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Proteomic Analysis of the Immunosuppressive Effects of Mesenchymal Stem Cells in a Rat Heart Transplantation Model

Analiza proteomiczna działania immunosupresyjnego mezenchymalnych komórek macierzystych na modelu zwierzęcym z wykorzystaniem serca szczurów

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article; G – other

Abstract

Background. Some reports suggest mesenchymal stem cells (MSCs) have immunosuppressive properties. However, conflicting evidence regarding the role of MSCs has emerged.

Objectives. To gain a better understanding of the immunosuppressive properties of mesenchymal stem cells (MSCs) in a rat heart transplantation model.

Material and Methods. MSCs were obtained from the femoral and tibial bone marrow of Sprague-Dawley rats and cultured. Heart-transplanted rats were allocated into a MSC-treated group and 2 control groups. On postoperative day 7, 1 rat was sacrificed and the pathological changes of heart tissues were assessed. Serum proteomic spectra were generated by surface-enhanced laser desorption/ionization-time-of-flight mass spectrometry (SELDI-TOF-MS).

Results. Rat MSCs displayed the typical spindle-shaped morphology in culture and significantly prolonged the graft survival up to 33.25 ± 2.54 days compared with controls (19.75 ± 1.56 and 11.16 ± 1.34 days, respectively). Pathological analysis showed the inflammatory cell infiltration in the MSC-treated group was significantly reduced. SELDI analysis showed that 5 protein/peptide peaks with M/Z 1272.33, 1986.65, 2323.42, 5375.59 and 12968.11 were up-regulated in the MSC-treated group ($P < 0.001$).

Conclusions. Donor-derived MSCs clearly alleviate acute rejection following heart transplantation in rats and significantly prolong the isograft survival time (*Adv Clin Exp Med* 2013, 22, 6, 785–794).

Key words: rat heart transplantation, immunosuppression, mesenchymal stem cells, SELDI ProteinChip, decision tree.

Mesenchymal stem cells (MSCs) are found in a variety of sources, including the skin, bone marrow and adipose tissue, and can be differentiated into chondrocytes, osteoblasts, and adipocytes *in vitro*; therefore, MSCs was considered to be potential candidates for cellular and genetic therapy.

Emerging evidence suggests the immunosuppressive function is one of the most important profiles of MSCs. It has been successfully applied in animal models and clinical trials. Different studies

have attributed the immunosuppressive effect of MSCs to different immunosuppressive factors. However, conflicting evidence regarding the immunosuppressive properties of MSCs has also been noted. The immunosuppressive mechanism was not well-understood, but several possible mechanisms have been advanced: 1) MSCs can modulate the functions of T-lymphocytes, B-lymphocytes, natural killer cells and dendritic cells; 2) MSCs can produce some soluble immunosuppressive factors;

3) MSCs highly express MHC I, but do not express the FAS ligand, the MHC μ and other costimulatory molecules. These characteristics make MSCs promising for the application in solid organ transplantation to prolong the graft survival.

To clarify the possible modulation mechanism of MSCs on the transplant rejection, a preclinical model of heart transplantation in rats was used and serum proteomic pattern was analyzed using the SELDI ProteinChip technology. Our results showed that donor-derived MSCs could significantly decrease the acute rejection following heart transplantation, prolong the survival time, and represent a promising candidate in clinical tissue transplantation.

Material and Methods

Animals

Healthy Sprague-Dawley (SD) and Wistar rats (160–200 g) were obtained from the Experimental Animal Center of Sun Yat-sen University, China. All animals were held under standard laboratory conditions at constant temperature, humidity, and light/dark cycles. They were fed with standard diet and had free access to tap water. All animals received care in compliance with the Principles of Laboratory Animal Care and the experimental protocol was approved by the local Animal Care and Research Committee.

Isolation and Culture of MSC

Male SD rats (3–4 weeks old) were sacrificed by cervical dislocation. Bone marrow cells were flushed out of the femoral and tibial cavities, washed twice with PBS buffer and re-suspended in DMEM medium (Gibco, Grand Islands, N.Y., USA) containing 10% fetal calf serum (FCS, Gibco). Following the determination of cell viability and numbers, bone marrow mononuclear cells were transferred to culture flasks with control medium consisting of DMEM with fetal bovine serum (10%), penicillin (100 U/mL) and streptomycin (100 μ g/mL). The re-suspended cells were incubated at 37°C with 5% CO₂, and the unattached cells were removed by medium replacement. The medium was changed every 24 h. When the cultures reached 50–70% confluence, the cells were harvested by centrifugation and washed twice with PBS buffer, then trypsinized by 0.25% trypsin and passaged. The cells at passage 3 were re-suspended in basal DMEM at a density of 1×10^6 cells/mL and used for transplantation.

Flow Cytometric Analysis of MSCs

The 3rd passage cells were collected by centrifugation at 600 g for 5 min at 25°C, washed twice with PBS buffer, digested with trypsin (0.25%) – EDTA (0.02%) and washed 3 times with PBS. Cell pellets were obtained by the centrifugation at 1000 rpm for 10 min, then re-suspended in PBS with 10% FBS. CD44-FITC and CD45-FITC antibodies (Pharmacia Corporation, USA) were respectively added and incubated in the dark at 4°C for 30 min. FITC-conjugated IgG antibodies served as controls. Then cells were centrifuged at 1400 rpm for 5 min, washed with PBS and fixed by 1% formaldehyde in PBS buffer for 10–30 min. Finally, cells were analyzed in a FACSCanto II Flow Cytometer.

Application of MSCs in a Rat Heart Transplantation Model

Rats were allocated to 3 groups: isograft group (group I, n = 8), from SD rats to SD rats; allograft group, from SD rats to Wistar rats and then divided into a control group (II, n = 8) and a MSC-treated group (III, n = 8). Abdominal cardiac transplantation was performed using the modified method as described. In brief, ketamine mixed with xylazine was given intraperitoneal injection for anesthesia. Donor hearts were perfused with chilled, heparinized saline via the *vena cava* and harvested after ligation of the *vena cava* and pulmonary veins. The aorta and pulmonary artery of donor hearts were anastomosed to the abdominal aorta and inferior *vena cava* of recipients using a microsurgical technique. Before the anesthesia recovery period, recipients (Group III) were injected with donor-derived MSCs (1.0×10^6 cells/rat) via the tail vein, followed by 3 additional doses each day for 3 consecutive days' post-transplantation. Group I and II were injected with PBS and used as control. All experimental groups were checked by daily abdominal palpation. 1 microliter of blood was obtained from the tail vein of each group (I, II and III) at Day 1, 3 and 5 after transplantation, respectively. The samples were stored at –20°C and used for protein-chip analysis.

Histopathological Examination

One rat from each group was sacrificed at 7 days post-transplantation. The transplanted hearts were removed, fixed in 4% formaldehyde and paraffin-embedded. Sections (5 μ m in thickness) were stained with hematoxylin and eosin for histopathological examination.

SELDI-TOF-MS Analysis

Mass-spectrum analysis was performed as previously described with some modifications. Briefly, all blood samples were thawed and centrifuged at 10,000 rpm for 5 min at 4°C. 10-microliter supernatant was diluted with 10 μ L U9 buffer (9 mol/L urea, 2% CHAPS, and 1% DTT). Then, the samples were continuously diluted 20-fold in binding buffer (50 mmol/L NaAc, pH 4). The weak cation exchange protein chips (CM10) (Ciphergen Biosystems) were put into a bioprocessor, and 200 μ L binding buffer was added to each well with gentle agitation at 600 rpm at 25°C for 5 min; this step was repeated once. Next, 100 μ L diluted sample was loaded on each spot. The processor was sealed and shaken at 600 rpm at 4°C for 1 hour to remove unbound samples. The chips were rinsed twice with 200 μ L binding buffer and 1 time with 200 μ L HPLC grade water, covered with 1 μ L saturated sinapinic acid (SPA) solution (prepared in 0.5% trifluoroacetic acid (TFA) and 50% acetonitrile) and air-dried at room temperature.

SELDI ProteinChip Analysis

The chips were scanned by the ProteinChip (Model PBS Iic) reader (Ciphergen). The parameters were as follows: mass range (0 to 50,000 Da), optimized mass-to-charge ratio (1,000 to 50,000 Da), laser intensity (240), laser shots (112), and sensitivity (8). All-in-one protein marker containing arg 8-vasopressin (1084.2 Da), somatostatin

(1637.9 Da), sovine insulin beta-chain (3495.9 Da), human insulin (5807.6 Da) and Hirudin BHVK (7033.61 Da) (Ciphergen Biosystems, USA) (Fig. 7) was used as the calibration. The peak intensities were normalized by the background subtraction. Protein peaks were calculated using the biomarker detection software (Ciphergen Biomarker Wizards, Ciphergen Biosystems, Inc). Spectra were analyzed using the ProteinChip (Version 3.2.1) in Biomarker Wizard software (Ciphergen Biosystems) as previously described. The baselines were normalized, subtracted with the signal-noise ratio threshold of 5. Protein expression patterns were clustered with GENE-E (<http://www.broadinstitute.org/cancer/software/GENE-E/index.html>).

Decision Tree Construction

The decision tree was constructed by Biomarker Patterns Software 5.0 (BPS; Ciphergen Biosystems). The splitting decisions were made based on the normalized peak intensities. The process was continued until terminal nodes were created. After cross-validation in test model, the decision tree was further confirmed using the test data that was independent of the training set.

Statistical Analysis

The data was expressed mean \pm standard deviation (SD). Significant differences in the peaks intensity of proteins between each group were

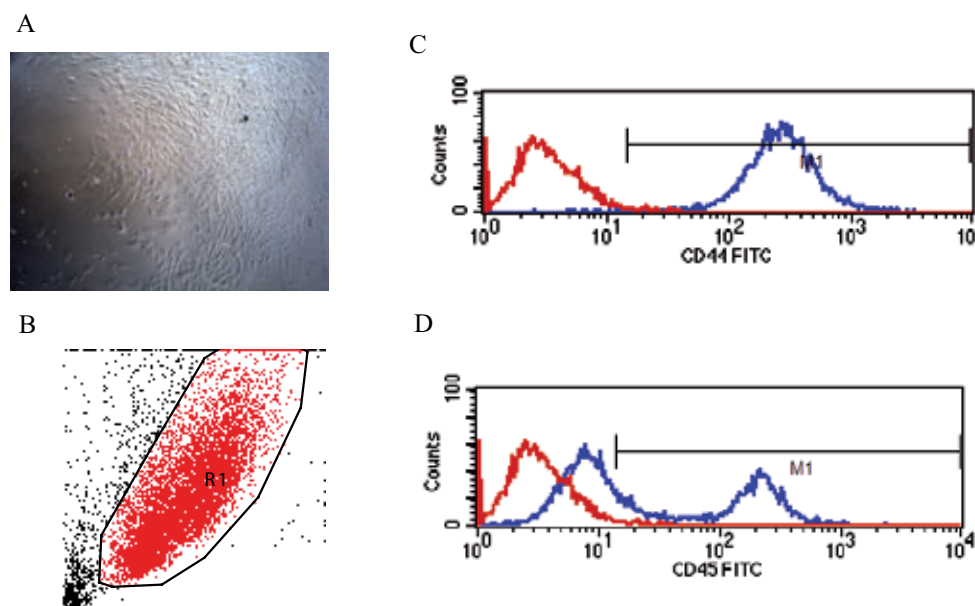


Fig. 1. Measenchymal stem cells from adult rat bone marrow were examined under microscopy and stained by hematoxylin-eosin. A: MSCs (cultured after 48 h) were observed under light microscopy at magnification ($\times 100$); B: FACS analysis of the third passage MSCs; C and D: The expression of CD44 (B), and CD45 (C) were evaluated by flow cytometry and the results were positive for CD44⁺ and negative for CD45⁻. The red line shows the negative control

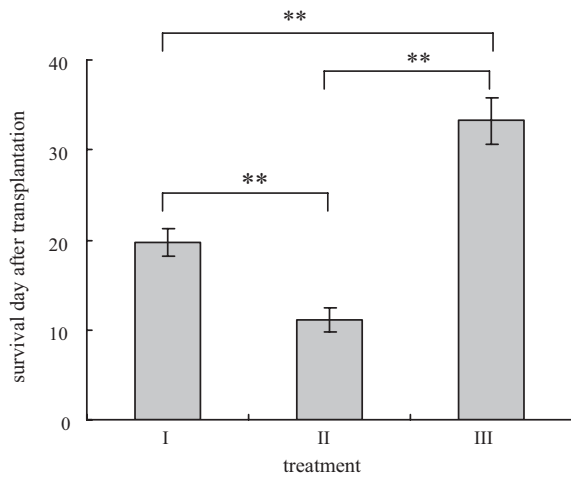


Fig. 2. Survival time of heart transplanted rats in different groups. Group I: The transplanted SD rats were treated with PBS buffer (SD rats as donors and recipients, $n = 16$); Group II and III: the SD rats were used as donor ($n = 8$ /each group) and Wistar rats as recipients ($n = 8$ /each group). The control group II was injected with PBS, while the experimental group III was injected with MSCs. ** $P < 0.01$

calculated. Statistical analysis among groups was performed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) multiple range test. The data that did not fit a normal distribution was analyzed by Kruskal-Wallis H test. All of the statistical analysis was carried out using SPSS version 16.0. Statistical significant difference was defined as $p < 0.01$.

Results

Characterization of Rat MSCs

After 48 hours culture, attached cells displayed an oval-, polygon- or short spindle-shaped fibroblastic morphology. One week later, on most of the cells there distinctly appeared a fibroblast-like, spindle-shaped morphology and these cells

exhibited an enlarged cell size, heterogeneous cell projection in different length, a large round nucleus with an unclear outline, and visible nucleoli. On the 12th day, the attached monolayer cells were harvested as the 1st passage. As the culture proceeded, the cells were distributed evenly with the same growth pattern, and presented a typical fibroblast-like morphology (Fig. 1A), which was consistent with the morphology of bone marrow-derived stem cells. Cell proliferation speed was increased to about 9 days. MSCs were still in latent phase after being subculture for 2 days, followed by logarithmical proliferation from day 3, and reached the growth platform at day 7.

Flow cytometry analysis confirmed that the cell population were consistent between samples and then were purified and homogeneous. Most of the cultured MSCs were positive for CD44 and negative for hematopoietic stem cell marker CD45 (Fig. 1C and 1D), which were in good agreement with the surface marker phenotype of MSCs.

Application of MSCs to an Allogeneic Heart Transplantation Model

To elucidate whether the immunomodulative effects of rat MSCs *in vivo*, MSCs were applied to a heart transplantation model. The process of donor-preparation and heart-harvesting took about 8 ± 2 min, and reception or vascular preparation took 4 ± 2 min. Abdominal aorta anastomosis time was 12 ± 2 min and inferior *vena cava* anastomosis time was 9 ± 2 min. The average cold ischemia time was 36 ± 3 min. The recipient rats usually took 2 ± 1 min to restart the blood supply of the heart and start ventricular fibrillation after operation. Taken together, the whole surgical procedure lasted 53 ± 7 min.

The mean allograft survival of control group I and II were 19.75 ± 1.56 days and 11.16 ± 1.34 days,

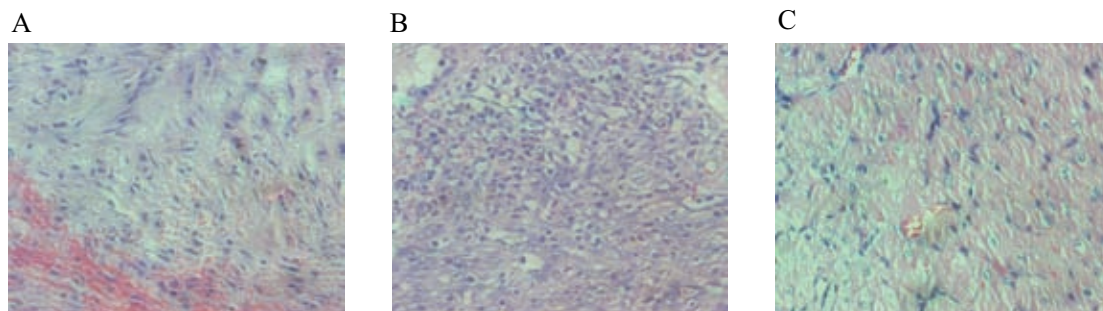


Fig. 3. Histological examination of heart tissue of the transplanted rats from different groups (HE stain $\mu \times 400$). A: The transplanted SD rats were treated with PBS (SD rats as donors and recipients, $n = 16$); B and C: the SD rats were used as donor ($n = 8$ /each group) and Wistar rats as recipients ($n = 8$ /each group). The control group II (B) was injected with PBS, while the experimental group III (C) was injected with MSCs

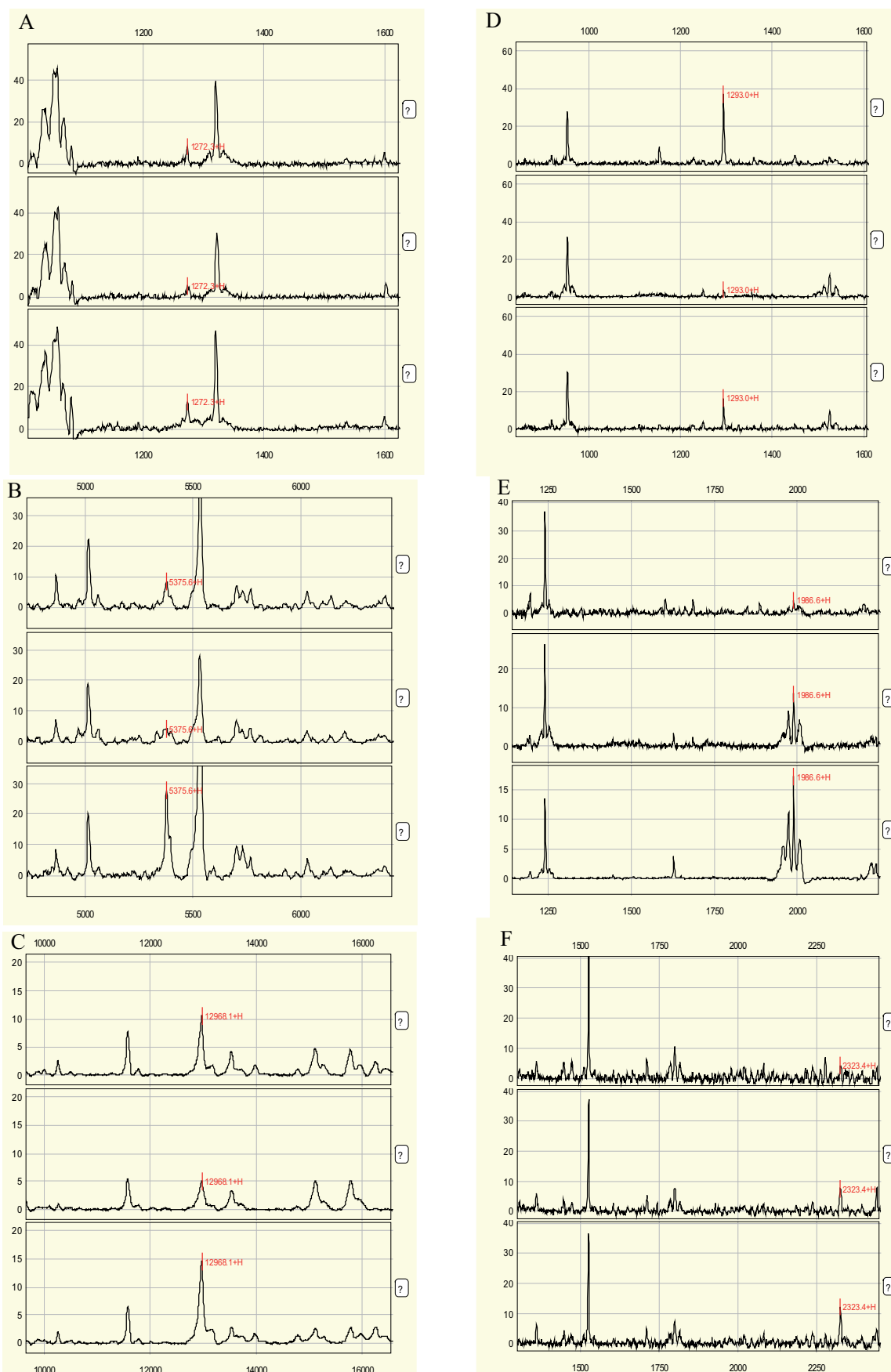


Fig. 4. Representative overview of protein profiling of serum from the heart transplanted rats. Group I: The transplanted SD rats (SD rats as donors and recipients, n = 16) were treated with PBS; Group II and III: the SD rats were used as donor (n = 8/each group) and Wistar rats as recipients (n = 8/each group). The control group II was injected with PBS, while the experimental group III was injected with MSCs. Differentially expressed proteins were (A) N¹⁰⁷, (B) N²²⁴, (C) N²⁵⁷, (D) N¹¹², (E) N¹⁷⁶ and (F) N¹⁸⁴. The x-axis represents the molecular mass calculation (mass-to-charge ratio [m/z]) and the y-axis represents the relative intensity. The peaks were indicated using red line and marked with m/z

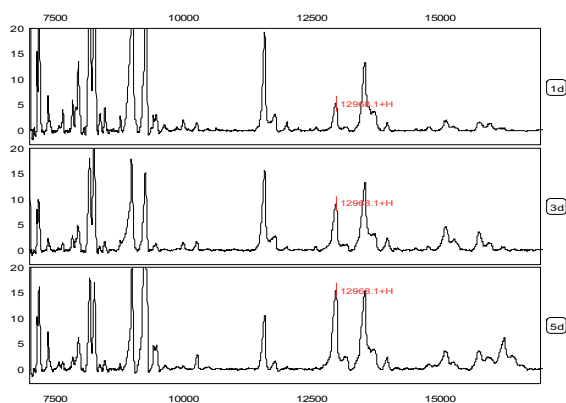


Fig. 5. Differential expression profile of protein N²⁵⁷ with m/z 12968.1 at different day after MSCs treatment for heart transplantation rats. The x-axis represents the molecular mass calculation (mass-to-charge ratio [m/z]) and the y-axis represents the relative intensity. The peaks were indicated by red line and marked with m/z

respectively. Donor-derived MSCs significantly prolonged graft survival up to 33.25 ± 2.54 days (Fig. 2). These results showed that rat donor-derived MSCs

effectively prolonged the graft survival compared to control group I and II ($p < 0.01$). Furthermore, graft rejections were significantly different between control group I and II treated with PBS ($p < 0.01$).

Histopathological Examination

The representative heart tissues of 7-day transplantation rats from different groups were shown in Fig. 3A–3C. Pathological examination revealed that in the control group I there was moderate disorder in myocardial tissue with moderate cellular swelling and inflammatory cell infiltration (Fig. 3A). In group II, there were cardiac cells necrosis, cellular swelling, sever acute rejection and inflammatory cell infiltration in myocardial interstitial (Fig. 3B). In contrast to these histopathological observations, in group III, pathological examination showed that there were no mild or moderate acute rejections and the transplanted heart has normal myocardial tissue structure with significantly alleviated inflammatory cell infiltration (Fig. 3C).

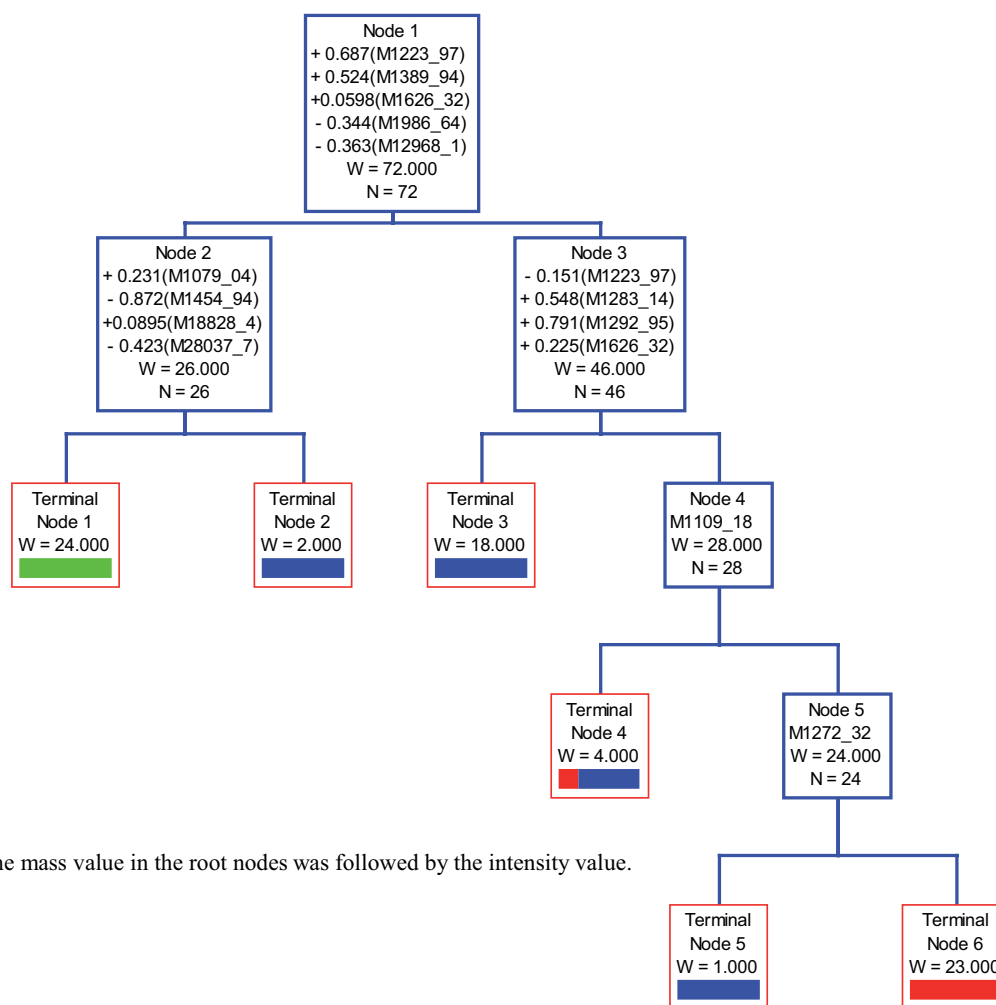


Fig. 6. Diagram of a decision tree for the classification of the PBS and MSCs treated heart transplanted rats in the learning dataset. The mass value in the root nodes was followed by the intensity value

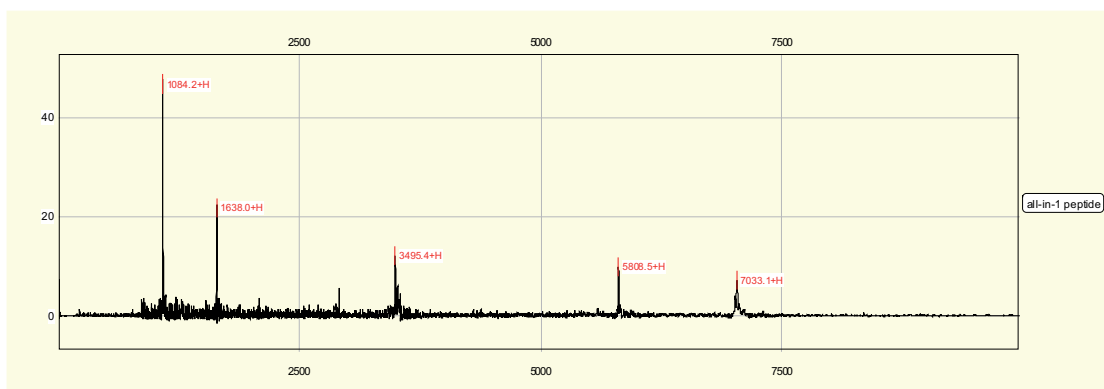
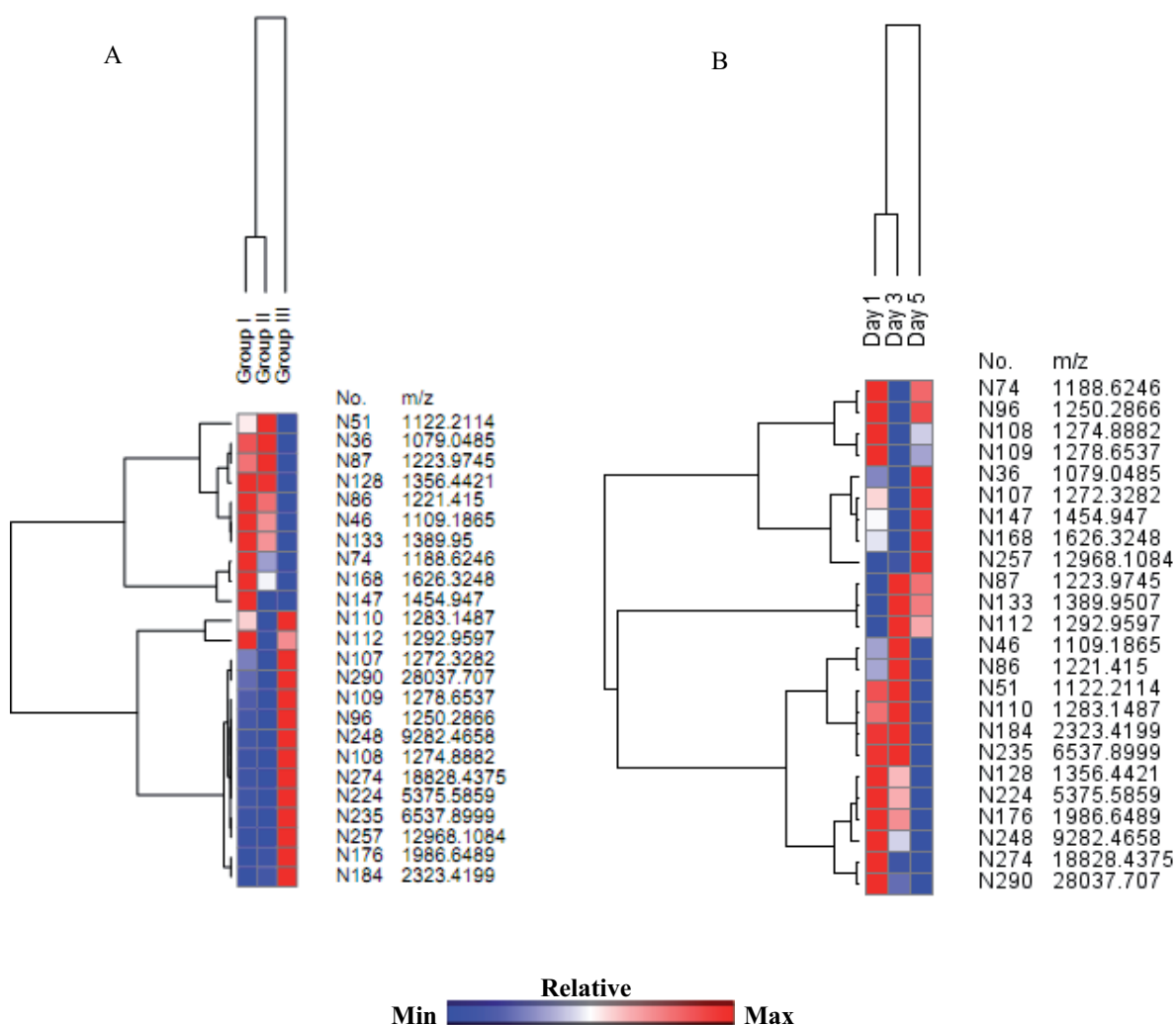


Fig. 7. All-in-one peptide reference standard used for optimization of the experimental conditions and evaluation of the reproducibility. Arg8-vasopressin (1084.2 Da), somatostatin (1637.9 Da), sovine insulin beta-chain (3495.9 Da) human insulin (5807.6 Da) and Hirudin BHVK (7033.61 Da)



Red represents maximal expression level and blue represents minimum expression level.

Fig. 8. A hierarchical clustering analysis of twenty four differentially expressed proteins/peptides in group-model (A) and day-model (B). Red represents maximal expression level and blue represents minimum expression level

Serum SELDI Profiles of MSCs Treated Rats vs. Controls

After noise filtering, clustering analysis revealed that 313 proteins were differentially expressed among different groups. Further analysis resulted in a subset of 83 proteins which were differentially expressed between MSCs treated samples and controls ($p < 0.05$). In this subset, 24 proteins were detected to be significantly different at $p < 0.01$ level. To search for the classification and comparability information of those differential protein/peptide peaks, a hierarchical clustering analysis among group I, II and III was performed. The results indicated that the relative expression level of some proteins was not significant difference between group I and II samples (Fig. 8). Further analysis showed 6 proteins were significantly difference among groups ($p < 0.001$) (Fig. 4). They were N¹⁰⁷ with m/z 1272.33 ($p = 3.36 \times 10^{-4}$), N¹¹² with m/z 1292.96 ($p = 2.44 \times 10^{-4}$); N¹⁷⁶ with m/z 1986.65 ($p = 3.09 \times 10^{-4}$), N¹⁸⁴ with m/z 2323.42 ($p = 4.08 \times 10^{-4}$), N²²⁴ with m/z 5375.59 ($p = 1.55 \times 10^{-4}$) and N²⁵⁷ m/z 12968.11 ($p = 3.25 \times 10^{-4}$).

To study the protein expression variations in MSCs treated groups, SELDI-TOF-MS data for day 1, 3 and 5 post-postoperative were analyzed by ANOVA. The results showed the expression pattern of N¹⁰⁷ was day 5 > day 1 > day 3. For N¹¹², the expression profile was day 1 > day 5 > day 3. N²⁵⁷ was up-regulated gradually from day 1–day 5 (Fig. 5). The expression of N¹⁷⁶, N²²⁴ and N²⁴⁸ were higher at day 1 and then declined with time (Fig. 8).

Decision Tree

After discriminatory analysis, 13 of 24 protein mass peaks (Table 1) were chosen by optimization to distinguish the differentially expressed serum proteins. The most optimal decision tree with the highest accuracy was established. (Fig. 6). The decision tree used 5 splitters with distinct masses and classified the samples into 6 nodes. The optimal decision tree manifested 95.8% (23/24), 100% (24/24) and 100% (24) accuracy for classifying group I, II and III, respectively.

Discussion

Immunologic rejection of a grafted organ remains a common problem after transplantation. Usually acute rejection is alleviated by using high doses of immunosuppressive drug with side-effects and severe toxicity. Therefore, it is imperative to find a safe and effective way to reduce immunologic rejection.

Table 1. Important peaks selected by the biomarker pattern software and used for decision tree analysis in grouping model

Peak, m/z	Score	
12968.1	100.00	
5375.58	91.00	
1272.32	86.74	
28037.7	65.94	
1274.88	61.81	
1283.14	38.71	
1292.95	33.17	
1389.94	25.10	
1109.18	25.10	
1278.65	20.04	
1454.94	13.99	
18828.4	12.93	
1188.62	12.12	

Note: Peaks were named by their mass to charge ratio (m/z). The most important peak was assigned an importance index of 100. The importance of other peaks was compared with the top peak and a number below 100 was given for each peak.

MSCs are considered a promising option for improving graft survival and achieving immunological tolerance without pharmacological immunosuppression. Several studies reported that MSCs can evade immune recognition and modulate immune responses. One of the first *in vivo* studies showed that systemic infusion of MSCs isolated from bone marrow prolonged the survival of allogeneic skin graft from 7 to 11 days in baboon. In other studies, Bartholomew et al. reported that a single infusion ($1-3 \times 10^6$ cells/kg) of allogeneic or third-part MSC prolonged skin graft survival in a baboon. In contrast, similar MSCs dosing (2×10^6 cells/rat) was applied before and repeatedly after the heart transplantation and significantly prolonged the survival of rat cardiac allograft. Further analysis based on real-time PCR showed that the induction of allograft tolerance through changing the Th1/Th2 balance. In a more recent study, Biancamaria et al. reported MSCs alone were able to prolong lung-graft survival time in rat model. On the contrary, Inoue S et al. reported MSCs alone could not prolong graft survival in a rat heart transplantation model. The different effects observed among different applications may be caused by the source, purity, site, dose and time of MSCs application, and the state of activation of cells for maximal toleragenic effects.

To develop MSC-based therapies for tolerance induction in clinical organ transplantation, the cellular mechanisms of their action must be understood thoroughly. Therefore, donor-derived MSCs (1×10^6 cells/rat) were evaluated in heart-transplanted rat model in the present study. Consistent with previous reports, donor-derived MSCs are able to alleviate inflammatory cell infiltration and significantly prolong graft survival.

83 proteins differentially expressed between experimental and control groups were identified by SELDI-TOF MS technology. Five of them were significantly up-regulated in the experimental group ($p < 0.001$). As serum protein profile alternates with immune rejection, we deemed that the up-regulated expression pattern of N²⁵⁷ would play an important role in immunosuppression or anti-inflammation. On the contrary, the expression pattern of N¹⁷⁶, N²²⁴ and N²⁴⁸ implied that they

negatively function in heart transplantation survival. Although biomarkers generated with SELDI-TOF technology were anonymous, the technology is very reliable in generating biomarkers signatures for the estimation of the total treatment effect on the serum proteome.

In conclusion, our results supported that donor-derived MSCs have immunosuppressive and/or anti-inflammatory properties *in vivo* and be able to prolong graft survival, opening new insights in the prevention and treatment of graft rejection in tissue and organ transplantation. However, further studies are needed to investigate the immunosuppressive mechanisms of MSCs based on the identification and characterization of these potential protein biomarkers, and the optimal conditions for handling stem cells. A careful evaluation of the harmful immunosuppressive effects of MSCs is also necessary.

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