Proteomic comparison between physiological degeneration and needle puncture model of disc generation disease

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Abstract

Background  The completeness of the intervertebral disc proteome is fundamental to the integrity and functionality of the intervertebral disc.

Methods  The 20 experimental rats were placed into two groups randomly, normal group (NG) and acupuncture pathological degeneration group-2 weeks (APDG-2w). The ten 24-month-old rats were grouped into physiological degeneration group (PDG). Magnetic resonance imaging, X-ray examination, histological staining (hematoxylin & eosin, safranin-O cartilage, and alcian blue staining), and immunohistochemical examination were carried out for assessing the degree of disc degeneration. Intervertebral disc was collected, and protein composition was determined by LC-MS, followed by bioinformatic analysis including significance analysis, subcellular localization prediction, protein domain prediction, GO function and KEGG pathway analysis, and protein interaction network construction. LC-PRM was done for protein quantification.

Results  Physiological degeneration and especially needle puncture decreased T2 signal intensity and intervertebral disc height. Results from hematoxylin & eosin, safranin-O, and alcian blue staining revealed that the annulus fibrosus apparently showed the wavy and collapsed fibrocartilage lamellas in APDG-2w and PDG groups. The contents of the nucleus pulposus were decreased in physiological degeneration group and APDG-2w group compared with NG. Results from immunohistochemical analysis suggested the degeneration of intervertebral disc and inflammation in APDG-2w and PDG groups. The protein composition and expression between needle puncture rat models and the physiological degeneration group showed significant difference.

Conclusions  Our studies produced point-reference datasets of normal rats, physiological degeneration rats, and needle puncture rat models, which is beneficial to subsequent pathological studies. There is differential expression of protein expression in degenerative discs with aging and acupuncture, which may be used as a potential discriminating index for different intervertebral degenerations.

Keywords  Disc degeneration disease · Proteome · Extracellular matrix

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Introduction

Disc degeneration is a pathological progression causing degeneration of the intervertebral discs, the soft connective tissue of the spine that shows a critical effect on spinal kinematics. The main role of the intervertebral disc is mechanical, transmitting, and releasing loads generated by muscle activity and body via the spine [1]. Degenerative process proceeds at the tissue, cellular, and molecular dimensions, resulting in significant morphological and physiological changes to the disc and ultimately decreasing its ability to withstand pressure loads [2]. It is now widely accepted that aging, genetic predisposition, poor lifestyle such as depressive symptoms, smoking, and obesity, and non-physiological mechanical loading promote disc degeneration [1, 3, 4]. Notably, significant degradation of aggrecan occurs during disc degeneration, which causes a loss of proteoglycans and tissue hydration [1, 2, 5, 6]. Subsequently, loss of glycosaminoglycans occurs, which in turn decreases osmotic pressure of the disc matrix [1, 2, 5, 6].

A number of pathological changes in the intervertebral disc are associated with disc degeneration, of which apoptosis, inflammation, and degradation of extracellular matrix are the most common [7–9]. Aging-induced degeneration causes an overall reduction in numbers and disrupts the balance of the intervertebral disc proteome [10, 11]. Cells from the intervertebral disc compartment generate proteins of different composition in the extracellular matrix in order to facilitate specific biomechanical requirements [12, 13]. The extracellular matrix is also defined as the matrisome, which houses the cells and facilitates their intercommunication by mediating the presentation and availability of signaling molecules [14]. However, aging causes that the nucleus pulposus appears less compliant and more fibrotic, eventually decreasing disc biomechanics [15]. Correspondingly, altered matrix stiffness changes in cell–matrix interactions and downstream transcriptional modulation and signaling transduction [16]. Thereafter, the alteration of the matrisome causes a vicious feedback loop and enhances cell degradation and extracellular matrix modification [17, 18]. Of interest, intervertebral disc generation was found to be associated with genetic risk factors involved in variants of gene encoding matrisome proteins, such as CHST3, ASPN and COL9A1, suggesting an important role of matrix proteins in disc function [19–21]. As a consequence, understanding the cellular and extracellular proteome is significant for understanding the pathogenesis and progressive mechanisms of intervertebral disc disease.

Low back pain results in more disability than any other diseases globally and imposes a huge economic burden because of the costs associated with lost time at work and medical care [22]. Nearly 80% of the population may experience low back pain, and the prevalence of intervertebral disc diseases is 15–45%, which may be caused by degenerative lumbar disc disease and generated discogenic pain [23]. Considering the severe burden of disc degeneration disease, several studies have established animal models to further investigate and treat this disease [24–28]. In order to analyze the availability of the rat model, the present study analyzes the extracellular matrix protein composition in terms of proteomics, which is a prerequisite for a fundamental understanding of intervertebral disc extracellular matrix protein composition and underlying pathophysiology.

Materials and methods

Animals

Before the initiation of animal experiments, this study obtained an approval from the Institutional Animal Care Use Committee of Soochow University (Soochow, China). Thirty Sprague–Dawley (SD) rats were used in this study. The 20 experimental rats were randomly divided into two groups, normal group (NG) and acupuncture pathological degeneration group-2 weeks (APDG-2w). The physiological degeneration group (PDG) included 10 24-month-old rats. In the NG group, there are 10 3-month-old rats. In the APDG-2w group, there are 10 3-month-old rats treated by acupuncture. In the PDG group, there are 10 24-month-old rats. All rats were housed in individual cages, fed water, and food freely, and their condition was monitored daily. All animal experiments were approved by the Animal Ethical and Welfare Committee of Soochow University (Approval No: SUDA20200507A01).

Establishment of disc degeneration rat model

The rats were anesthetized after inhalation of isoflurane. The location of the intervertebral space was determined by digital palpation, followed by confirmation via counting the vertebrae from sacral region. Radiographs of rat tails were captured using an AMX-3-portable X-ray equipment (GE Healthcare, Piscataway, NJ, USA) at a penetration of 50 kV, an exposure of 5 mA, and a distance of 30 inches. X-rays were captured using an AMX-3-portable X-ray equipment (GE Healthcare, Piscataway, NJ, USA) at a penetration of 50 kV, an exposure of 5 mA, and a distance of 30 inches. X-rays were captured prior to the puncture procedure as a baseline examination of the disc height. The skin was disinfected with povidone–iodine solution. In the APDG, the intervertebral disc of the tail vertebra was completely penetrated by the 21G needle percutaneous. A degeneration model was created after 2 weeks, and we determined the levels of injured tail disc segments by using intraoperative
radiographic fluoroscopy. We used a hollow 21-G needle and the needle penetrated the both sides of skin completely. The needle rotated 360 degrees after puncture and stayed for 30 s.

**Magnetic resonance imaging**

In the needle puncture research, a 0.3-T imager was used for magnetic resonance imaging assays. The instruction was equipped with a quadrature extremity coil receiver (Hitachi Medical System, Tokyo, Japan). T2-weighted slices in the sagittal plane were measured with the following parameters: 4 excitations; field of view of 260; 256 (h) × 128 (v) matrix; a fast spin-echo sequence with a repetition time of 4,000 ms, and an echo time of 120 ms. The thickness of the section was about 1 mm, and the gap was 1 mm. The obtained magnetic resonance images were assessed using ImageJ software (Media Cyber, Rockville, MD, USA).

**X-ray assessment**

The rats were anesthetized as described above. Radiographs of the tails of all rats were captured before or after puncture procedure. The baseline measurement was a preoperative radiograph. The level of anesthesia in each rat was maintained at 2 L/min oxygen flow and 2% isoflurane concentration during filming to maintain a similar level of muscle relaxation, which was aimed to reduce the effect of muscle contraction on disc height. Muscle relaxation was detected by checking for loss of muscle tone in the lower extremity. The rat is placed in the prone position with the tail straightened and placed on custom V-shaped platform so that the vertical beam reaches the tail and was focused at the target level. Epson Perfection V750 Pro (Epson, Long Beach, CA, USA) was used to scan radiographs that were stored. Two spine surgeons blinded to needle size and follow-up time independently measured all images. Scion software (Scion Corp., Frederick, MD, USA) was used to interpret and analyze the digitalized radiographs. The disc height index (DHI) was used to present the average intervertebral disc height [29]. DHI was calculated by averaging the measurements of anterior (a), middle (b), and posterior (c) parts of the intervertebral disc height. The average height of adjacent vertebral bodies is (d + e + f + g + h + i)/6. Alteration of DHI was suggested as (%DHI = 2 × (a + b + c)/ (d + e + f + g + h + i) × 100%. DHI helps minimize the inter-subject variations in overall size of the vertebral column, enabling inter-subject analyses [30].

**Histological staining**

Tissues were fixed in 10% neutral-buffered formalin supplemented with 10% cetylpyridinium chloride. After decalcification in Cal-Ex II Fixative/Decalifier (Fisher Scientific, Pittsburgh, PA, USA), the tissues were embedded in paraffin and sectioned into slices with 6 µm thickness. Cellular components of the sections were stained with hematoxylin and eosin (H&E), and proteoglycans were stained with safranin-O. The sections were deparaffinized and rehydrated, followed by being stained with H&E staining kit (Solarbio, Beijing, China), safranin-O cartilage staining kit (Solarbio), and alcian blue staining kit (Solarbio) according to the instructions. The images were captured using a light microscopy (Leica, Wetzlar, Germany) with 50× and 40× magnifications.

**Immunohistochemical examination**

The isolated intervertebral disc tissues were fixed in 4% paraformaldehyde, followed by decalcification, dehydration, clearing using dimethylbenzene, and being embedded in paraffin using Leica embedder (Leica). The Sects. (5 µm) were maintained in 3% hydrogen peroxide for 10 min, followed by being sealed with 5% bovine serum albumin for 30 min at 37 °C. The primary antibodies against COL1, COL2, MMP3, and TNF-α (1:1,000, Servicebio, Wuhan, China) were incubated with the sections overnight at 4 °C. After reaction with diaminobenzidine-based peroxidase substrate (Beyotime, Shanghai, China), the images were captured using the light microscopy and quantified using ImageJ.

**Protein examination by LC–MS method**

**Protein extraction, digestion, and separation**

Proteins were extracted using SDT (4% SDS, 1 mM DTT, 100 mM Tris–HCl, pH7.6) and detected with BCA Protein Assay Kit (Bio-Rad, Hercules, CA, USA). Proteins were digested according to filter-aided sample preparation procedure. The digested peptides were desalted using C18 Cartridges (Empore™ SPE Cartridges C18). Then the extract was concentrated by vacuum centrifugation. For examination, the extract was reconstituted in 40 µL of formic acid (0.1%, v/v). Finally, appropriately 20 µg of protein was redissolved in 5× loading buffer and incubated in a boiling water bath for 5 min. Next proteins were subjected to separation by 12.5% SDS-PAGE gel and visualized by Coomassie Blue R-250 staining.

**LC–MS analysis**

A Q Exactive mass spectrometer (Thermo Scientific) was used to perform LC–MS analysis in a positive ion mode. A reverse phase trap column (100 µm × 2 cm, nanoViper C18, Thermo Scientific) was equipped with a C18-reverse phase analytical column (3 µm resin, 75 µm inner diameter,
Thermo Scientific). The separation procedure was done with a linear gradient of buffer B (0.1% formic acid and 84% acetonitrile) and buffer A (0.1% formic acid). The flow rate was 300 nL/min. The most abundant precursor ions were chosen from the survey scan 300–1800 m/z. The survey scan was obtained with a resolution of 70,000 (m/z 200). The resolution of HCD was 17,500 (m/z 200). The isolation width was set to 2 m/z. Dynamic exclusion duration was 40 s. Maximum injection time was 10 min. Automatic gain control target was 3e6. Normalized collision energy was 30 eV. The underfill ratio was 0.1%.

**Protein identification and quantification**

The MS raw data were searched using the MaxQuant 1.5.3.17 software for identification and quantitation analysis. Protein quantification was carried out with intensity-based absolute quantification method. Related parameters and instructions are given in Table 1.

**Bioinformatic analysis**

**Clustering analysis of protein**

The quantitative results of proteins were normalized. Then, samples and proteins were grouped simultaneously using the ComplexHeatmap R package version 3.4. The distance algorithm chosen was Euclidean, the linkage was average linkage, and then a hierarchical clustering heatmap was illustrated.

**Subcellular localization analysis**

CELLO (http://cello.life.nctu.edu.tw/) was used for subcellular localization prediction. The CELLO method is based on a multi-class support vector machine learning approach. This machine learning approach collected protein sequence data and their known subcellular localization from public databases.

**Protein domain analysis**

Protein domain analysis was performed using Pfam database. This database collects protein families and presents them in the form of multiple sequences and hidden Markov model. InterProScan software package was used to characterize the function of targeted sequences and annotate protein domain.

**GO function annotation**

GO annotation was performed using Blast2GO. The annotation process includes sequence alignment, GO entry, and function annotation by InterProScan.

**KEGG pathway analysis**

The target protein was annotated based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

| Table 1 | Related parameters and instructions used for protein identification and quantification |
|---------|---------------------------------------------------------------------------------------|
| Item    | Value                                                                 |
| Enzyme  | Trypsin                                                               |
| Max missed cleavages | 2                             |
| Fixed modifications | Carbamidomethyl (C), Oxidation (M)                     |
| Variable modifications | 6 ppm, 20 ppm, 20 ppm |
| Main search | uniprot_mouse_76417 represents: “uniprot,” public database (http://www.uniprot.org/) |
| First search | Reverse                     |
| PRM/MS tolerance | True                          |
| Database pattern | Protein FDR ≤ 0.01            |
| Include contaminants | Peptide FDR ≤ 0.01               |
| Peptides used for protein quantification | Use razor and unique peptides            |
| Time window (match between runs) | 2 min                             |
| Protein quantification | Label-free quantification 1 |
| min. ratio count |                                                                      |
Enrichment analysis

The enrichment analysis of GO annotation was performed using Fisher’s exact test, as well as KEGG pathways and protein domain.

Protein interaction network analysis

CytoScape software version 3.2.1 was based on the STRING database and IntAct (http://www.ebi.ac.uk/intact/main.xhtml) database. CytoScape software was used to generate the interaction networks of the target proteins. The direct and indirect interactions of the target proteins were presented.

Protein confirmation by LC-PRM method

Appropriately 200 μg protein extracts were mixed in DDT with a final concentration of 100 mM. The mixture was boiled for 15 min. After refrigeration, the mixture was added with 200 μL UA buffer. The supernatant was discarded after centrifugation (14,000 × g, 30 min). Next, 100 μL of IAA (iodoacetamide) (50 mM IAA in UA) was supplemented. The mixture was vibrated for 60 s (600 rpm) and maintained in the dark for 30 min. After centrifugation (14,000 × g, 20 min) three times, appropriately 100 μL NH₄HCO₃ buffer (50 mM) was added. The mixture was then centrifugated (14,000 × g, 20 min) twice. The mixture was added with 40 μL NH₄HCO₃ buffer containing trypsin (1:50), vibrated for 1 min (600 rpm), and incubated for 16 h 37°C. The supernatant was collected by centrifugation (14,000 × g, 30 min). After lyophilization, the obtained free-dried powder was redissolved in 0.1% formaldehyde and detected its absorbance under 280 nm. The protein extract (1 μg) was added with 20 fmol standard peptides and then separated using HPLC in buffer A (0.1% formic acid) and buffer B (0.1% formic acid and 84% acetonitrile). The flow rate for separation was 0.3 μL/min. The procedure was as follows, from 5 to 10% B (0–2 min), from 10 to 30% B (2–45 min), from 30 to 100% B (45–55 min), and 100% B (55–60 min). A Q Exactive mass spectrometer in a positive mode was used for PRM analysis. The scanning range was 300–1,800 m/z. The resolution was 60,000 (m/z 200). The fixed first mass was 100 m/z. The isolated window was 1.6 Th, AGC target 3 × 10⁶, maximum IT 120 ms, MS2 activation type HCD, MS resolution 30,000 (m/z 200), and normalized collision energy 27. The obtained PRM data were analyzed using Skyline 3.5.0.

Data and statistical analysis

All the data were presented as the mean ± standard error. Data were tested for normality using the Shapiro–Wilk test. The analysis of variance (ANOVA) was performed to evaluate the significance of differences among means of data, with a post hoc test Fisher PLSD. P-values less than 0.05 were defined as significance for all assays.

Results

Imaging evaluation, histological analysis, and immunohistochemical staining

T2 signal intensity and disc height index were measured by magnetic resonance and X-ray, respectively. The obtained images by magnetic resonance are presented in Fig. 1A–C, and T2 signal intensity of NG, APDG-2w, and PDG groups was scored by three spinal surgeons. Figure 1D shows that APDG-2w and PDG group displayed decreased T2 signal intensity compared with NG group, while PDG group showed improved T2 signal intensity in relative to APDG-2w group (p < 0.01, p < 0.001). The images by X-ray were conducted for NG, APDG-2w, and PDG groups (Fig. 1E–G). The calculated disc height index is detailed in Fig. 1H, suggesting that needle puncture treatment or physiological degeneration significantly decreased DHI compared with NG group (p < 0.01), while PDG displayed an improvement in DHI compared to APDG-2w (p < 0.05). The results from histological sections by hematoxylin and eosin staining and histological scores manifested a change in morphology from normal degenerated to severely degenerated (Fig. 2A–B). The structure of intervertebral disc degenerated, and the arrangement of annulus fibrosus was destroyed in APDG-2w and PDG groups compared with NG. The discs of NG group possessed an intact annulus fibrosus showing a well-defined border between the nucleus pulposus and annulus fibrosus. Besides, the annulus fibrosus showed an intact morphology that is U-shaped in the posterior aspect and slightly convex in the anterior aspect. As for the APDG-2w, the annulus fibrosus apparently showed the wavy and collapsed fibro-cartilage lamellas, and the contents of the nucleus pulposus were reduced. The decrease in nucleus pulposus contributed to the wavy appearance suggesting fewer small chondrocyte-like cells and large vacuoles in PDG group. The boundary between nucleus pulposus and disc is less pronounced than that in a normal disc (Fig. 2A–B). Additionally, images of safranin-O staining and alcian blue staining suggested the decrease in proteoglycan in nucleus pulposus, indicating disc degeneration (Fig. 2C–D) Immunohistochemical analysis showed that COL2 protein was significantly degenerated, while MPP3 and TNF-α were accumulated in APDG-2w and PDG compared with NG (Fig. 3). Together, needle puncture-induced disc generation and physiological degeneration were associated with collagen degeneration and inflammation.
Analysis of differentially expressed proteins

Here, we detected 1008 proteins in NG samples, 1327 proteins in APDG-2w samples, and 1101 proteins in PDG samples (Fig. 4A). To analyze the overlap of the detected proteins between groups, a Venn diagram was used to analyze the overlap of the detected proteins between NG and APDG-2w, NG and PDG, as well as APDG-2w and PDG. Notably, Fig. 4A shows that 698 proteins were both determined in NG, APDG-2w, and PDG groups. In detail, there were 100 proteins up-regulated in APDG-2w and 82 proteins down-regulated in APDG-2w compared to PDG group (Fig. 4B). Compared with NG group, 51 proteins were elevated and 29 proteins were decreased in PDG group. Here 70 proteins were highly expressed and 61 proteins were low expressed in APDG-2w sample (Fig. 4B). Protein names and their corresponding protein-coding genes are provided in Supplementary File 1. To demonstrate the significant differences of proteins between the groups, co-expressed proteins were plotted as volcanoes with two factors: fold change of protein expression and p-values. Among them, significantly up-regulated proteins in APDG-2w group were marked in red (FC > 2.0, p < 0.05), significantly down-regulated proteins in blue (FC < 0.5, p < 0.05), and proteins with no significant expression change in gray, in relative to PDG group (Fig. 4C). Figure 4D and Fig. 4E represent the distribution of protein expression in PDG and APDG-2w compared with NG. In order to analyze the protein expression patterns of inter-groups, the differentially expressed proteins were grouped and categorized using a hierarchical clustering algorithm and presented in the form of heatmap. Figure 5A–C shows the grouping rationality and reveals the significant effects of physiological degeneration and needle puncture on the sample clustering. As shown, with the screening criteria of fold change more than 2.0 and p-value less than 0.05, the obtained differentially expressed proteins effectively differentiated the groups.

Subcellular localization and domain analysis of differentially expressed proteins

All proteins were analyzed for subcellular localization using the CELLO. The results were output and are shown in Fig. 6A (APDG-2w vs. NG), Fig. 6B (PDG vs. NG), and Fig. 6C (APDG-2w vs. PDG). Further, to reveal the domain characteristics of proteins, Fisher’s exact test was carried out.
Fig. 2 Needle puncture-caused histological alterations. A Hema
toxylin and eosin-stained sections showing disc (5.0×) with nucleus pulposus (40×) and annulus fibrosus (40×) in NG, APDG-2w, and PDG groups; B Histological score of the typical histological change in NG, APDG-2w, and PDG groups. C Safranin-O-stained and D alcian blue-stained sections of the intact disc (50×), nucleus pulposus (400×), and annulus fibrosus (400×) in NG, APDG-2w, and PDG groups. **p<0.01 and ***p<0.001. NG, normal group; PDG, physiological degeneration group; APDG-2w, acupuncture pathological degeneration group-2 weeks

Fig. 3 Immunohistochemical evaluation of COL1, COL2, MMP3, and TNF-α in NG, APDG-2w, and PDG groups. COL1, collagen type 1; COL2, collagen type 2; MMP3, matrix metalloproteinase 3; TNF-α, tumor necrosis factor alpha; NG, normal group; PDG, physiological degeneration group; APDG-2w, acupuncture pathological degeneration group-2 weeks
out. Compared with NG group, the differentially expressed proteins in APDG-2w were categorized as lipocalin/cytosolic fatty-acid binding protein family (Fig. 6D). In terms of PDG samples, the differentially expressed proteins showed leucine-rich repeat N-terminal domain compared with NG samples (Fig. 6E). Compared with PDG, the differentially expressed proteins in APDG-2w were predicted as lipocalin/cytosolic fatty-acid binding protein family (Fig. 6F).

GO annotation and KEGG enrichment analysis

Further, Blast2Go software was used for annotation of all differentially expressed proteins at the secondary functional annotation level. The results presented in Fig. 7A showed that 457 differentially expressed proteins in APDG-2w may be involved in cellular process, 426 for binding, and 459 for cell part, compared with NG. As for APDG-2w, 246 proteins participate in regulating cellular process, 211 in binding, and 230 in cell compared with NG (Fig. 7B). In terms of the differentially expressed proteins between APDG-2w and PDG, 556 proteins were associated with cellular process, 520 for binding, and 552 for cell part (Fig. 7C). As shown in Fig. 7D, the differentially expressed proteins in APDG-2w mostly participated in staphylococcus aureus infection and complement and coagulation cascades, compared with NG. Figure 7E shows that the differentially expressed proteins in

![Diagram](image-url)
PGD were more associated with AGE-RAGE signaling pathway in diabetic complications than in NG. As suggested in Fig. 7F, APDG-2w samples showed differentially expressed proteins associated with staphylococcus aureus infection compared with PDG group.

Protein interaction network analysis

Combining the results of pathway annotation and protein interaction network analysis contributes a more comprehensive and systematic model of cellular activity from a molecular dimension, facilitating mining and in-depth study of molecular mechanisms. Here, in the PPI interoperability network, based on the principle of topology identification, the proteins with high aggregation in the interoperability network graph were divided into different clusters, and then each type of cluster was functionally grouped. The differentially expressed proteins between APDG-2w and NG consisted the interaction network as shown in Fig. 8, and the clustering proteins are provided in Supplementary file 2. As for PDG and NG, the proteins are illustrated in Fig. 8 and provided in Supplementary file 3 in detail.
Figure 8 and Supplementary file 4 show the differentially expressed proteins between APDG-2w and PDG. Next, LC-PRM method was used to confirm the expression of Mapk3, Acan, Mmp10, and Mmp2 protein. As suggested in Fig. 9, APDG-2w and PDG groups showed significant decreases in Acan (p < 0.05) and increases in Mapk3 (p < 0.05), while Mmp10 and Mmp2 protein was elevated in APDG-2w compared to NG and PDG groups (p < 0.01, p < 0.001). Collectively, APDG triggered alterations of proteome in the intervertebral disc, and these proteins interacted with each other to form a regulatory network.

Discussion

To simulate human intervertebral disc degeneration, needle puncture is widely applied for establishing animal models, such as sheep, rabbit, canine, pig, and rodents [24–28]. Given its availability and inexpensiveness, the rat caudal disc is a preferred option recently. With respect to the effect of initial injury severity, a previous study has standardized the process of needle injury in a rat caudal disc model [31]. Based on this method, we established a rat model of severe disc degeneration and analyzed the changes in proteome of intervertebral disc caused by needle injury. The obtained results revealed the importance of differentially expressed proteins for the diagnosis of disc degeneration.

As one of the most crucial public health conundrum, disc degeneration disease frequently causes low back pain [2]. This disease is characterized by a complex succession of progressive morphological, biomechanical, and biochemical changes in the intervertebral disc, leading to the development of discogenic low back pain, and in some cases, causing impairment of mechanical function [2, 32, 33]. To investigate and understand the pathogenesis and pathophysiology, it is necessary to develop animal models for intervertebral disc degeneration [34, 35]. In our study, we inserted a needle through the nucleus pulposus. Meanwhile, we used older rats kept normally for 24 months with 3-month-old rats as controls. When compared with normal rats, the differences in injured discs were obviously displayed in APDG-2w and PDG, according to the radiographic and histological results. However, the severity of disc injury was different between APDG-2w and PDG. Studies have demonstrated that needle puncture causes degenerative discs and biomechanical-deteriorated discs [34–36]. The puncture process generates tissue herniation, which causes biomechanical and biochemical deterioration within the intervertebral disc [36–39]. From the results obtained, we effectively constructed the disc degeneration model.
Fig. 7 Gene ontology annotation and KEGG pathway analysis for differentially expressed proteins. Statistical graph of GO annotations of the differentially expressed proteins between A NG and APDG-2w, B NG and PDG, as well as C APDG-2w and PDG; KEGG pathway analysis of the differentially expressed proteins between D NG and APDG-2w, E NG and PDG, as well as F APDG-2w and PDG. NG, normal group; PDG, physiological degeneration group; APDG-2w, acupuncture pathological degeneration group-2 weeks. GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Fig. 8 The categorization chart of the interaction network. The network consisted of the differentially expressed proteins between A NG and APDG-2w, B NG and PDG, and C APDG-2w and PDG. NG, normal group; APDG-2w, acupuncture pathological degeneration group-2 weeks; PDG, physiological degeneration group; NG, normal group.
Of note, we detected the up-regulation of MMP3 and TNF-α in APDG-2w and PDG groups compared with NG. Further, LC-PRM continually confirmed the down-regulation of Acan, as well as the up-regulation of Mapk3, Mmp10, and Mmp2. Intervertebral discs are complex anatomical structures conferring the spine flexibility [37]. Its inner score nucleus pulposus consists of a loose network of collagen fibers, aggrecan, and water [40]. The hallmark of progressive intervertebral disc degeneration is the incapacity of nucleus pulposus to sustain normal homeostatic tissue remodeling [41, 42]. The initiation of the degenerative process is characterized by the disappearance of the large vacuolated notochordal-like cells in the nucleus pulposus [43]. Next, proteoglycans are reduced, and collagen synthesis is conversed [44]. However, the synthesis and activity of MMP are increased, and proteoglycans are degenerated, resulting in a reduced loading capacity and height of the intervertebral discs [45]. Inflammation and related signaling pathways have been identified as important factors in the onset and progression of disc degeneration [46]. Inflammatory molecules like TNF-α have been reported to trigger or initiate intervertebral discs degeneration since proinflammatory mediators affect autophagy, senescence, and apoptosis of disc cells, as well as decrease anabolic proteins like aggrecan and collagen [47–49].

The completeness and function of the intervertebral disc strongly rely on its spatiotemporal proteome, and the change in spatiotemporal proteome is to a great extent caused by age.
Although our studies showed that APDG-2w and PDG both exhibited disc injury, comparisons between proteome of these two groups produced a difference in proteomic profiles of APDG-2w and PDG. We revealed a dataset of the differentially expressed proteins comprising 182 proteins; thereinto, 82 proteins were down-regulated and 100 proteins were up-regulated in APDG-2w compared with PDG. Domain analysis, GO annotation and KEGG pathway analysis revealed that the differentially expressed proteins are mainly associated with Staphylococcus aureus infection. It has been validated that anaerobic low-virulence bacteria cause degenerative discogenic disease, and Staphylococcus aureus induces the pyogenic discitis [51, 52]. Studies suggested that low-grade infection plays effects on herniation and inflammation [53–55]. It was conjectured that the differentially expressed proteins may affect disc injury associated with inflammation reaction. Previous study has shown that the findings in proteomics analysis might be a possible diagnostic tool for intervertebral disc patients [56, 57].

In this study, protein interaction network maps were constructed for differentially expressed proteins based on protein interaction relationships in STRING or IntAct databases using CytoScape software. In protein–protein interaction networks, highly aggregated proteins may often have the same or similar functions and perform biological functions through synergistic interactions [58, 59]. Therefore, based on the principle of topology identification, the proteins with high aggregation in the interactions network graph are classified into different clusters. This study obtained 3 clusters composed by the differentially expressed proteins between APDG-2w and PDG. These proteins collaborate with each other to regulate cellular immunity and inflammatory responses, which has also been elucidated in a previous review [60]. What’s more, inflammation reaction is associated with pain mechanisms, as well as contributes to the pathogenesis of disc degeneration. The presence of inflammatory mediators can be systemically determined in the blood, and these biomarkers show a potential to be applied as novel markers for diagnosing and treating patients with disc degeneration diseases.

Conclusions

In conclusions, this study produced point-reference datasets of normal rats, physiological degeneration rats, and needle puncture rat models. In dependence on a methodological framework, the protein composition and cellular functions were revealed. We found differences in protein composition and expression between needle puncture rat models and physiological degeneration group. This result facilitates the screening of similar proteins in the model and pathology groups of disc degeneration, which is beneficial to subsequent pathological studies.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical approval All animal experiments were approved by the Animal Ethical and Welfare Committee of SooChow University.

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