Hyperbaric Oxygen Therapy-Induced Molecular and Pathway Changes in a Rat Model of Spinal Cord Injury: A Proteomic Analysis

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Abstract
Hyperbaric Oxygen Therapy (HBOT) has definitive therapeutic effects on spinal cord injury (SCI), but its mechanism of action is still unclear. Here, we’ve conducted a systemic proteomic analysis to identify differentially expressed proteins (DEPs) between SCI rats and HBOT + SCI rats. The function clustering analysis showed that the top enriched pathways of DEPs include oxygen transport activity, oxygen binding, and regulation of T cell proliferation. The results of functional and signal pathway analyses indicated that metabolic pathways, thermogenesis, LXR/RXR activation, acute phase response signaling, and the intrinsic prothrombin pathway in the SCI + HBOT group was higher than SCI group.

Keywords
proteomic, hyperbaric oxygen therapy, spinal cord injury, isobaric tags for relative and absolute quantification

Introduction
Spinal cord injury (SCI) is a severe disease that causes neural dysfunction and disability.¹,² The impacts of SCI mainly include two aspects: (1) physical damage caused by hyperflexion or dislocation of the spine³ and (2) physiological disorders caused by spinal cord infection or tumors.⁴ Clinically, surgical treatment can relieve primary injuries such as spinal cord compression, but it cannot reverse the pathological changes that have already taken place.⁵,⁶

Hyperbaric oxygen therapy (HBOT) has been widely used to improve the outcomes of SCI by increasing blood oxygen concentration,⁷,⁸ especially in SCI patients. It has been reported that HBOT can protect the spinal cord cells, improve the hypoxic condition of tissues, and promote the recovery of spinal cord functions.⁹,¹⁰ Although HBOT possesses a definite therapeutic effect, its mechanism has not been fully elucidated, leading us to probe the molecular mechanisms which are involved in SCI.¹¹ Our previous studies showed that HBOT protects against SCI in rats by regulating the expression of inflammation-related proteins and inhibiting endoplasmic reticulum stress.¹²,¹³ However, the underlying changes at the proteomic level under SCI and HBOT conditions have not been studied.

Here, we delve further into determining the underlying changes of SCI before and after HBOT at the proteomic level. In this study, isobaric tags for relative and absolute quantification (iTRAQ) was used to conduct the proteomic analysis in SCI rat model before and after HBOT aiming to further explore the mechanism of HBOT on SCI.

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Methods

Animal Experiments

Ethical Approval. The experiment protocol was approved by the Committee of the Ethics of Animal Experiments of Capital Medical University (Protocol ID: 2010-D-013). All surgeries were performed under pentobarbital sodium anesthesia with additional efforts made to ensure animal welfare.

Animal Care

Healthy adult male Sprague–Dawley rats (250-300g) were purchased from Beijing Vital River Laboratory Animal Technology Company (Beijing, China) and housed in individual cages in a temperature-controlled setting with 12 hr light and dark cycles and non-restricted access to food and distilled water.

Rat Model of Acute SCI

A baseline behavioral assessment was conducted before the surgery. Acute SCI surgeries were performed using the modified Allen’s test, as previously described. During the surgery, the rats were anesthetized with intraperitoneal injection of pentobarbital sodium (90 mg/kg) following 12 hours of preoperative fasting of food and water. A dorsal laminectomy was performed at the center of the T9 spinous process to expose the spinal cord under sterile conditions. Subsequently, each rat sustained a contusive SCI from a Spinal Cord Impactor (W.M. Keck Center for Collaborative Neuroscience, Rutgers, NJ, USA), with a 10 g impact rod vertically dropped from a height of 5 cm, which then impinged on the spinal cord in a circular zone with a 2 mm diameter. The dwell time for each injury was 10 s, which was sufficient to cause a moderate contusion. The release weight, height of drop, and velocity of each SCI were determined using the Spinal Cord Impactor software (version 7.5). The signs of the successful infliction of SCI were as follows: spastic swinging of rat tails, retraction of the lower limbs and a torso-like flutter, and flaccid paralysis of both hind extremities. In the sham-operated control group, the spinal cord was exposed in the same manner as described earlier, but without the contusive SCI procedure.

Experimental Grouping

Forty-five rats were randomly assigned to one of the following three groups (n = 15 in each group) according to random number table: (1) spinal cord injury (SCI) and (2) spinal cord injury + hyperbaric oxygen therapy (HBOT). The rats in each group were then randomly divided into five subgroups for harvesting at various endpoints of postoperative day (POD) 0, 7, 14, 21, and 28. For the sham (SH) group, only a laminectomy was performed without either SCI procedure or HBOT treatment. For the SCI group, no treatment was given. For the HBOT group, HBOT was performed after the surgery. In our previous study, animals were evaluated with the Basso-Bette-Bresnahan locomotor rating scale to determine the effects of HBOT treatment on neurological recovery after SCI. The results suggest that HBOT can improve the motor function of rats with SCI.

Hyperbaric Oxygen Intervention

For HBOT treatment, rats in the HBOT group were exposed to 100% O2 at 2.0 ATA for 1 hour in a hyperbaric chamber (701 Space Research Institute, Beijing, China) twice a day at 12-hour intervals for three consecutive days and thereafter, once a day, starting from POD 0 to POD 28. Compression and decompression of gas were carried out at a rate of .2 kg.cm⁻².min⁻¹. During HBOT exposure, oxygen and carbon dioxide contents were continuously monitored and maintained at ≥ 98% and ≤ 0.3%, respectively. The temperature of the chamber was maintained at a range of 22°C-25°C. After exposure to HBOT, the rats were maintained in a normoxic environment. For the sham group, the rats were treated with normobaric air at 1.0 ATA with 21% O2 at an ambient temperature of 22°C-25°C.

Sample Preparation

At desired time points (POD 0, 7, 14, 21, and 28), the rats were euthanized in a CO2 chamber. The spinal segments of the injured sites were then harvested carefully. Each sample was stored in liquid nitrogen and prepared for iTRAQ.

Extraction and Quantification of Proteins. The procedures of protein extraction are as follows: the tissue samples were frozen by liquid nitrogen and then smashed into powder. Lysis buffer was added to the samples with the ratio of 1:10 (W/V). The mixtures were vortexed and then sonicated for 60 seconds. Then, the mixtures were incubated at room temperature for 30 minutes. The samples were then centrifuged at 15000 × g at 10°C for 20 minutes. Finally, the extracted supernatant was stored at −80°C for further use.

The Bradford method was used to determine the concentration of extracted protein {Nicolas, 2017 #2040}. To ensure that test solutions were within the linear range, the original solutions were diluted with lysis buffer. 10 μL of diluted samples were mixed with 300 μL of protein quantitative dye. The mixtures were incubated in the dark for 20 minutes. The absorbance at 595 nm of samples were measured. The protein concentration was calculated according to the standard curves.

Protein Digestion

After quantification, 200 μg of protein solution was put in a centrifuge tube. DTT at a final concentration of 25 mM was added at 60°C for a one-hour reaction. Then iodoacetamide at
a final concentration of 50 mM was added at the room temperature for 10-minute incubation. The alkylated proteins were transferred to an ultrafiltration tube (10 K) and then centrifuged at 12 000 r/min for 20 minutes. The solution at the bottom of the tube was discarded. Subsequently, 100 μL of dissolution buffer was added and centrifuged for another 20 minutes. Same as above, the solution at the bottom of the tube was discarded. This procedure was repeated for 3 times. The samples were transferred to a new collection tube, and trypsin (1 μg for 50 μg of proteins) was added. The mixtures were incubated at 37°C overnight. After the incubation, all samples were centrifuged at 12 000 r/min for 20 minutes. The peptides generated from digested proteins were enriched at the bottom of the collecting tube. After three repeated operations, the peptide solutions were combined.

### iTRAQ Labeling

The iTRAQ reagent was centrifuged to the bottom of the tube. 150 μL of isopropyl alcohol was added to the tube. The solution was vortexed and then centrifuged again. 50 μL of sample (100 μg of hydrolysate) was transferred into a new tube. Followed by a similar procedure of vortex and centrifugation, the mixture was incubated at room temperature for 2 hours. After the incubation, 100 μL of water was added to terminate the reaction. After vortex, the labeled sample was centrifuged to the bottom of the tube. All samples were lyophilized.

### LC-MS/MS Analysis

A chromatographic column of Durashell-C18 (4.6 mm × 250 mm, 5 μm, 100 Å) was used for peptide separation. Mobile phase A was composed of 98% double-distilled water and 2% acetonitrile (pH = 10). Mobile phase B consisted of 98% acetonitrile and 2% water (pH = 10). The samples were dissolved in 100 μL of mobile phase A. The solution was centrifuged at 14 000 × g and the supernatant were used for analysis. The injection volume was 100 μL. The flow rate was 1 mL/min. The elution program was set as follows: (B%): 0-13 min, 5-8%; 13–90 min, 8-30%; 90–100 min, 30–50%; 100–105 min, 50–95%; 105–115 min, 95–95%; 115–116 min, 95–5%.

A Q-Exactive mass spectrometry with an EASY-Spray source was used for analysis. The related parameters are as following: Spray voltage, 2.3 KV; Capillary temperature, 320°C; DP, 100; the resolution of full MS, 70 000 FWHM; full scan AGC target, 3 × 106; full scan max. IT, 20 ms; Scan range, 300–1800 m/z; the resolution of dd-MS2, 17 500 FWHM; AGC target, 1 × 105; max. IT, 120 ms; intensity threshold, 8.30 × 103; fragmentation methods, HCD; NCE, 32%; Top N, 20.

### Data Analysis

The selection of the database was based on designated species, database annotation completeness and sequence reliability. In this experiment, the database was selected from UniProt (https://www.uniprot.org/). The mass data was processed by Mascot and Scaffold software {Antharavally, 2011 #2041}. Two-sample tests of Perseus 1.5.3.1 (https://www.maxquant.org) was used to compare samples. The criteria for screening differential proteins were P value ≤ .05 and ratio ≥ 1.2 (ratio ≤ .83). The Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis (https://david.abcc.ncifcrf.gov) {Huang da, 2009 #2060} {Huang da, 2009 #2061} was applied to delineate the biological process, cellular component and molecular functions on the basis of Gene Ontology (GO). Ingenuity Pathway Analysis (IPA) software (https://www.ingenuity.com/) was adopted to conduct the pathway and network analysis of differential proteins {Thomas, 2010 #2042}. The PPI networks were constructed using String (https://string-db.org/), a flexible web interface for constructing protein–protein interaction (PPI) network. The gene-miRNA interactions and signaling network of DEPs were constructed by NetworkAnalyst 3.0 tool (https://www.networkanalyst.ca/).

### Results

#### iTRAQ Data and Identification of Differentially Expressed Proteins

To reveal the potential molecular mechanism of HBOT for SCI, the iTRAQ were performed to compare the proteomes of SCI group and SCI + HBOT group on POD 7, 14, 21, and 28, respectively. The schematic diagram of iTRAQ is shown in Figure 1. A variety of isotopes were used to label the N terminal of proteins or polypeptides, and the proteomics of 8 samples can be simultaneously analyzed. By combining high-resolution mass spectrometry with database search, 50,758 specific peptides were assigned to 1746 proteins. A total of 46 differentially expressed proteins were identified between the SCI group and SCI + HBOT group. Of these 46 proteins, a total of 6 proteins...
were found to be significantly different at POD 7 (P < .05). A total of 9, 18, and 13 were found at POD 7, 14, 21, and 28, respectively. The quantitative analysis and identification of proteins altered in the SCI + HBOT group samples are summarized in Table 1 and Table 2. Among the DEPs at these four time points, there were only three overlapping proteins. The three proteins are LUM (Lumican), IGG2A (Ig gamma-2A chain C region), and D3ZVB7 (Osteoglycin) on POD 21 and POD 14 (Figure 2). In addition, the most DEPs between SCI and SCI + HBOT groups were observed on POD 21. Therefore, we will analyze the effect of HBOT on SCI therapy by focusing on the DEPs on POD 21.

**GO and KEGG Enrichment Analyses of DEPs**

The proteins which expressed differentially between SCI and SCI + HBOT groups on POD 21 were sub-grouped and analyzed under their molecular biological processes, cellular components and functions, as shown in Figure 3(A)-(C) respectively. In terms of biological process, most proteins were associated with the cellular process (22%, Figure 3(A)). The cellular component analysis showed that 29% was from the cell, 23% was extracellular region, and 18% was extracellular matrix (Figure 3(B)). Under the molecular function category, 50% and 25% proteins were related to binding and catalytic activity function, respectively (Figure 3(C)). A further protein GO and clustering analysis

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**Table 1. Differentially Expressed Proteins at POD 7, 14 Between SCI + HBOT Group and SCI Group.**

| Accession Number | SCI + HBOT 7 vs SCI 7 up/down | SCI + HBOT 14 vs SCI 14 up/down |
|------------------|-------------------------------|---------------------------------|
| D4A0M2           | .80 Down                       |                                 |
| A0A0G2JSP8       | .81 Down                       |                                 |
| P15429           | .81 Down                       |                                 |
| A0A0H2UHM3       | .82 Down                       |                                 |
| QSM7T7           | .82 Down                       |                                 |
| P01946           | 1.25 Up                        |                                 |
|                 |                                |                                 |
| A0A0H2UHF8       | .67 Down                       |                                 |
| F8WGA3           | .77 Down                       |                                 |
| MYL3             | .78 Down                       |                                 |
| CAH3             | 1.20 Up                        |                                 |
| LUM              | 1.24 Up                        |                                 |
| IGG2A            | 1.33 Up                        |                                 |
| F1LNH3           | 1.36 Up                        |                                 |
| D3ZUL3           | 1.38 Up                        |                                 |
| D3ZVB7           | 1.40 Up                        |                                 |
shows that these differential proteins are related to many molecular functions such as oxygen transporter activity, oxygen binding, heme binding, and tetrapyrrole binding (Figure 3(G)). Moreover, the biological processes such as response to oxygen transport, gas transport, and regulation of T cell proliferation were influenced by HBOT (Figure 3(E)).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that the proteins which expressed differentially between SCI and SCI + HBOT groups on POD 21 participate primarily in the metabolic pathways and thermogenesis, etc. (Figure 4). These results suggest a widespread impact of HBOT for SCI on the global proteomics.

### Results of IPA Analysis

The cellular and signaling pathways that these proteins are involved were utilized in IPA analysis in order to identify protein targets of interest. The abundances of differentially expressed proteins (DEPs) in SCI and SCI + HBOT groups were also compared in relation to their signaling pathways (Figure 4). After hyperbaric oxygen therapy, LXR/RXR activation, acute phase response signaling and the intrinsic prothrombin pathway was abnormally activated.

#### DEPs Networks of PPI, Gene-miRNA and Signaling

DEPs reflects the specific influence of HBOT on SCI, thus the DEP-derived-protein-protein interaction (PPI) network was assembled based on STRING (Figure 5). As shown in Figure 6(A), these proteins generally have no protein interactions. Among them, Spt1 (Spectrin, alpha, erythrocytic 1, D4A678), Sptb (Spectrin, beta, erythrocytic, Q6XDA0), Slc4a1 (Solute carrier family 4 (anion exchanger), member 1, B3AT), Hba1 (Hemoglobin subunit alpha-1/2, HBA), and Hbb-b1 (Hemoglobin subunit beta-2-like, Q62669), are related to erythrocyte.

![Figure 2. Identification and quantitation of differentially expressed proteins by mass spectrometry trials between SCI groups and SCI + HBOT group on POD 7, 14, 21, 28.](image)

| Accession Number | SCI + HBOT 21 vs SCI 21 up/down | SCI + HBOT 21 vs SCI 21 up/down Accession Number | SCI + HBOT 28 vs SCI 28 up/down | SCI + HBOT 28 vs SCI 28 up/down Accession Number |
|------------------|----------------------------------|-----------------------------------------------|----------------------------------|-----------------------------------------------|
| G3V8D2           | .59 Down                          | POSTN                                         | .66 Down                         |                                               |
| A0A0G2JWD2       | .68 Down                          | ORM1                                          | .73 Down                         |                                               |
| IGG2A            | .70 Down                          | KNG1                                          | .73 Down                         |                                               |
| R9PKZB           | .78 Down                          | MAP1                                          | .73 Down                         |                                               |
| PRLIO            | .80 Down                          | HP                                            | .75 Down                         |                                               |
| A0A0G2K079       | .81 Down                          | PRX                                           | .76 Down                         |                                               |
| THNS2            | 1.20 Up                           | LOC299282                                     | .78 Down                         |                                               |
| D4A678           | 1.21 Up                           | NEWGENE_621 351                               | .80 Down                         |                                               |
| Q6xda0           | 1.21 Up                           | C9                                            | .81 Down                         |                                               |
| LUM              | 1.23 Up                           | FGG                                           | .82 Down                         |                                               |
| B3AT             | 1.27 Up                           | LAMBI                                         | .82 Down                         |                                               |
| G3V7W1           | 1.27 Up                           | OGN                                           | .82 Down                         |                                               |
| D4A9D6           | 1.33 Up                           | ERMN                                          | 1.21 Up                          |                                               |
| D3ZVB7           | 1.41 Up                           |                                               |                                 |                                               |
| HBB2             | 1.43 Up                           |                                               |                                 |                                               |
| LEG5             | 1.49 Up                           |                                               |                                 |                                               |
| Q62669           | 1.49 Up                           |                                               |                                 |                                               |
| HBA              | 1.59 Up                           |                                               |                                 |                                               |

### Table 2. Differentially Expressed Proteins at POD 21, 28 Between SCI + HBOT Group and SCI Group.
Figure 3. GO functional classification and enrichment analysis of differentially expressed proteins (DEPs). The DEPs were grouped and analyzed under their biological processes (A), cellular components (B) and molecular functions (C). DEPs are shown in the order of statistical significances under the grouping of their biological processes.

Figure 4. Significantly enriched KEGG pathways for differentially expressed proteins (DEPs).
Figure 5. IPA biological pathway analysis for differentially expressed proteins (DEPs). The signaling pathways where proteins with differential SCI + HBOT vs SCI changes as well as with highest correlations to their pathways are shown. The indicated P value was calculated based on their correlation to the particular signaling pathway. The ratio indicates the percentage of the number of differential protein as compared to the total number of proteins in that group.

Figure 6. The differentially expressed proteins (DEPs) profiles. The protein–protein interaction (PPI) network of differentially expressed proteins (DEPs) (A). The gene-miRNA interactions of DEPs (B). The signaling network of DEPs (C).
Discussion

In this study, we used iTRAQ strategy to evaluate the differentially expressed proteins induced by HBOT treatment. A total of 1746 proteins were identified by high-precision mass spectrometry. The largest number of differentially expressed protein were observed on POD 21 between SCI groups and SCI + HBOT groups.

Therefore, we analyzed the effect of HBOT on SCI outcomes by focusing on the DEPs at POD 21. These proteins are mostly involved in oxygen binding, oxygen transporter activity, heme binding, tetrapyrrole binding, and cellular processes including oxygen transport, gas transport, and the regulation of T cell proliferation. Our results suggest that hyperbaric oxygen therapy increases the oxygen binding capacity of hemoglobin and promotes oxygen transport, which is helpful in alleviating anoxic injuries caused by SCI. We found that the upregulation of differentially expressed proteins was associated with wound healing and platelet aggregation. There is evidence that hyperbaric oxygen can contribute to wound healing and regulate oxidative metabolism in platelet aggregation.

The down-regulated proteins in the SCI + HBOT group were found to be associated with neuronal differentiation, metal ion binding, molecular chaperone interaction, and ubiquitin related protein homeostasis, suggesting that these proteins may be markers of spinal cord injury.

One of the dominant functions of these proteins is the regulation of T cell proliferation. Besides, T cell regulatory protein SPTA1 (D4A678) was increased in the SCI + HBOT group, therefore it was reasonable to assume that the mechanism of HBOT was correlated with the boosting of body immunity, in which T cell proliferation might play an important role. As with many other physical injuries, patients with SCI display acutely elevated plasma levels of pro-inflammatory cytokines, such as IL-6 and soluble IL-2 receptors. Reduced natural killer cell count and cytotoxic activity was also seen in SCI patients. In rats, pro-inflammatory cytokines are increased along with anxiety- and/or depression-like behaviors after SCI. When the signaling of IL-6 was inhibited in mice after SCI, the survival rate of transplanted bone marrow stromal cells was increased. Thus, an impaired or suppressed immune response may be the case overall after SCI. This may lead to decreased immunity response against subsequent infections as seen in mice.

Through IPA analysis, we found that LXR/RXR activation, acute phase response signaling, and the intrinsic prothrombin pathway was activated in the SCI + HBOT group. Our results suggest that these pathways are closely related to SCI and the prothrombin-related proteins may be potential therapeutic targets.

We found HBA (Hemoglobin subunit alpha- 1/2) expression was mostly increased after HBOT treatment and regulates important signal factors such as TGFβ1 and STAT5A. TGFβ1 is a pleiotropic cytokine, which is associated with wound repair. TGF-beta1 inhibition has been reported to promote functional recovery after spinal cord injury. It has been reported that activation of the JAK/STAT5 pathway was deleterious after spinal cord injury. These results suggest that HBA participates in the therapeutic effects of HBOT on SCI. HBA can also regulate other signaling factors including Cpr1, Epsa1, and Cpm, suggesting that these signal pathways may be closely associated with SCI outcomes and its potential therapeutic targets.

Besides, we found that rno-mir-9a-5p and rno-mir-125b-5p were involved in the regulation of Tnhs2 (THNS2, O-phospho-l-threonine phospho-lyase) and Lgals5 (LEG5, Lectin, galactose binding, soluble 5). Overexpression of mir-9a-5p has been reported to improve neurological function after spinal cord ischemia/reperfusion injury and may alleviate blood-spinal cord barrier (BSCB) disruption, neuro-inflammation, and apoptosis through MAPK3 or NotCH2-mediated signaling pathways. It has been found that serum exosomal Mir-125b-5p is a specific and easily detected diagnostic marker of acute spinal cord injury. So assumingly, rno-mir-9a-5p and rno-mir-125b-5p may be important targets for HBOT in SCI treating.

Author Contributions

Conceptualization, Z.L., X.-M.H and J.-W.T.; methodology, Z.L., X.-M.H and J.-W.T.; investigation, Z.L., X.-M.H, X.-H.L and L.-L.M; writing—original draft preparation, Z.L., X.-M.H and J.-W.T.; writing—review and editing, Z.L., X.-M.H, J.-W.T.; supervision, X.-H.L and L.-L.M; funding acquisition, J.-W.T. All authors have read and agreed to the published version of the manuscript.

Declaration of Conflicting Interests

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