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Isobaric Tagging for Relative and Absolute Protein Quantification (iTRAQ)-Based Quantitative Proteomics Analysis of Differentially Expressed Proteins 1 Week After Spinal Cord Injury in a Rat Model

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Background: Spinal cord injury (SCI) is a devastating trauma of the central nervous system (CNS), with high levels of morbidity, disability, and mortality. One week after SCI may be a critical time for treatment. Changes in protein expression have crucial functions in nervous system diseases, although the effects of changes occurring 1 week after SCI on patient outcomes are unclear.

Material/Methods: Protein expression was examined in a rat contusive SCI model 1 week after SCI. Differentially expressed proteins (DEPs) were identified by isobaric tagging for relative and absolute protein quantification (iTRAQ)-coupled liquid chromatography tandem-mass spectrometry (LC-MS/MS) proteomics analysis. Gene Ontology (GO) analysis was performed to identify the biological processes, molecular functions, and cellular component terms of the identified DEPs, and the Kyoto Encyclopedia of Genes and Genomes (KEGG) was used to identify key enriched pathways. Protein-protein interaction (PPI) networks were analyzed to identify the top 10 high-degree core proteins.

Results: Of the 295 DEPs identified, 204 (69.15%) were upregulated and 91 (30.85%) were downregulated 1 week after injury. The main cellular components, molecular functions, biological processes, and pathways identified may be crucial mechanisms involved in SCI. The top 10 high-degree core proteins were complement component C3 (C3), alpha-2-HS-glycoprotein (Ahsg), T-kininogen 1 (Kng1), Serpinc1 protein (Serpinc1), apolipoprotein A-I (Apoa1), serum albumin (Alb), disulfide-isomerase protein (P4hb), transport protein Sec61 subunit alpha isoform 1 (Sec61a1), serotransferrin (Tf), and 60S ribosomal protein L15 (Rpl15).

Conclusions: The proteins identified in this study may provide potential targets for diagnosis and treatment 1 week after SCI.

MeSH Keywords: **Protein Interaction Maps • Proteomics • Spinal Cord Injuries**

Full-text PDF: <https://www.medscimonit.com/abstract/index/idArt/924266>

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Background

Spinal cord injury (SCI) is a serious traumatic injury with high levels of morbidity, disability, and mortality, as well as many complications [1]. SCI is usually caused by high-energy impact. In developed countries such as the United States, most SCIs are caused by motor vehicle accidents [2], whereas in developing countries such as China, SCIs are more frequently caused by falls [3]. Due to high-energy damage, primary SCIs include damage to the blood spinal cord barrier (BSCB), ischemic and related cell injury, cell necrosis, apoptosis, and alarmin release. This may be followed by a series of secondary injuries, including inflammatory responses, extravasation of infiltrating leukocytes, and proliferation of and morphological changes to the glia, which ultimately leads to the development of a chronically axon-inhibitory structure called the glial scar [4]. Secondary SCI injury can be divided into several pathophysiologic stages, including acute stage (<48 h), subacute stage (48 h to 14 days), moderate stage (14 days to 6 months), and chronic stage (>6 months) [5]. Ischemia and excitotoxicity during the subacute phase lead to a loss of intracellular and extracellular ion homeostasis. The imbalance of calcium regulation in neurons and glial cells is the main mediator of cell death. In addition, increased expression of hypertrophic astrocytes and glial fibrillary acidic protein (GFAP) also participate in formation of the glial scar, a physical and chemical barrier that limits functional recovery [6]. The subacute period is a potential crucial treatment phase [7]. Previous studies published by our research team identified key proteins and predicted critical pathways 2 weeks after SCI [8]. Although several proposed treatment methods, including surgery, drug treatment, and cell therapy, have been associated with positive effects, few patients experience complete neurological functional recovery, and there is currently no effective or universally accepted treatment for SCI [9]. Determination of protein expression 1 week after SCI may establish a spectrum of differentially expressed proteins (DEP), enabling better understanding of the molecular mechanisms and potential therapeutic targets of SCI.

Proteomics is a science of studying the composition and changes of protein at the cell, tissue, organ, or organism level. Proteomics is of great significance in the early diagnosis, subtyping, monitoring of process and curative effect, prognosis, and molecular mechanism of disease. Recent advances in proteomics technology have enabled the use of animal disease models or biological samples from clinical observational studies to study differential proteomics in SCI [10]. Although traditional molecular biology techniques such as PCR and western blotting have been used to analyze the pathological mechanisms of SCI, it remains difficult to accurately identify the biomolecules that play important roles in dynamic changes of the microenvironment after SCI and in the evolution of the many biological processes and key signal pathways that

are activated through the progression of injury. Isobaric tagging for relative and absolute protein quantification (iTRAQ) is a method with high throughput, stability, and sensitivity to sample properties, and can be used to evaluate the pathological mechanisms associated with SCI [11].

The present study utilized iTRAQ-coupled liquid chromatography tandem-mass spectrometry (LC-MS/MS) proteomics analysis to compare protein expression levels 1 week after induction of SCI in a rat model with protein expression in control rats. These findings may establish a profile of differentially expressed proteins 1 week after SCI. In addition, these finding may help clarify the potential molecular pathological mechanisms underlying SCI, aid in further exploration of therapeutic targets, and provide a basis for clinical treatment.

Material and Methods

Experimental animals

Eighteen adult female Wistar rats weighing 190-220 g were obtained from the Military Medical Sciences Academy (Tianjin, China). All animal procedures in this study were approved by the Animal Experimental Ethics Inspection Committee of Tianjin Medical University and conformed to the approved guidelines on the protection and use of experimental animals. All Wistar rats were adapted to preoperative conditions for 7 days in a controlled environment ($23\pm 1^\circ\text{C}$) under a 12-h dark/light cycle and were allowed water and food ad libitum.

Model of spinal cord contusion injury

The 18 rats were randomly divided into 2 equal groups. Rats in the control group did not undergo surgery but only underwent laminectomy at the level of the tenth thoracic vertebra (T10) prior to tissue extraction. Rats in the injured group were administered a contusive SCI at T10 using the New York University (NYU) impactor model II (10 g \times 25 mm) as well as undergoing laminectomy at the T10 level. After SCI, the hindlimbs of rats in the injured group exhibited involuntary twitches and tail wagging, conforming to the described standard for this model [12]. The bladder of each rat in the injured group was artificially emptied twice per day. All rats were sacrificed 1 week later, and a 10-mm length of spinal cord tissue, including the center of the SCI, was subsequently removed.

Protein extraction

Protein extraction, iTRAQ labeling, and LC-MS/MS were performed as described [8]. Spinal cord samples from 3 rats in each group, weighing approximately 200 mg, were stored at -80°C . Each sample was incubated in 300 μL lysis buffer (8 M

urea, 50 mM Tris, pH 8.0, 10 mM DTT, 2 mM EDTA, 1% NP40, 1% NaDOC, and 1% proteinase inhibitors), homogenized at 800 rpm, and subjected to ultrasound on ice to dissolve the sample. The samples were subsequently centrifuged at 20 000 g for 10 min at 4°C. The protein concentration of the interlayer solution was determined using a 2D Quant kit (GE Healthcare) after repeated centrifugation. A 30- μ g aliquot of protein of each sample was subjected to 12% SDS-PAGE.

iTRAQ labeling

A 500- μ g aliquot of each protein sample was withdrawn, and differences in volume were made up by the addition of lysis buffer. The samples were incubated at 30°C for 40 min with 5 mM DTT (Solarbio, Biotechnology) to reduce the disulfide bonds. After cooling to room temperature, the samples were incubated in the dark for 40 min with 40 mM iodoacetamide (Sigma, BioUltra) to alkylate the free sulfhydryl groups. An 8-fold volume of acetone pre-cooled to -20°C was added to each sample to precipitate the proteins, followed by incubation overnight at -20°C. The samples were centrifuged at 20 000 g for 10 min at 4°C, the liquid was discarded, and the pellet was rinsed with 80% acetone pre-cooled to -20°C and incubated at -20 °C for 1 h. The pellets were rinsed once with pre-cooled acetone, the supernatant was discarded, the proteins were precipitated at room temperature for 10 min, and the residual acetone was volatilized. Each protein pellet was resuspended in 300 μ l triethylammonium bicarbonate (TEAB, 0.1 M, Sigma, BioUltra) and ultrasonically dissolved at 4°C. Trypsin (5 μ g) was added to each solution, and the solutions were incubated overnight at 37°C. Enzymolysis was terminated by the addition of 1% trifluoroacetic acid (TFA; Sigma, HPLC), followed by desalting on a C18 SPE (Phenomenex) column. The eluted peptides were dried using a matched vacuum concentrator.

In addition, the peptide fragments were dissolved in an appropriate amount of 0.5 M TEAB, with 50 μ g of each sample labeled with TMT reagent (Pierce) at room temperature for 1 h.

LC-MS/MS

After drying, the labeled peptide fragments were redissolved in liquid phase solution A; 25 μ g of each of the 2 samples were added to each replication group, followed by centrifugation at 20 000 g for 10 min. Each supernatant was loaded onto a C18 column and antigradient separation was performed by high-performance liquid chromatography (HPLC; Shimadzu, LC20AD) under alkaline conditions. Approximately 20 of the above peptide fragments were combined into 7 components after elution according to the peak-appearing extended protein, and they were dried using a vacuum concentrator. Each of the 7 predissociated fractions was dissolved in liquid phase

solution A, and the supernatants were centrifuged at 20 000 g for 10 min. Samples were analyzed on an LC (Dionex, Ultimate RSLCnano 3000)-MS (Thermo Scientific, Q Exariented plus) system, with each 1- μ g fragment requiring 1 h. The LC-MS system was also used to analyze 7 predissociated components in each group. Qualitative calculations were performed using Mascot software.

Protein identification and quantitation

The raw data were processed and converted using the Mascot search engine (Matrix Science, v.2.3.02) and mascot generic file with Proteome Discoverer (Thermo Scientific, v1.4.1.14). A total of 29 982 sequences of tandem-mass spectra were retrieved using a rat reference proteome. The mass error of precursor ions was set at 10 PPM and fragment ions at 0.02 Da. Enzyme specificity was determined by trypsin and 2 missed cleavages were allowed. The iTRAQ 8-plex tag on Lys, the N-terminal peptide, and the carbamidomethylation tag on Cys were specified as fixed modifications, whereas iTRAQ 8-plex tag on Tyr and oxidation on Met were specified as variable modification. False discovery rates (FDR) were estimated from a Decoy (reverse) database. The algorithm percolator and PSMs (Peptide-Spectrum Match) were used to revalue the calculated results using a *P* value <0.05 and an *e*-value <0.05. Thus, to be included, each protein must contain at least 2 identical unique peptides. The type of protein ratio was weighted, and all were normalized to the median.

GO analysis

Investigation of the roles of the identified DEPs were determined using Gene Ontology (GO) analysis. The biological processes (BP), cellular components (CC), and molecular functions (MF) were analyzed. GO analyses were performed by R software with the cluster Profiler package, and Fisher's exact tests were performed to analyze distinct terms. A *P* value <0.05 was defined as statistically significant.

KEGG analysis

The roles of the identified DEPs were explored by pathway enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG). The pathways corresponding to the process were analyzed by KEGG analysis using R software with the cluster Profiler package to conduct KEGG analyses, and distinct pathways were evaluated by Fisher's exact test. A *P* value <0.05 indicated statistical significance.

PPI network construction

The Search Tool for the Retrieval of Interacting Genes (STRING) database has been shown to indicate protein-protein

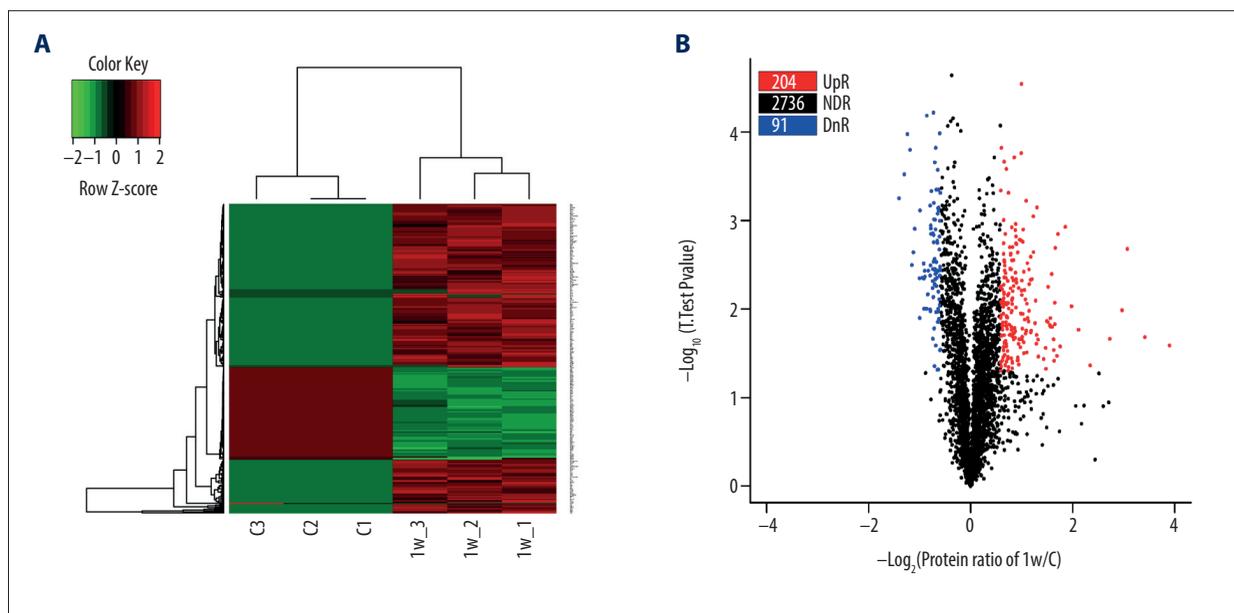


Figure 1. Heatmap and Volcano plot of the differentially expressed proteins (DEPs). **(A)** Heatmap of DEPs, in which red represents upregulated DEPs and green represents downregulated DEPs. **(B)** Volcano plot of DEPs. The X axis is the fold change (\log_2), and the Y axis represents the P value ($-\log_{10}$). Red points (fold change >1.5) indicate upregulated proteins, and blue points (fold change <-1.5) indicate downregulated proteins.

interactions (PPIs) [13]. In the present study, the PPIs of the DEPs were analyzed by the STRING database using the cut-off value of a confidence score ≥ 0.7 . PPI network results were analyzed with Cytoscape software [14].

Western blotting (WB)

Western blotting was performed as described [8], with slight modifications. Spinal cord tissues were lysed on mice using the RIPA lysis buffer, supplemented with protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The samples were subjected to ultrasound treatment to reduce their viscosity, and their protein concentrations were determined by bicinchoninic acid assays (Pierce, Rockford, IL, USA). Equal aliquots of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were incubated overnight at 4°C with primary antibodies against GAPDH (1: 4000, Abcam), T-kininogen 1 (Kng1) (1: 1000, Proteintech), sodium- and chloride-dependent GABA transporter 3 (Gat3) (1: 1000, Abcam), and sodium/potassium-transporting ATPase subunit beta (Atp1b2) (1: 1000, Proteintech). After washing, the membranes were incubated with secondary anti-rabbit or anti-mouse antibody (1: 10 000, Abcam) labeled with horseradish peroxidase, followed by incubation with enhanced chemiluminescence substrates (Pierce) and visualization of the immunoreactive bands. Results were quantified using Image J software, and band intensities were normalized to those of GAPDH.

Statistical analysis

Statistical analyses were performed using SPSS17.0 and R software. Fold-changes >1.5 and P values <0.05 were considered statistically significant.

Results

iTRAQ data

iTRAQ analysis of spinal cord samples 1 week after SCI identified 295 DEPs; of these proteins, 204 (69.15%) were upregulated and 91 (30.85%) were downregulated in SCI compared with control samples. A heatmap is shown in Figure 1A and a volcano plot in Figure 1B. The top 10 downregulated and upregulated DEPs are listed in detail in Table 1.

GO and KEGG enrichment analysis

Target proteins were annotated by GO and KEGG enrichment. GO term annotation results indicated that the DEPs were enriched in enzyme inhibitor activity, response to stress, defense response, regeneration, response to steroid hormone, neurotransmitter transport, and inflammatory response (Figure 2A). Of these, enzyme inhibitor activity, response to stress, defense response, regeneration, response to steroid hormone, and inflammatory response were the upregulated GO terms, whereas neurotransmitter transporter activity was the downregulated

Table 1. The top 10 upregulated and downregulated proteins.

Protein	Gene name	P value	1wvsC ratio	Protein	Gene name	P value	1wvsC ratio
Upregulated proteins				Downregulated proteins			
P00697	<i>Lyz1</i>	0.0259	14.95	P31647	<i>Gat3</i>	0.000561	0.38
P01048	<i>Kng1</i>	0.0207	10.69	P06686	<i>Atp1a2</i>	0.000301	0.41
P02764	<i>Orm1</i>	0.00209	8.42	P13638	<i>Atp1b2</i>	0.000106	0.42
P02650	<i>ApoE</i>	0.0103	7.83	G3V8Q2	<i>Ina</i>	0.00016	0.44
P70623	<i>Fabp4</i>	0.0217	6.64	Q63754	<i>Sncb</i>	0.00318	0.45
P08699	<i>Lgals3</i>	0.0433	5.08	P23978	<i>Slc6a1</i>	0.00229	0.46
P08932	<i>Kng2</i>	0.0171	4.34	P24329	<i>Tst</i>	0.00124	0.47
P31000	<i>Vim</i>	0.0093	3.94	F1LNK0	<i>Map2</i>	0.00077	0.5
Q6IN22	<i>Ctsb</i>	0.00118	3.62	P29101	<i>Syt2</i>	0.00449	0.5
Q6AXR4	<i>Hexb</i>	0.0265	3.38	Q9J166	<i>Slc4a4</i>	0.0126	0.5

GO term (Figure 2B, 2C). KEGG enrichment analysis showed that complement and coagulation cascades, *Staphylococcus aureus* infection, lysosome, carbohydrate digestion and absorption, mineral absorption, systemic lupus erythematosus, ribosome, proximal tubule bicarbonate reclamation, antigen processing and presentation, phagosome, glycosaminoglycan degradation, pertussis, thyroid hormone synthesis, and leishmaniasis were enriched pathways (Figure 3A). Of these, complement and coagulation cascades, *Staphylococcus aureus* infection, lysosome, systemic lupus erythematosus, ribosome, antigen processing and presentation, phagosome, glycosaminoglycan degradation, pertussis, and leishmaniasis were upregulated, whereas carbohydrate digestion and absorption, mineral absorption, proximal tubule bicarbonate reclamation, and thyroid hormone were downregulated pathways (Figure 3B, 3C).

PPI network analysis

PPI network analysis of the DEPs showed 228 nodes and 899 connections (Figure 4). The top 10 core DEPs were complement component C3 (C3), alpha-2-HS-glycoprotein (Ahsg), T-kininogen 1 (Kng1), Serpinc1 protein (Serpinc1), apolipoprotein A-I (Apoa1), serum albumin (Alb), disulfide-isomerase protein (P4hb), transport protein Sec61 subunit alpha isoform 1 (Sec61a1), serotransferrin (Tf), and 60S ribosomal protein L15 (Rpl15). C3 displayed the highest node degree among these 36 genes. All of these DEPs were upregulated. The core genes are listed in detail in Table 2.

Western blotting

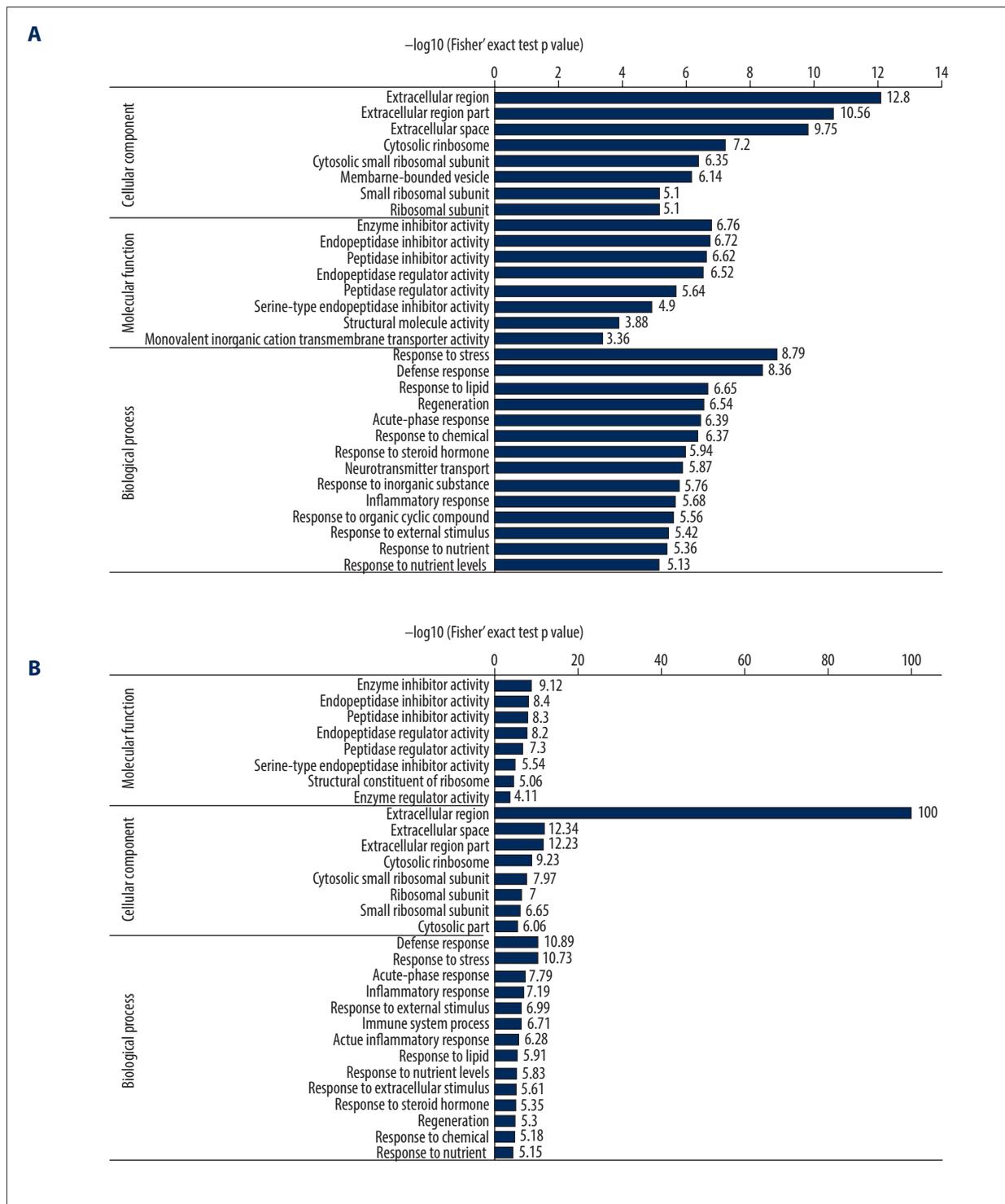
Western blotting experiments were performed to validate the changes in expression of 3 of the top 10 downregulated and upregulated DEPs. The expression of Kng1 increased significantly, whereas the expression of Gat3 and Atp1b2 decreased

significantly, in the injured compared with the control group (Figure 5, $P < 0.05$).

Discussion

SCI is a serious CNS trauma that leads to long-term neurological injury and other complications in patients, along with a heavy socioeconomic burden [15,16]. The pathophysiology of SCI is very complex, as it affects the nervous, immune, respiratory, and circulatory systems, as well as other systemic functions [17,18]. One week after SCI may be the optimal treatment window, and pathological changes at this stage may be crucial in the secondary injury of SCI [6]. Nevertheless, few studies have explored the expression of proteins 1 week after SCI. The present study found significantly different protein expression levels in spinal cord tissue after SCI. GO and KEGG analyses were performed to identify the functions of these DEPs, and the STRING database was used to investigate a series of core proteins that may be critical 1 week after SCI.

GO enrichment analyses of the DEPs revealed that enzyme inhibitor activity, response to stress, defense response, regeneration, response to steroid hormones, neurotransmitter transport, and inflammatory response were significantly enriched in spinal cords of rats with SCI. Of these, enzyme inhibitor activity, response to stress, defense response, regeneration, response to steroid hormones, and inflammatory response were upregulated, whereas neurotransmitter transporter activity was downregulated. Sildenafil citrate, a phosphodiesterase inhibitor, was found to exert a neuroprotective effect on the spinal cord after ischemia/reperfusion injury by attenuating inflammatory mediators [19]. Our team's previous study showed that a synthetic serine protease inhibitor, nafamostat mesylate, could attenuate inflammation and apoptosis and promote locomotor



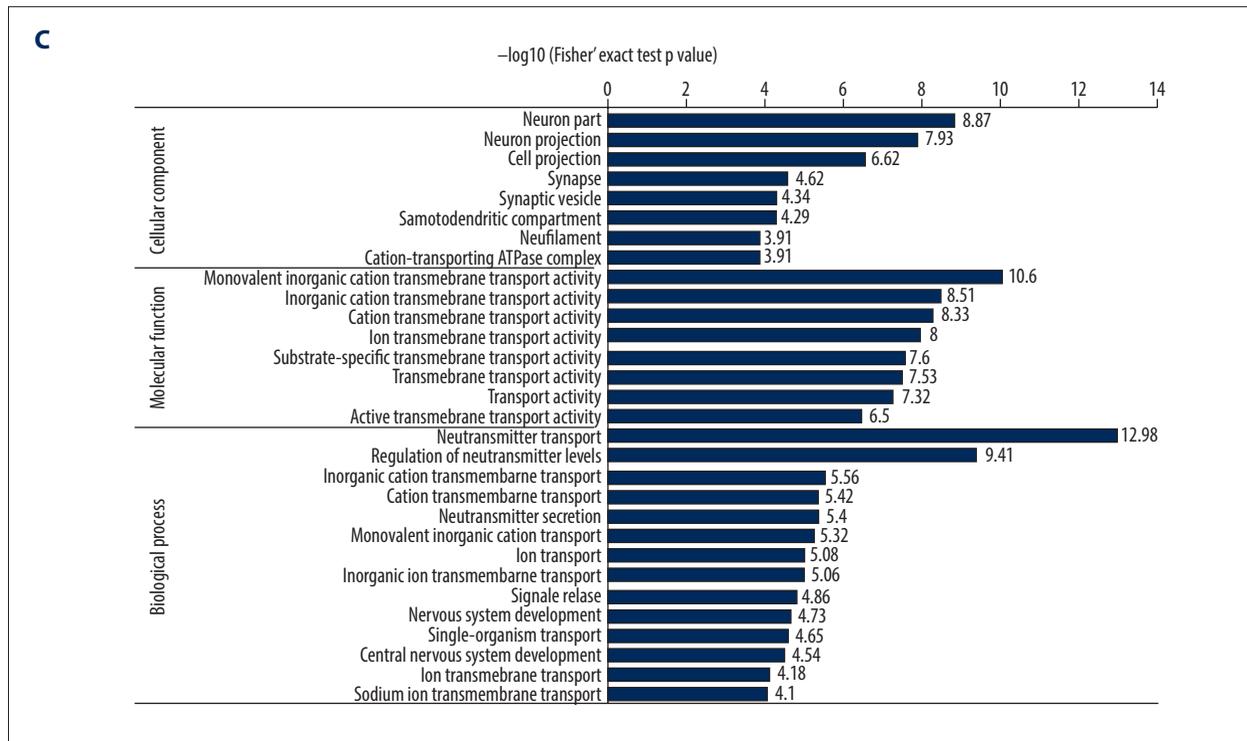
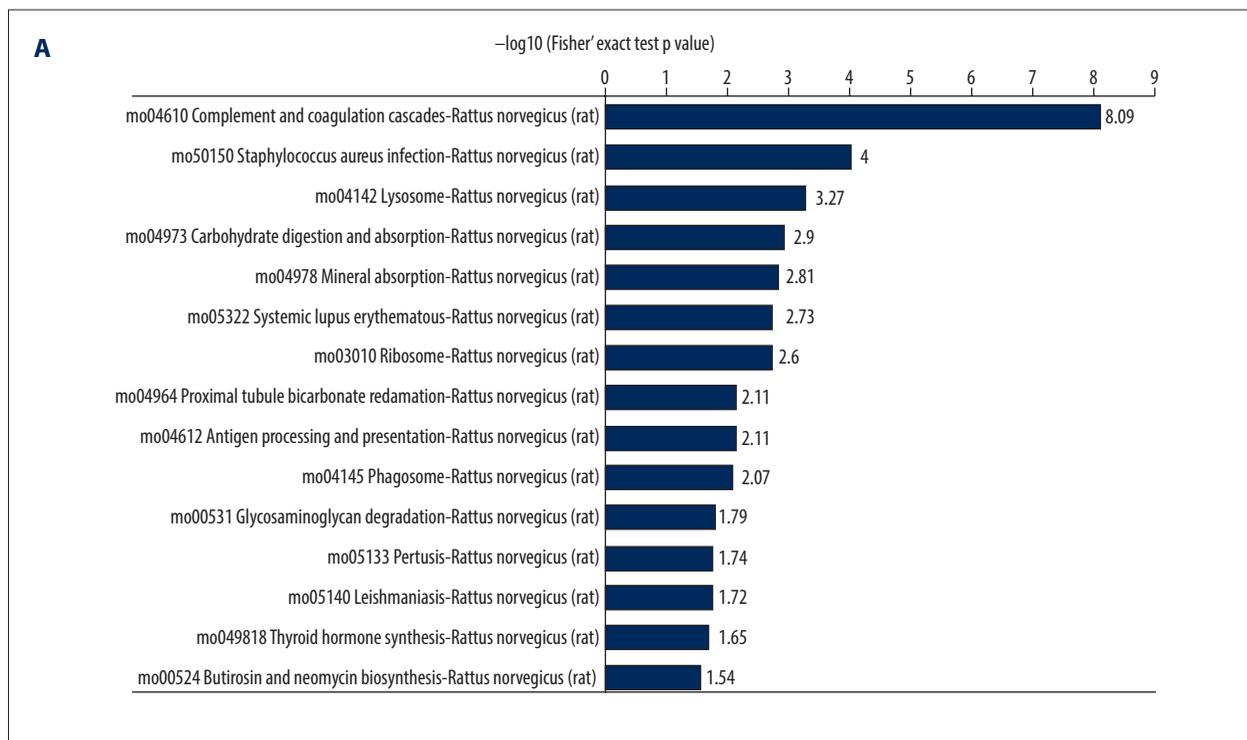


Figure 2. Gene Ontology (GO) term enrichment of differentially expressed proteins (DEPs). **(A)** All enriched GO terms of the DEPs in the 2 groups of rats. DEPs were categorized according to biological processes, cellular components and molecular functions. **(B, C)** Enriched terms upregulated **(B)** and downregulated **(C)** in the SCI compared with the control group.



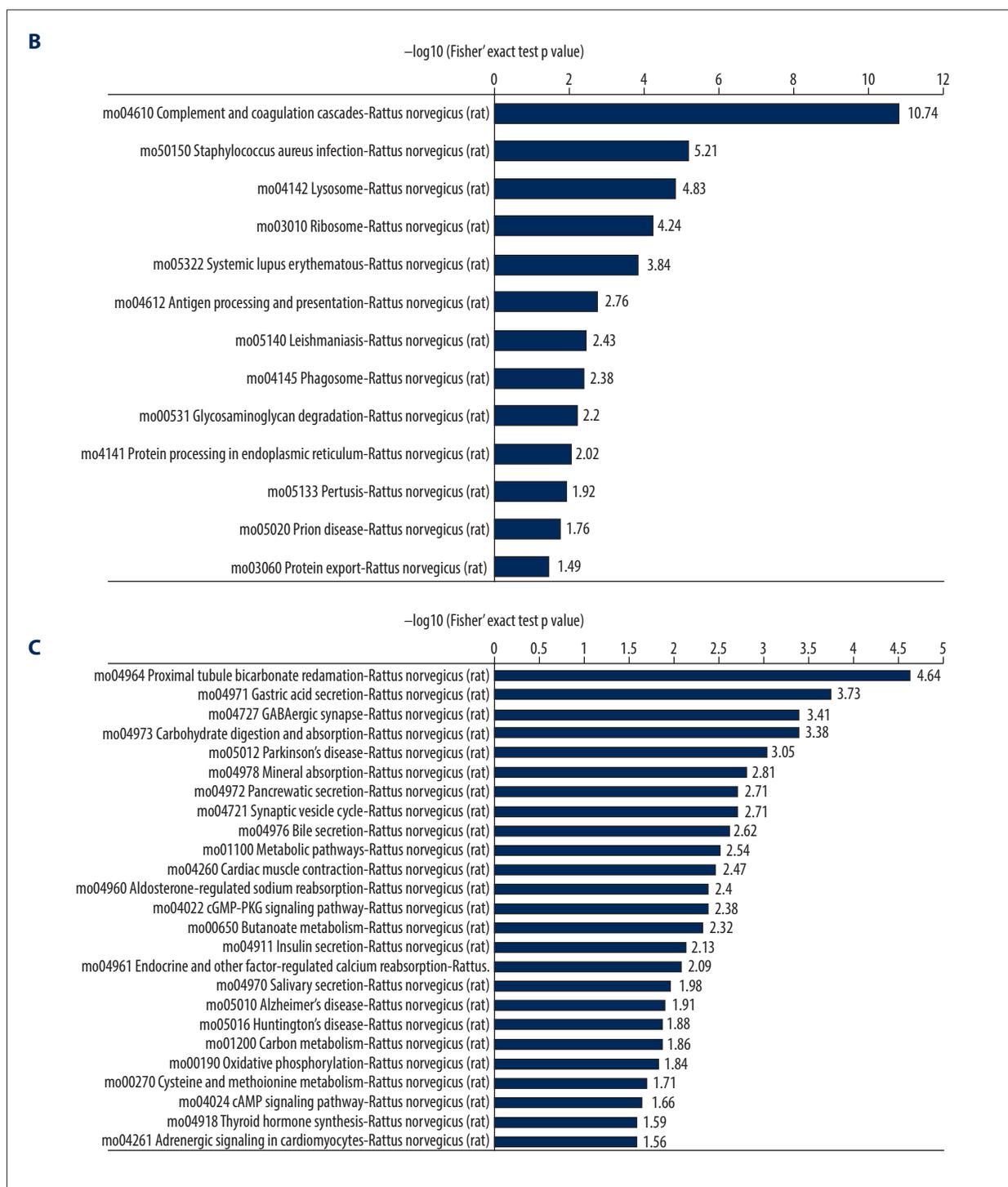


Figure 3. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment of the differentially expressed proteins (DEPs). (A) All enriched KEGG pathways of the DEPs in the 2 groups of rats. (B, C) Enriched pathways of DEPs upregulated (B) and downregulated (C) in the SCI compared with the control group.

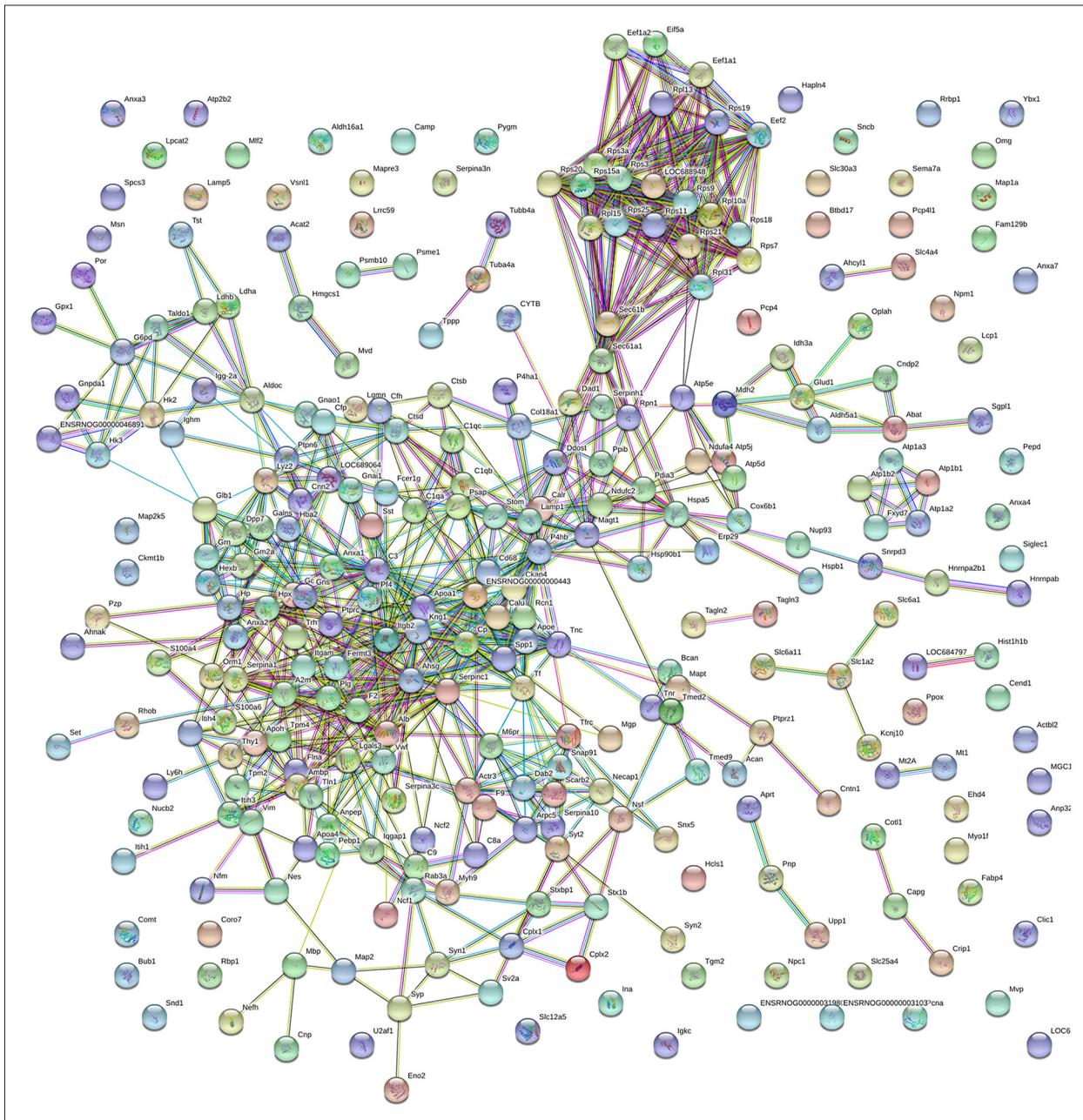


Figure 4. Protein–protein interaction (PPI) analysis of differentially expressed proteins (DEPs). Nodes indicate proteins, whereas lines indicate interactions between proteins.

Table 2. The core genes and their corresponding degrees.

Gene	Degree	Gene	Degree	Gene	Degree	Gene	Degree
<i>C3</i>	36	<i>Alb</i>	27	<i>Ckap4</i>	22	<i>Rps9</i>	20
<i>Ahsg</i>	35	<i>P4hb</i>	25	<i>Rps3a</i>	21	<i>Rpl31</i>	20
<i>Kng1</i>	31	<i>Sec61a1</i>	23	<i>Rps3</i>	21	<i>LOC688948</i>	20
<i>Serpinc1</i>	28	<i>Tf</i>	22	<i>Rps21</i>	21	<i>Rps18</i>	20
<i>Apoa1</i>	27	<i>Rpl15</i>	22	<i>Rps15a</i>	20	<i>Cp</i>	20

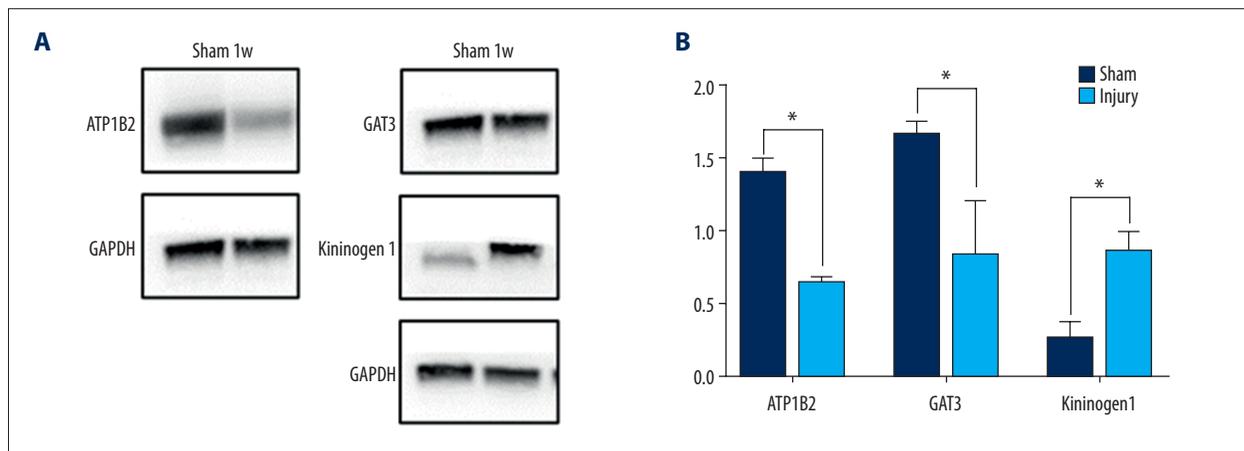


Figure 5. Western blotting of differentially expressed proteins (DEPs). (A) Expression of T-kininogen 1 (Kng1), sodium- and chloride-dependent GABA transporter 3 (Gat3) and sodium/potassium-transporting ATPase subunit beta (Atp1b2) in the SCI and control groups of rats. GAPDH levels were loading control. (B) Quantification of expression of DEPs. * $P < 0.05$.

recovery after SCI [20]. Other biological events play an early protective role, such as the defense response caused by the response to stress. Nevertheless, stress pathway modulation has been reported to be detrimental or ineffective for functional recovery after SCI [21]. Methylprednisolone is frequently used to treat SCI, but it has deleterious long-term adverse effects [22]. However, high-dose methylprednisolone administered within 8 h of SCI has been reported to be effective [23]. A recent study showed that gonadal hormones can reduce the degeneration of motor neurons and muscle morphology after SCI [24], supporting the role of steroid hormones as neurotherapeutics for treatment of injuries to the nervous system. The recovery of neurological function after injury may be affected by changes in neurotransmitter transport in the spinal cord. Although glutamic acid is one of the most important excitatory amino acids in the CNS, playing crucial roles in neurogenesis and the induction of synaptic plasticity, glutamic acid can mediate neurotoxicity under pathological conditions, including trauma, degeneration and nervous system ischemia, by binding to its receptor and causing neurological dysfunction [25]. Immune system activity is significantly increased during the subacute stage after SCI, promoting a more complex inflammatory response. Cellular inflammation after SCI has complex, time-dependent functions. Macrophages/microglial cells peak 7 days after injury, further aggravating SCI [26]. However, infiltrating macrophages were shown to shift from proinflammatory to anti-inflammatory states over time [27,28]. In addition, blocking chemoattractant C5a-mediated inflammation 14 days after SCI reduced motor recovery and myelination of the damaged spinal cord, indicating that the late inflammatory response is associated with repair function [29].

KEGG analysis showed that pathways enriched 1 week after SCI included complement and coagulation cascades, *S. aureus* infection, lysosome, carbohydrate digestion and absorption,

mineral absorption, systemic lupus erythematosus, ribosome, proximal tubule bicarbonate reclamation, antigen processing and presentation, phagosome, glycosaminoglycan degradation, pertussis, leishmaniasis, and thyroid hormone synthesis. Of these, the complement and coagulation cascades, *S. aureus* infection, lysosome, systemic lupus erythematosus, ribosome, antigen processing and presentation, phagosome, glycosaminoglycan degradation, pertussis, and leishmaniasis pathways were upregulated, whereas the carbohydrate digestion and absorption, mineral absorption, proximal tubule bicarbonate reclamation, and thyroid hormone pathways were downregulated. Complement and coagulation cascades are a group of proteins with enzymatic activity in human and vertebrate serum and tissue fluid. The complement system extensively participates in the body's defensive responses and immune regulation. This system can also mediate immunopathological damage, such as inflammation, and is an important biological effect system and effect amplification system *in vivo*. Primary injury to the CNS can activate the complement system, whereas complement-mediated inflammatory responses are associated with secondary injury. An excessive inflammatory response can lead to tissue ischemia, hypoxia, and the release of neurotoxic mediators, resulting in neuronal cell death and ultimately leading to and aggravating secondary injury. The complement system was shown to be activated in a rat impact SCI model, with activated molecular fragments expressed in neurons and astrocytes for more than 6 weeks [30]. In addition, inhibition of the classical and bypass pathways of complement activation reduced areas of tissue damage and complement sediment and leukocyte infiltration in the injured tissue, as well as reducing damage to the animal's motor function [31]. Administration of C5aR antagonists 14 days after SCI in a rat spinal cord contusion model was found to increase neuron demyelination and macrophage infiltration and to aggravate microglial activation, rat motor

function, and pathological damage to tissue [29]. Taken together, the results of these studies indicate that the complement system plays a dual role in SCI, in that complement activation exacerbates secondary injury while also promoting injury repair at a certain stage of SCI. We previously reported showed that lysosome and phagosome signaling pathways were up-regulated 14 days after SCI [8], suggesting that the lysosome and phagosome pathways may be closely related to the regulation of autophagy. The present study also found that these 2 pathways were upregulated, indicating that autophagy may play vital roles 1 week after SCI.

Analysis of the PPI network showed that C3, Ahsg, Kng1, Serpinc1, Apoa1, Alb, P4hb, Sec61a1, Tf, and Rpl15 were the top 10 core proteins altered 1 week after SCI and may be important in the pathological processes of SCI secondary injury. Inflammation is an important pathological reaction in secondary injury of SCI. It can activate the complement system, thus further enhancing inflammation and aggravating secondary injury after SCI [32]. C3 is an essential component of the complement cascade. Sensory axon regeneration after dorsal hemisection with peripheral conditioning lesions was shown to be significantly greater in the spinal cord of C3^{-/-} mice than of wild-type C3^{+/+} mice [33]. C3 deficiency can reduce inflammation by inhibiting TNF- α expression and AST activation, thus attenuating secondary injury, improving neural regeneration, and promoting functional recovery after SCI [34]. Ahsg may be a risk factor for neurogenic heterotopic ossification (NHO) in patients with SCI [35]. Apolipoprotein plays an important physiological role in lipoprotein metabolism. Apoa1 is the main structural protein of high-density lipoprotein (HDL) and is abundant in cerebrospinal fluid [36]. Apoa1 may also improve tissue injury accompanied by uncontrolled inflammation and promote axonal repair by controlling inflammatory activity after CNS injury [36]. Indeed, Apoa1 was reported to promote neuronal healing after injury by activating the ERK pathway and actin polymerization [37]. Albumin has been shown to significantly improve motor and sensory recovery in rats, and albumin oleic acid therapy not only can help restore motor function but can also relieve symptoms after SCI, such as pain [38–41]. In addition, a recent study showed that serum albumin is an independent indicator of long-term neurological

prognosis in SCI, suggesting that serum albumin may be prognostic of injury and stratification in clinical trials [42]. Iron (Fe), an essential element in all types of cells and organisms, is carried by Tf in interstitial fluids and plasma. A large amount of iron is released after SCI, resulting in increased formation of free radicals, which may aggravate tissue injury. As a carrier of iron, Tf may play a crucial role in the process [43].

Taken together with our previous findings, the present study further determined the key proteins and signal pathways that are involved 1 week after SCI, providing a more credible basis for understanding these pathological mechanisms. Although iTRAQ quantification is currently the most widely used technique in quantitative proteomics, it still has biological limitations. For example, the iTRAQ reagent can label almost all proteins and is easily contaminated by impurities in the sample and buffer. In addition, the sample currently studied was relatively small. Also, the data obtained from this rat model of SCI cannot be applied clinically, suggesting that similar studies be performed in non-human primates. Despite these limitations, the results presented here will enhance understanding of the molecular pathology of SCI after 1 week. Future studies should assess protein expression during other stages of SCI. Genomics, transcriptomics, and proteomics will also be combined to further explore and verify the pathways and molecular mechanisms 1 week after SCI.

Conclusions

In this study, iTRAQ-coupled LC-MS/MS proteomics analysis identified 295 DEPs in a rat model 1 week after SCI. A comprehensive bioinformatics analysis identified the key pathways that may be crucial mechanisms for the pathogenesis of SCI. Analysis indicated that C3, Ahsg, Kng1, Serpinc1, Apoa1, Alb, P4hb, Sec61a1, Tf, and Rpl15 may contribute to the pathogenesis 1 week after SCI.

Conflict of interests

None.

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