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Fabrication of a Novel Hybrid Scaffold for Tissue Engineered Heart Valve*

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Summary: The aim of this study was to fabricate biomatrix/polymer hybrid scaffolds using an electrospinning technique. Then tissue engineered heart valves were engineered by seeding mesenchymal stromal cells (MSCs) onto the scaffolds. The effects of the hybrid scaffolds on the proliferation of seed cells, formation of extracellular matrix and mechanical properties of tissue engineered heart valves were investigated. MSCs were obtained from rats. Porcine aortic heart valves were decellularized, coated with poly(3-hydroxybutyrate-co-4-hydroxybutyrate) using an electrospinning technique, and reseeded and cultured over a time period of 14 days. In control group, the decellularized valve scaffolds were reseeded and cultured over an equivalent time period. Specimens of each group were examined histologically (hematoxylin-eosin [HE] staining, immunohistostaining, and scanning electron microscopy), biochemically (DNA and 4-hydroxyproline) and mechanically. The results showed that recellularization was comparable to the specimens of hybrid scaffolds and controls. The specimens of hybrid scaffolds and controls revealed comparable amounts of cell mass and 4-hydroxyproline ($P>0.05$). However, the specimens of hybrid scaffolds showed a significant increase in mechanical strength, compared to the controls ($P<0.05$). This study demonstrated the superiority of the hybrid scaffolds to increase the mechanical strength of tissue engineered heart valves. And compared to the decellularized valve scaffolds, the hybrid scaffolds showed similar effects on the proliferation of MSCs and formation of extracellular matrix. It was believed that the hybrid scaffolds could be used for the construction of tissue engineered heart valves.

Key words: tissue engineered heart valve; hybrid scaffold; electrospinning; mesenchymal stem cells

Heart valve tissue engineering represents a new multidisciplinary approach which combines basic research such as matrix, developmental and cell biology, biochemistry, and materials science with clinical disciplines such as surgery and immunology to replace diseased natural valves. Successful construction of tissue engineered heart valve (TEHV) depends on three basic elements: scaffold materials (matrix), seed cells, the interaction of the matrix and cells. The scaffolds ought to be biomimetic. In general, two approaches to the formation of TEHV can be distinguished by the nature of the scaffolding material: (1) bioresorbable polymer scaffold, and (2) decellularized valve scaffold[1]. Although elaborate manufacturing techniques using stereolithographic anatomical models have been used for polymeric heart valve scaffolds, this approach so far could not reproduce the functional complex 3-dimensional composite heart valve architecture and has limitations in cellular adhesion[2, 3]. The use of a decellularized valve scaffold offers the advantages of its innate anatomical architecture and remains the active sites for cell adhesion and growth factors, but its mechanical strength is decreased[4-7]. Because of the shortcomings of the two scaffolds, current studies of scaffolds are relatively stagnant. In this study, the two previous approaches were merged in a novel biomatrix/polymer hybrid scaffold in order to utilize the advantage of both decellularized valve scaffold and bioresorbable polymer scaffold. Hybrid scaffolds were fabricated from decellularized valve scaffolds and coated with P3/4HB using an electrospinning technique. Then TEHVs were engineered by seeding mesenchymal stem cells (MSCs) onto the hybrid scaffolds. And the effects of the hybrid scaffolds on the proliferation of MSCs, formation of extracellular matrix (ECM) and mechanical properties of TEHVs were investigated.

1 MATERIALS AND METHODS

1.1 MSCs Isolation and Culture

Whole bone marrow was aspirated from the tibia and femur of adult S-D rats (body weight 150–200 g).

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MSCs were isolated from rat bone marrow by washing in phosphate buffered saline (PBS) for 10 min at 1500 r/min. The supernatant was discarded, and the cells were resuspended in PBS. A low-density cell fraction of bone marrow was obtained by centrifugation of the cell suspension over a Ficoll step gradient (density 1.077 g/mL) (Sigma, USA) at 1500 r/min for 10 min. The nucleated cells were collected from the interface, diluted with two volumes of PBS, and centrifuged at 1500 r/min for 10 min. The cells were resuspended, counted, and inoculated in a density of at $2\times10^5/cm^2$.

The isolated cell fraction was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, HyClone, USA), penicillin (100 U/mL), and streptomycin (100 U/mL) in tissue flasks, and left to adhere at 37ºC for 4–5 h. The non-adherent cells were removed, while MSCs adhered, spread, and grew. Medium was replaced at 24th and 72nd h, and then every 3 days. Daily growth was observed under the phase-contrast microscopy. The cells were serially passaged, and expanded in a humidified incubator (37ºC, 5% CO_2). Sufficient cell number for cell seeding on scaffolds was obtained after 21–28 days. Cells were characterized before valve implantation.

1.2 Preparation of Decellularized Valve Scaffolds

Fresh porcine aortic valves were obtained from the local slaughterhouse. Hearts were obtained under cleaning conditions, and valve leaflets were excised. The leaflets were immediately stored in PBS at 4°C. Within 30 min, the leaflets were incubated in DMEM at 4°C for 12 h with 100 U/mL penicillin, 400 μg/mL gentamicin, and 500 μg/mL Amphotercin B for sterilization. Then the leaflets were placed in a solution of 0.05% trypsin and 0.02% EDTA at 37°C for 12 h in PBS. The leaflets were subsequently treated with 1% tert-octylphenylpolyoxyethylen (Triton X-100, Sigma, USA) in PBS for 48 h at 4°C. Then the leaflets were treated with RNase (20 μg/mL, Sigma, USA) and DNase (200 μg/mL, Sigma, USA) in PBS for 1 h at 4°C. All steps were conducted in an atmosphere of 5% CO_2 and 95% air at 37°C under continuous shaking. In the end, decellularized valve scaffolds were created. The decellularized valve scaffolds were washed with PBS several times to remove residual substances and then stored in PBS at 4°C before further processing and seeding[8].

1.3 Preparation of Hybrid Scaffolds

Solution was prepared by dissolving the purified poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P3/4HB, PHA 3444, TEPHA Inc., GB) (0.2 g) in dichloromethane (10 mL) at room temperature with stirring for 3 h. Electrospinning was carried out with a high voltage power supply set (DC high-voltage generator, BMEI Co, Ltd, China) at 20 kV with a collection distance between 15 and 20 cm. A 5 mL glass syringe with a tip-blunt needle (0.9 mm in diameter) was used to store the polymer solution for electrospinning[9]. Polymeric fibers were collected on the surface of the decellularized valve scaffolds until the required thickness of membrane was achieved. In the end, the decellularized valve scaffolds were coated with P3/4HB fibers, and hybrid scaffolds were created.

1.4 Cell Seeding and *in vitro* Culture

Hybrid scaffolds served as experimental group, decellularized valve scaffolds as control group. MSCs were seeded onto the scaffolds of two groups with an approximate cell density of $5\times10^6/cm^2$. Then the reseeded hybrid scaffolds and decellularized valve scaffolds were cultured in DMEM supplemented with 10% FBS. All reseeded scaffolds were cultured *in vitro* for 14 days in the humidified incubator (37ºC, 5% CO_2).

1.5 Cell Characterization

A single cell suspension of MSCs was prepared for flow cytometry. 1×10^6 cells in 100 mL PBS plus bovine serum albumin (BSA, Sigma, USA) were incubated with saturating concentrations of monoclonal antibodies CD29-FITC (Caltag, USA), CD 44-FITC, CD 14-FITC and CD 45-FITC (Beckon Dickinson, USA). Samples were analyzed with the flow cytometer FACS-Calibur (Becton Dickinson Immunocytometry Systems, USA). Data analysis was performed with the CELL QUEST software program (Becton Dickinson Immunocytometry Systems, USA).

Immunocytometry was performed by incubation with monoclonal mouse antibodies for α-SMA (Sigma, USA) and vimentin (NeoMarkers, USA)[10]. Incubation with a secondary biotin-labeled goat-anti-mouse IgG antibody (Sigma, USA) was performed, and the signal was developed with the avidin-peroxidase system (ABC kit, Vector Lab, USA). Prior to intracellular staining, permeabilization of the cells was performed by incubation with 0.1% Triton for 10 min. Cells were examined by fluorescence microscopy.

1.6 Histomorphology

After being cultured *in vitro* for 14 days, specimens of two groups (n=6 each) were fixed in neutral formalin for 15 min, and embedded in paraffin for hematoxylin-eosin (HE) staining. Immunohistostaining (n=6 each) was carried out using immunofluorescence techniques. Monoclonal primary antibodies were α-SMA. A FITC-conjugated rabbit anti-mouse antibody (Sigma, USA) served as secondary antibodies. Negative controls with the absence of the primary antibodies were demonstrated. Sections were analyzed and documented by fluorescence microscopy. The hybrid scaffolds (n=6 each) and specimens of two groups (n=6 each) were examined by scanning electron microscopy (SEM) (Hitachis-520 ESEM, Japan) for microstructure and surface morphology.

1.7 Biochemical Assays

After being cultured *in vitro* for 14 days, biochemical assays were performed for analysis of cellular and extracellular components of specimens of two groups (n=4 each). Crude protein extracts were prepared by manual disruption of the entire specimen using a pistil and mortar. Total DNA was isolated and purified using a QIAamp DNA Mini Kit 50 (Qiagen, USA). And the absorbance (A) values were measured using an spectro-

photometry at wavelength of 260 nm[11]. Total 4-hydroxyproline (4-OHP) determination was performed using a hydroxyproline detection kit (Jiancheng Bioengineering Institute, China).

1.8 Mechanical Tests

The mechanical tests were performed on an AGS-J electronical universal test at ambient temperature. Specimens were attached at both ends to a nontraumatic clamp and subjected to tensile loading to failure[12].

1.9 Statistical Analysis

All values were expressed as $\bar{x}\pm s$. The significance of differences between specimens of two groups was estimated by analysis of variance (Independent-Samples *t* Test) using the commercially available software package SPSS for windows, version 12.0. *P* values less than 0.05 were considered statistically significant.

2 RESULTS

2.1 Characterization of the MSCs

Flow cytometry characterization of rat MSCs demonstrated that these cells expressed CD29 (94.82%) and CD44 (93.59%), and did not express the leukocyte common antigen CD14 (0.49%) or CD45 (2.00%) (fig. 1).

MSCs at low density displayed the spindle-shaped morphology characteristic of fibroblast-myofibroblast cell lineage. Immunocytochemistry showed the expression of a-SMA and vimentin by MSCs (fig. 2).

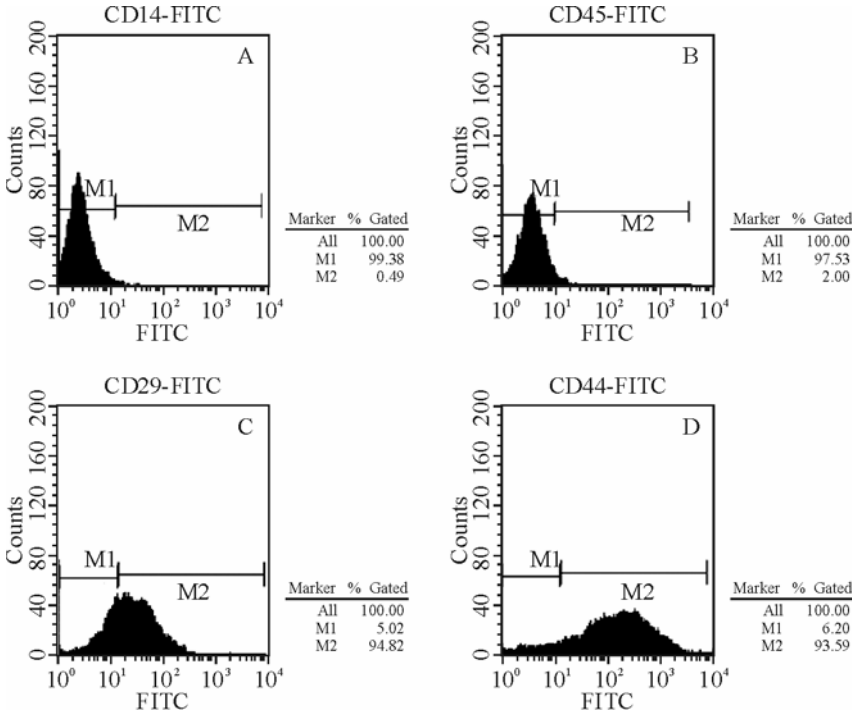

Fig. 1 Flow cytometry of rat MSCs
A: CD14-FITC; B: CD45-FITC; C: CD29-FITC; D: CD44-FITC

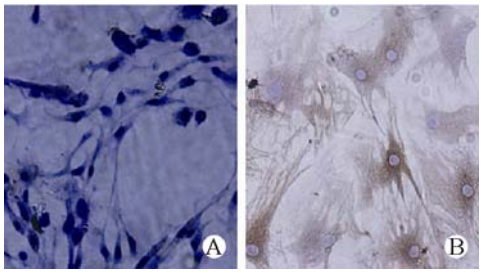

Fig. 2 Immunocytochemistry of rat MSCs
A: α-SMA (×100); B: vimentin (×100)

2.2 Histomorphological Analysis of Engineered Heart Valve

A comparable cell lining of the entire surface of the hybrid scaffolds and controls was demonstrated by HE staining and immunostaining (fig. 3A). MSCs were seen well attached to the scaffolds of two groups (fig. 3B). Cells in all specimens showed a strong expression of α-SMA throughout the entire surface of the scaffolds (fig. 3C, and 3D). The electrospun membrane of P3/4HB firmly combined with the surface of the decellularized valve scaffold and showed uniform structure (fig. 4A, and 4B). And confluent MSCs clusters of the entire surface of the hybrid scaffolds and controls could be demonstrated by SEM (fig. 4C, and 4D).

2.3 Biochemical Assays

Analysis of the hybrid scaffolds, as well as the controls, showed almost comparable DNA contents and amounts of 4-OHP ($P>0.05$, table 1).

Table 1 Assays for quantification of cell mass (DNA) and extracellular matrix components

Groups	DNA (ng/mg)	4-OHP (μg/mg)
Experimental	46.66 ± 4.10	5.45±0.37
Control	47.36±3.02	5.57±0.24

All sample data are shown according to equivalent dry weight in mg

2.4 Mechanical Tests

Compared to the controls, mechanical testing of the hybrid scaffolds demonstrated a significant increase in the Max-load, Max-stress and elastic modulus (P<0.05). And there was no statistically significant difference in Max-strain between two groups (P>0.05, table 2).

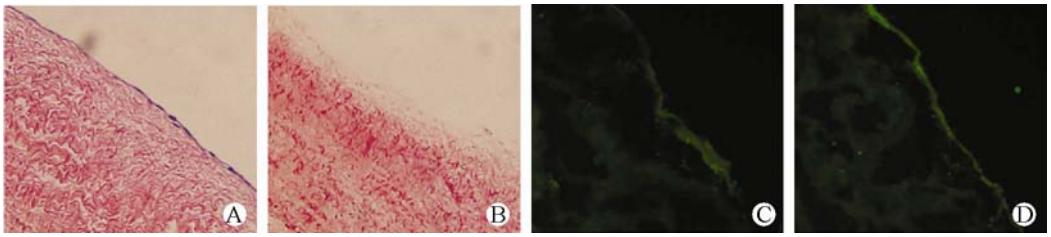

Fig. 3 Specimens of the control group (A) and hybrid scaffolds group (B) (HE×100), and specimens of the control group (C) and hybrid scaffolds group (D) (Immunohistochemical staining×100)

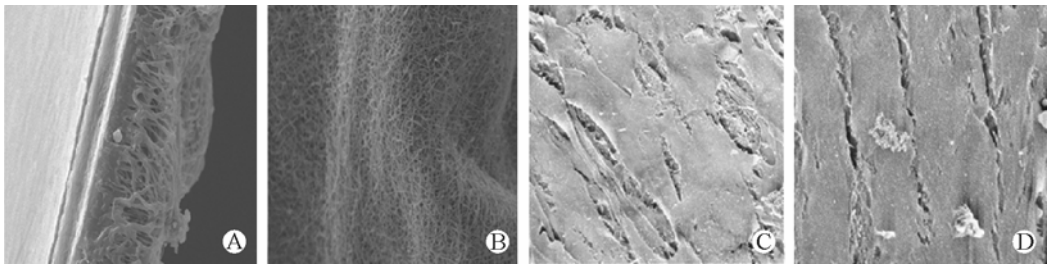

Fig. 4 Under SEM, transverse view of the hybrid scaffold (A, ×600), superficial view of the hybrid scaffold (B, ×150), specimens of the control group (C, ×700), and specimens of the hybrid scaffolds group (D, ×700)

Table 2 Mechanical tests ($\bar{x}$±s)

Groups	Max-load (N)	Max-stress (N/mm^2)	Max-strain (%)	Elastic modulus (N/mm^2)
Experimental	12.086±1.532*	2.446±0.173*	37.462±3.209	115.665±16.854*
Control	8.180±1.152	1.904±0.092	38.083±2.636	77.512±13.639

*P<0.05 versus control group

3 DISCUSSION

Scaffolds, primarily providing anchoring substrate for cells, are the key element to influence the cellular behaviors including proliferation, differentiation, and morphogenesis of new tissue in tissue engineering. We believe that hybrid scaffolds can utilize the advantages of both bioresorable polymer scaffold and decellularized valve scaffold. First, the scaffords are fabricated from decellularized valve scaffolds, so that the bioinformation and complex 3-dimensional architecture of heart valve are retained. Second, the controllability of polymers in mechanical function, degradation, components, and process is retained. Third, the growth factors can be added to the scaffolds. In this study, hybrid scaffolds were fabricated from decellularized valve scaffolds and coated with P3/4HB using an electrospinning technique. And this study focused on the effects of the hybrid scaffolds on the proliferation of seed cells, formation of extracellular matrix and mechanical properties of TEHV.

In this study, MSCs were selected as seed cells. Flow cytometric analysis indicated that MSCs expressed CD29 and CD44, and did not express the leukocyte common antigen CD45 or CD14 expressed by monocytes, which distinguishes them from the much more numerous hematopoietic cell fraction of the marrow. Moreover, when cultured at low density, MSCs display the spindle-shaped morphology characteristic of fibroblast-myofibroblast cell lineage. They also express α-SMA and vimentin displayed by resting interstitial cell population of valves in culture. These findings demonstrated that MSCs isolated from rat bone marrow could be used to provide cells of fibroblast-myofibroblast lineage for the interstitial cell fraction of a substitute heart valve. Overall, morphology and ultra-structure analysis showed good cell-scaffold adhesion and MSCs growth in two groups. A compact layer of MSCs that expressed α-SMA in the hybrid scaffolds and controls was demonstrated by immunohistostaining. The complete and comparable repopulation of the hybrid scaffolds and controls was proven by HE staining and SEM. The hybrid scaffolds and controls revealed comparable DNA contents and amounts of 4-OHP. Our results showed that as compared to decellularized valve scaffolds, the hybrid scaffolds revealed similar effects on the proliferation of MSCs and formation of extracellular matrix.

The biopolymers used in this study were P3/4HB. A number of studies indicated a good biocompatibility of P3/4HB[13, 14]. Because of this characteristic, our results showed that the electrospun membrane of P3/4HB firmly combined with the surface of the decellularized valve scaffold. And the good biocompatibility of P3/4HB is benefit to the cell–P3/4HB–decellularized valve scaffold

interaction. In addition, the mechanical properties of P3/4HB, such as pliability and elasticity, make them very suitable for application in soft tissue engineering[13, 14]. Electrospinning is a useful technique for producing nonwoven, porous, three-dimensional scaffolds containing fibers, ranging in diameter from tens of microns to tens of nanometers[15, 16]. This technology offers the potential for controlling the composition, structure, and mechanical properties of biomaterials. In addition, the potential of applying nano-scale scaffolds in tissue engineering is significant since it is believed that the nano-scale dimension provides a well-defined architecture with high surface area-to-volume ratio. The fibers produced from P3/4HB randomly deposited on the surface of decellularized valve scaffolds, and formed membrane structure with uniform thickness. And compared to the controls, mechanical testing of the hybrid scaffolds demonstrated a significant increase in the Max-load, Max-stress and elastic modulus. These results demonstrated that TEHV engineered by hybrid scaffolds showed a significant increase in mechanical strength. This could be attributed to the following reasons: (1) after the coating with P3/4HB, the thickness of the scaffolds was increased. And the increase in strength of TEHV was partly related to the increase in thickness; (2) the P3/4HB based tissues showed supraphysiologic mechanical strength[15, 16], and (3) the anisotropy of the P3/4HB fibers that carried part of the stress and leaded to an increase in tensile strength under load.

In conclusion, hybrid scaffolds were fabricated from decellularized porcine aortic heart valve scaffolds and coated with P3/4HB using an electrospinning technique. Then TEHVs were engineered by seeding MSCs onto the hybrid scaffolds. We found the superiority of the hybrid scaffolds to increase the mechanical strength of TEHVs. And as compared to the controls, the hybrid scaffolds showed similar effects on the proliferation of MSCs and formation of extracellular matrix. We believe that the hybrid scaffolds could be useful for the construction of TEHVs.

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