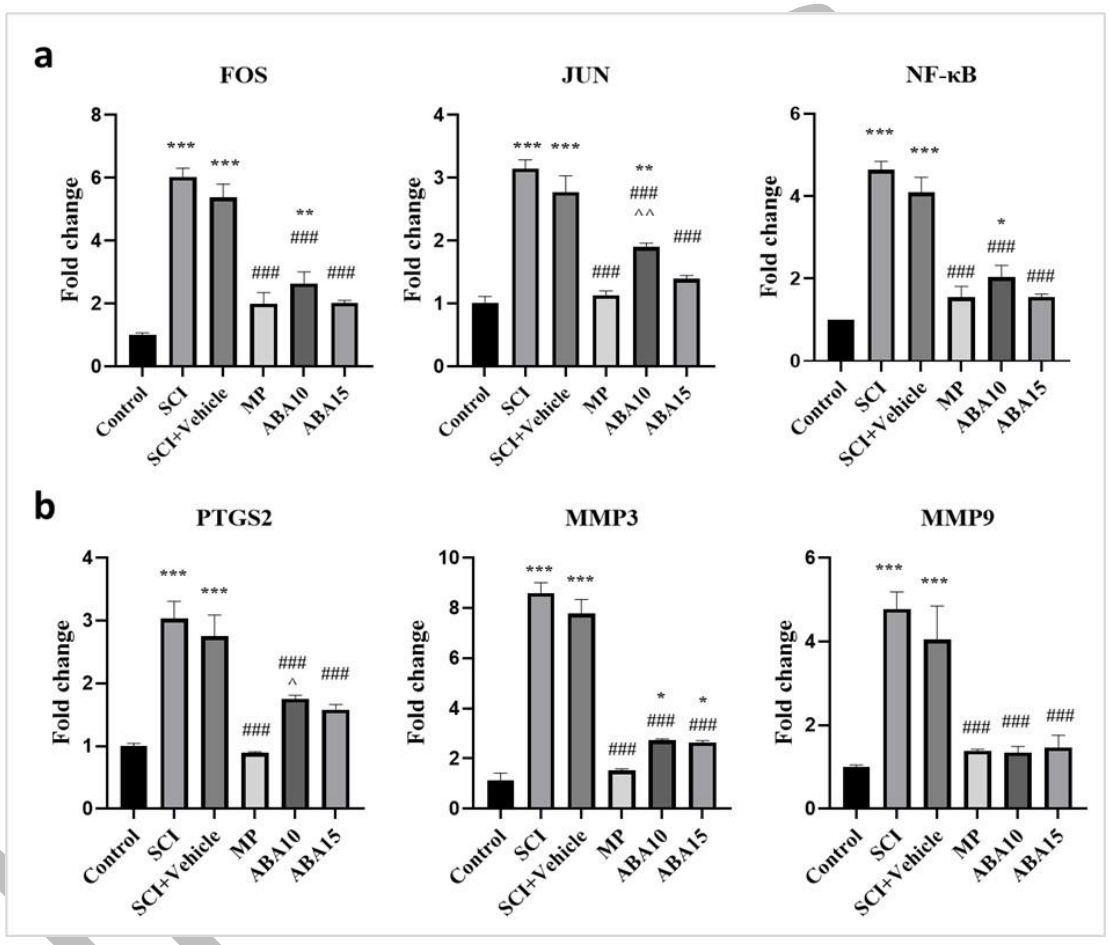

Figure 3. The expression level of enzymes and matrix remodeling genes in rats with spinal cord injury (SCI), in comparison to SCI rats who received methylprednisolone (MP) and abscisic acid (ABA, 10 µg and 15 µg). * significant differences compared to the control group (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$); ### significant differences compared to the SCI and SCI+Vehicle groups at $p \leq 0.001$; ^ significant differences compared to the MP group (^$p \leq 0.05$, ^^$p \leq 0.01$)

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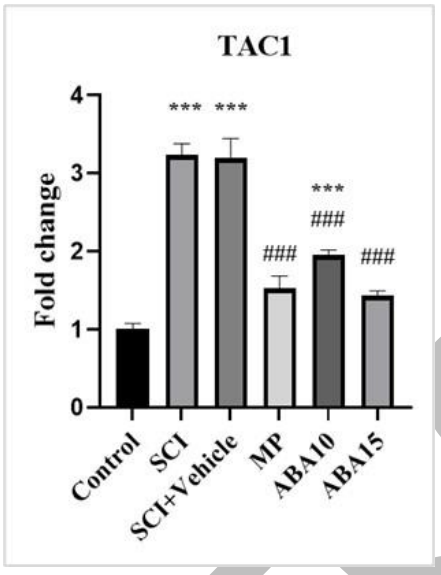

Figure 4. The expression level of enzymes and matrix remodeling genes in rats with spinal cord injury (SCI), in comparison to SCI rats who received methylprednisolone (MP) and abscisic acid (ABA 10 µg and 15 µg). * significant differences compared to the control group at $p \leq 0.001$; ### significant differences compared to the SCI and SCI+Vehicle groups at $p \leq 0.001$**

Bioinformatic Analysis

Gene Network Analysis

The STRING database PPI analysis of the target genes associated with spinal cord injury resulted in a network comprising 18 nodes and 73 edges (Figure 5). Here, each node represents a protein encoded by a target gene, and each edge represents a predicted or known interaction between two proteins. The fact that no edge is connected to a node such as *ADRA1A* and *ADRB1* indicates that interactions between this node and others do not exist, or at least their interacting confidence scores were ≤0.4. Also, the modulators for interacting with these genes may not exist in this network. The top 10 genes are listed in Table 2 based on their degree. Among these, the *FOS, IL-1B*, and *IL-6* nodes exhibited the greatest degree and are considered the most important genes in this network.

Table 2. The top 10 genes in the PPI network of the 18 genes involved in SCI.

No	Node	Full name of the node	Degree
1	*FOS*	FOS proto-oncogene, AP-1 transcription factor subunit	14.0
2	*IL-1β*	Interleukin 1 beta	13.0
3	*IL-6*	Interleukin 6	12.0
4	*MMP9*	Matrix metallopeptidase 9	11.0
5	*TLR4*	Toll-like receptor 4	11.0
6	*PTGS2*	Prostaglandin-endoperoxide synthase 2	11.0
7	*CCL2 (MCP)*	C-C motif chemokine ligand 2	11.0
8	*TLR2*	Toll-like receptor 4	10.0
9	*JUN*	Jun proto-oncogene, AP-1 transcription factor subunit	10.0
10	*TAC1*	Tachykinin, precursor 1	10.0

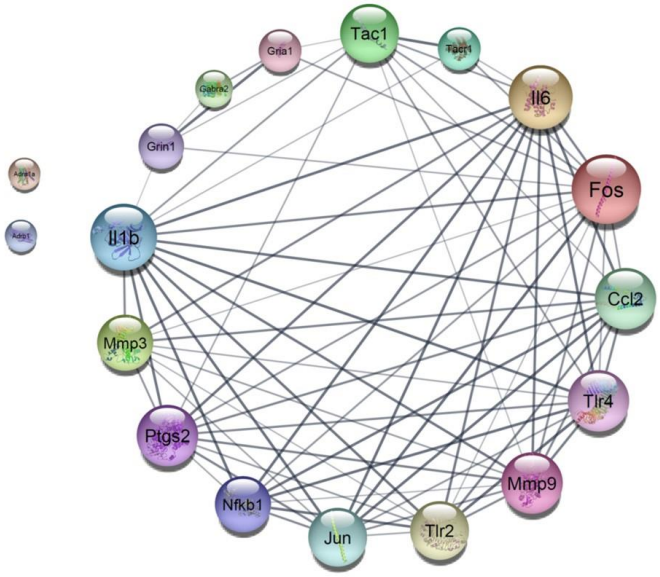

Figure 5. The PPI network of 18 genes involved in spinal cord injury. The size of each node shows the number of edges received from other nodes. The edges represent the predicted functional associations, and the color saturation of the edges represents the confidence score of a functional association

Gene Ontology (GO) Analysis

GO term enrichment analyses of proteins related to the SCI were conducted for biological processes (BP), cellular components (CC), and molecular functions (MF). The top 10 terms for BP, MF, and CC are listed and shown in Figure 6. Enrichment analysis of biological processes (BP) revealed that these proteins are involved in cellular responses and metabolic processes, notably the positive regulation of matrix metallopeptidase secretion.

The enriched GO terms for cellular components (CC) predominantly relate to the synapse-associated extracellular matrix and the NMDA-selective glutamate receptor complex, which exhibited the most significant p-values. This suggests that the target proteins may influence synaptic integrity and neurotransmission, factors critical in neuronal recovery post-SCI. In addition, most of the targeted genes involved in the TLR1 protein complex, IL6 receptor complex, and NF-κB complex all participate in inflammatory responses.

The MFs are influenced by the interacting proteins that are mostly related to the receptor bindings and activities, indicating that ABA may modulate receptor-mediated signaling pathways involved in SCI. Notably, the Rich Factor—a ratio indicating the proportion of target genes involved in a specific pathway—was high for pathways such as prostaglandin-endoperoxide synthase activity and receptor binding (substance P, neuromedin K, diacyl lipopeptide). This suggests that a substantial fraction of our target genes participate in these significant biological functions. Rich Factor is calculated by taking the number of these genes and dividing it by the total number of genes that are part of that pathway. A higher Rich Factor indicates a greater level of pathway enrichment.

Biological Pathways of Target Genes

To further understand the biological functions of the target genes, we performed KEGG enrichment analysis, revealing that 18 target genes were enriched in 30 pathways (Figure 7), notably in inflammatory signaling pathways. According to the analysis, 9 targeted genes are involved in IL-17 and TNF signaling pathways. In addition, the Toll-like receptor signaling pathway and neuroactive ligand-receptor interaction consist of 7 genes that are targeted and reveal important roles of these genes in the inflammatory response and neuroactivational responsibilities.

These findings suggest that the target genes play important roles in inflammatory responses and neuroactivation, providing insights into how ABA may exert neuroprotective effects through modulation of these pathways.

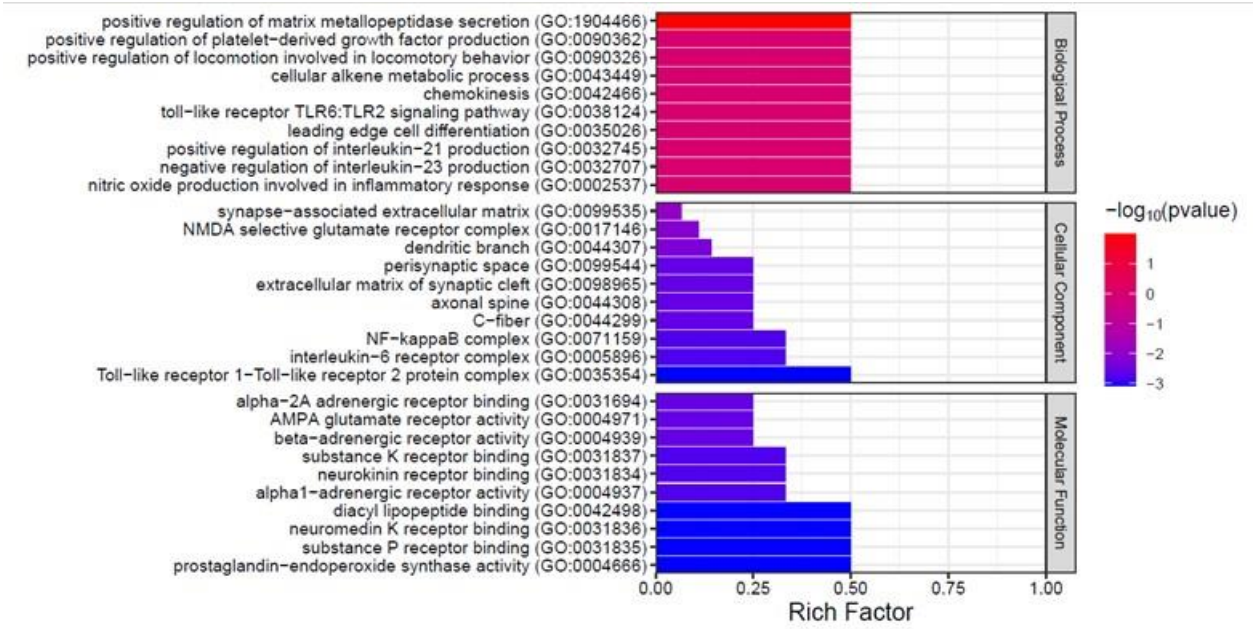

Figure 6. Histogram of top 10 enriched GO terms for SCI-related gene members. The Y-axis represents GO Terms and three GO biological processes (BP), cellular components (CC), and molecular functions (MF), which are shown in 3 boxes. The X-axis represents the Rich Factor of GO terms. All GO terms have a $p \leq 0.05$.

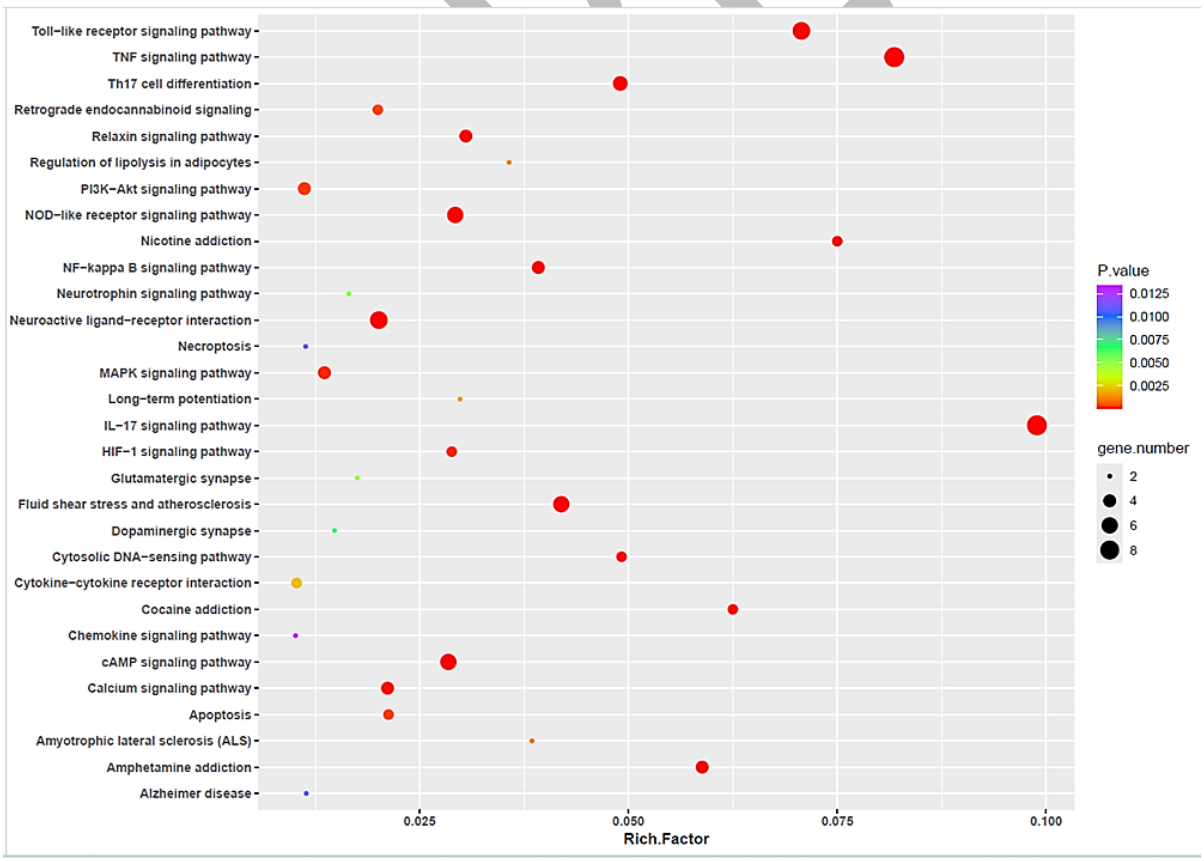

Figure 7. The top 30 terms in the KEGG pathway enrichment. The X-axis represents the Rich Factor. The number of genes enriched in the pathway is represented by the size of the dots, and the change in *p* value is displayed from red to purple

DISCUSSION

Although abscisic acid (ABA) is traditionally known as a plant hormone, recent discoveries have unveiled its potential roles in animal physiological functions. Nevertheless, there is still a lack of comprehensive understanding of ABA's activities in animal systems, particularly its function in modulating gene expression in response to injury. The present research study delved into the impact of SCI on gene expression in rats and the effect of ABA on these genes by combining two approaches: the experimental studies, including the evaluation of the expression of 18 genes potentially associated with SCI in rats, and in-silico analyses, including protein-protein interaction and gene ontology analysis. Intriguingly, the findings from the investigations are quite intriguing and suggest a multifaceted mechanism of action for ABA in mitigating the detrimental effects of SCI.

As our results showed, SCI caused a significant elevation in the proinflammatory cytokines and receptors, such as *IL-6, IL-1b, MCP, TLR2*, and *TLR4*. While ABA administration downregulated these genes in injured rats. These factors are produced by resident immune cells, glial cells, and neurons in response to inflammatory signals or tissue damage.[23] *TLR4* and *TLR2* are primarily expressed in microglia and macrophages in the central nervous system, and their activation can be triggered by damage-associated molecular patterns (DAMPs) released from injured cells. The activation of *TLR4* and *TLR2* leads to the production of pro-inflammatory cytokines (such as IL-1β and IL-6), chemokines, and reactive oxygen species, contributing to the inflammatory response in the spinal cord.[24,25] Also, it has been shown that cytokines and TLR agonists can increase the expression of chemokine ligands, such as *MCP* (or *CCL2*), which go on to attract immune cells to the site of inflammation.[26] We also observed that following spinal cord injury, *IL-6* and *IL-1β* levels increased. This may initially contribute to the inflammatory response and potentially influence neuronal function and survival. Based on previous studies, acute IL-6 and IL-1β production is important for initiating immune responses and tissue repair. However, their chronic or excessive signaling can lead to sustained inflammation and tissue damage,[27,28] exacerbating the SCI. Downregulation of these genes in ABA-treated rats suggests that ABA has anti-inflammatory properties, which could help reduce the secondary damage caused by post-SCI inflammation.

Based on our results, the expression of *NMDA* (or *GRIN1*), *AMPA* (or *GRIA1*), *NK1R* (or *Tacr1*), and substance P (or *TAC1*) increased in the rats with SCI, while treatment with ABA led to a considerable reduction in the expression of these receptors. GRIN1 is a critical subunit of N-methyl-D-aspartate (NMDA) receptors, which are ligand-gated ion channels. The overactivation of Grin1 receptors can lead to an excessive glutamate release, resulting in excitotoxicity.[29] In addition, it has been demonstrated that excessive activation of NMDA receptors directly triggers mitochondrial apoptosis in many organs.[30] Also, glutamate ionotropic receptor AMPA type subunit 1 (GRIA1) belongs to a family of AMPA receptors, which are the predominant excitatory neurotransmitter receptors in the mammalian brain and are activated in a variety of normal neurophysiological processes.[31] The overexpression and activation of AMPA1 receptors can contribute to excitotoxicity, similar to the NMDA receptors.[32] This can also cause an influx of calcium and Na+ ions into neurons, leading to the release of pro-inflammatory molecules and activation of immune cells, resulting in cell damage and death[33,34] in the spinal cord. Likewise, *NK-1R* is expressed in neurons, glial cells, and immune cells, and its activation by substance P contributes to the modulation of pain transmission and neuroinflammation.[35] Substance P is a neuropeptide that plays a key role in pain transmission and modulation in the nervous system.[36] Activation of NK-1R by substance P in the spinal cord can lead to the release of inflammatory mediators, such as cytokines and chemokines, and the sensitization of neurons involved in pain processing.[37,38] Given that these receptors are involved in excitatory neurotransmission, it seems that ABA's ability to reduce their expression might protect neurons from excitotoxic damage. Furthermore, decreased expression of *TAC1* indicates that ABA may also possess analgesic properties, alleviating pain related to SCI. To support these observations, behavioral tests conducted on rats with SCI demonstrated that ABA treatment resulted in a prolonged response delay to painful heat stimuli and improved the rats' mobility.[39]

On the other hand, we observed that ABA could increase adrenergic receptor levels (ADRA1A, ADRB1, and AGBRA2), which were downregulated in SCI rats. *ADRA1A* and *ADRB1* are expressed in different regions

and cell types, including neurons and glial cells. Activation of ADRA1A in the spinal cord can modulate sensory processing, pain transmission, motor, and autonomic functions. Following SCI, stimulation of adrenergic receptors can lead to the contraction of smooth muscle in blood vessels, resulting in vasoconstriction.[40] This can affect blood flow in the spinal cord and other tissues, influencing overall perfusion and oxygenation. These receptors' activation can also modulate neurotransmitter release in the spinal cord, affecting the transmission of pain signals and sensory information. Additionally, their signaling may influence motor neuron activity and contribute to motor control and coordination.[40,41] In addition, the GABA-A receptors (containing the GABRA2 subunit) are primarily responsible for mediating the inhibitory effects of GABA in the spinal cord, which help regulate neuronal excitability and maintain the balance between excitation and inhibition in the nervous system. GABA-A receptors play a role in modulating sensory processing, motor control, and pain perception.[42] Considering that these genes are involved in the adrenergic signaling pathway, which can modulate various physiological responses, including neuroprotection and anti-inflammatory effects, the increased levels of these receptors in ABA-treated rats suggest that ABA might enhance neuroprotective signaling pathways.

As our results suggested, ABA treatment could reverse the increased expression of the transcription factors and inflammatory mediators *FOS, JUN*, and *NF-κB*. Both FOS and Jun proteins are regarded as immediate early genes. The Fos protein is a nuclear transcription factor that forms dimers with other proteins, such as Jun proteins, a key component of the AP-1 (Activator Protein-1) transcription factor complex, to regulate gene expression in response to various stimuli.[43] These protein expressions can be induced in neurons of the spinal cord in response to noxious stimuli, such as pain or injury, and are involved in the processing and modulation of sensory information.[44] The Fos and Jun proteins can also cooperate with NF-κB, and they mutually increase each other's transcriptional activity.[45] Activation of NF-κB in glial cells, such as microglia and astrocytes, can lead to the production of pro-inflammatory cytokines, chemokines, and other inflammatory mediators in response to various stimuli, contributing to neuroinflammation in spinal cord-related conditions.[46] Dysregulation of NF-κB1 signaling in the spinal cord has been implicated in various neurological disorders, including spinal cord injury, neuroinflammation, and neurodegenerative diseases.[47,48] Downregulation of these genes in ABA-treated rats indicates that ABA may suppress pathways that lead to inflammation and cell death.

We observed that ABA administrations caused a reduction in matrix metalloproteinases and prostaglandin synthase. Matrix metalloproteinases, MMP-3 and MMP-9, are upregulated following injury and contribute to the breakdown of the blood-spinal cord barrier, leading to increased permeability and infiltration of immune cells into the spinal cord. This can further exacerbate inflammation and tissue damage.[49] Also, prostaglandin-endoperoxide synthase 2 (Ptgs2), or COX-2, is an enzyme that catalyzes the production of prostaglandins, which are lipid signaling molecules involved in inflammation and pain responses. As previously reported, COX-2 levels increase in cerebral stroke, traumatic brain injury, and the neurodegeneration process, leading to pathological changes, including blood-brain barrier breakdown and cerebral edema.[50] Additionally, COX-2 is highly expressed in inflammation and correlates with inflammatory factors such as IL-1β, IL-6, and TNF-α.[51] COX2 also plays a role in spinal cord injury-induced neuropathic pain, and its elevated expression has been reported in the lumbar region following thoracic spinal injury [52]. Based on the fact that these enzymes are involved in tissue remodeling and inflammation, their reduction indicates that ABA might help in preserving the structural integrity of the spinal cord and reducing inflammation.

The protein-protein interaction, gene ontology, and KEGG enrichment analysis highlight the central role of certain genes and pathways in the network of interactions related to SCI and the effects of ABA administration. The degree of a gene in the PPI context refers to the number of direct interactions it has with other proteins, indicating its importance in the network. Based on our findings, 10 key genes with high interaction degrees were *FOS, IL-1β, IL6, MMP9, TLR4, PTGS2, CCL2, TLR2, JUN*, and *TAC1*, respectively. The high degree of interaction for these genes underscores their central roles in the network of responses to SCI.

The downregulation of these key genes by ABA suggests a coordinated suppression of multiple inflammatory and stress-related pathways. This broad-spectrum modulation likely contributes to the overall

neuroprotective and anti-inflammatory effects observed with ABA treatment. Although further analysis is needed to fully understand how ABA can ameliorate SCI, the following mechanisms of action can be suggested: Anti-inflammatory effects: the downregulation of *IL-1β, IL-6, TLR2, TLR4, PTGS2*, and *CCL2* by ABA indicates a strong anti-inflammatory response, reducing the recruitment and activation of immune cells and the production of inflammatory mediators. Neuroprotection activity: the reduction in *FOS, JUN,* and *MMP9* expression suggests that ABA helps protect neurons from stress and damage, preserving tissue integrity. Finally, pain reduction effects: the decreased expression of *TAC1* points to potential analgesic effects, reducing pain associated with SCI.

In this study, SCI induced an alteration in the gene expression of inflammatory factors, neural signaling components, and adrenergic and gamma-aminobutyric acid receptors in rats. However, administration of ABA mitigated these effects. ABA downregulated pro-inflammatory genes and neural signaling components while upregulating adrenergic receptors and a gamma-aminobutyric acid receptor. Among the regulated genes, *FOS, IL-1β, IL-6, MMP9*, and *TLR4* play central roles in the network. Functional analyses revealed potential impacts on cellular responses, metabolic processes, and synapse-associated extracellular matrix components. Notably, these genes were enriched in inflammatory signaling pathways according to KEGG analysis.

STATEMENT OF ETHICS

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Kerman University of Medical Sciences (No. IR.KMU.REC. 1399.09.6).

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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DATA AVAILABILITY

Upon reasonable request (Maryam Rezaeezade Roukerd; email: rzrm88@gmail.com)

AI ASSISTANCE DISCLOSURE

Not applicable.

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