Preliminary fabrication of tissue engineered veins containing valves using bone marrow mesenchymal stem cells and biodegradable scaffolds *in vitro*

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Abstract

BACKGROUND: Chronic venous insufficiency is a major health problem worldwide. Clinical treatments include venous valve repair and venous segment containing valve transplantation. However, these are invasive procedures, and the supply of vein containing valves is limited. Significant progress in the fields of tissue engineering and regenerative medicine has been made towards the creation of tissue engineered vascular grafts for the repair of damaged or malformed vessels. It has been reported that using tissue engineering, a tissue engineered vein containing valves constructed with self-derived endothelial cells and allogeneic acellular matrices can provide the complex physiological valve structure and mechanical stability, but this elicited an immunogenic response.

OBJECTIVE: To create a viable and functional vein containing valves, which has the ability to grow, repair, and imitate natural tissues.

METHODS: Bone marrow mesenchymal stem cells were obtained from Beagle dogs by density gradient centrifugation and adherence methods. Bone marrow mesenchymal stem cells were cultured *in vitro*. Following isolation and culture the cells were examined using flow cytometry and identified by direct induction towards the osteogenic and adipogenic lineages. We fabricated biodegradable venous scaffold containing valves using the method of injection molding combined with thermally induced phase separation. Based on the self-made cast, a three-dimensional biodegradable vein scaffold containing valves was constructed from poly(lactic-co-glycolic acid). Morphological structure was tested. Bone marrow mesenchymal stem cells were used as seed cells to be seeded onto the lumen of the tissue engineered vein scaffold containing valves *in vitro* and then incubated for 2 weeks.

RESULTS AND CONCLUSION: Scanning electron microscopy images showed that the scaffold demonstrated sufficient porosity. Cultured cells expressed mesenchymal cell markers, CD44 and CD29, but did not express hematopoietic cell markers, CD34 and CD45 at the same time point. Scaffolds were nontoxic to cells and were favorable for the growth and migration of bone marrow mesenchymal stem cells. Cells attached on the surface of poly(lactic-co-glycolic acid) scaffolds formed a confluent layer after incubation. The cellular constructs were tested *in vitro*, and the valve leaflets were functionally capable of opening and closing when stimulated. These results suggested that the tissue engineered vein containing valves have been successfully constructed by using a three-dimensional poly(lactic-co-glycolic acid) scaffold and bone marrow mesenchymal stem cells as seed cells. Tissue engineered vein containing valves is potentially useful for the substitution and regeneration of vein valves.

Subject headings: biocompatible materials; venous insufficiency; mesenchymal stem cells; venous valves
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INTRODUCTION

Chronic venous insufficiency is a highly prevalent and costly medical condition that affects the lower extremity venous system[1]. There are congenital, primary, and secondary causes for chronic venous insufficiency. Venous reflux is primarily responsible for the symptoms in chronic venous insufficiency which consist of swelling, bleeding, ulcerations, and severe pain.

Current clinical therapies for chronic venous insufficiency include external compression therapy and surgical treatments such as superficial vein surgery, vein valve repair, transposition and transplantation of vein valves. In addition, studies have shown that various vein valve replacements, such as prosthetic venous valves fabricated using pellethane, or luteraldehyde-fixed umbilical cord segments, bileafed mechanical valves or small intestinal submucosa grafts[2-4] are efficacious. Although these vein valves initially appeared to treat chronic venous insufficiency, all vein valve prostheses are non-viable structures and lack the ability to grow, repair, or remodel, unlike viable vein valves.

Significant progress in tissue engineering and regenerative medicine has been made towards the creation of tissue engineered vascular grafts for the repair of damaged or malformed vessels. Tissue engineering is a new approach for regenerating vein containing valves using cells and scaffolds. In an attempt to reconstruct a tissue-engineered venous valve, Teebken *et al* [5] seeded myofibroblasts and endothelial cells in decellularized allogeneic ovine veins. After the matrix was successfully repopulated with myofibroblasts and endothelial cells, the tissue-engineered venous valves were implanted into the ovine external jugular vein. Patent tissue engineered grafts were indistinguishable from autografts with minor inflammatory reactions. Reflux was caused by neo-intima formation related to the tissue engineered graft. This experimental tissue engineered vein containing valves has demonstrated favorable patency rates, but the main drawback is the immunological rejection of allogeneic implants.

The ideal cell source should be non-immunogenic, functional and easy to achieve and expand in culture, and thus, we could use autologous vascular stem cells. Non-immunogenic autologous endothelial cells and smooth muscle cells isolated from vascular are the first choice for tissue engineered vascular graft, but the harvested cells are limited in quantity and proliferation capacity. Compared with autologous vascular cells, bone marrow mesenchymal stem cells are well characterized, readily available, and have no restrictions in using, Previous studies have revealed that mesenchymal stem cells can differentiate into endothelial cells and smooth muscle cells *in vivo* or *in vitro* [6-7].

Our laboratory has focused on tissue engineered approaches to construct veins containing valves. The major goal of our group is to create a viable and functional vein containing valves, which is able to grow, repair, and imitate natural tissues. In the present study, we used poly(lactic-co-glycolic acid) (PLGA) copolymers as a biodegradable scaffold and bone marrow mesenchymal stem cells as seed cells for the construction of veins containing valves. The morphologic, biochemical, and mechanical characteristics of the tissue engineered veins containing valves were also investigated.

MATERIALS AND METHODS

Design

A biomaterial construction study.

Time and setting

From January 2010 to December 2011, the study was completed in Center for Stem Cell Biology and Tissue Engineering, Sun Yat-sen University, China.

Materials

Animals

Beagle dogs, male, conventional animals, weight ranges from 12–18 kg and age ranges from 2–2.5 years, provided by the Institute of Pharmaceutical Industry (license No. SYXK (Yue) 2007-0081), Guangzhou, China. Dogs were handled in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, formulated by the Ministry of Science and Technology of China[8]

Biomaterials

PLGA (75/25) provided by State Key Laboratory of Optoelectronic Materials and Technologies, Sun Yat-sen University, China.

The main reagents and apparatus:

Reagent and instrument	Source
PBS, low glucose-Dulbecco's modified Eagle's medium (LG-DMEM), fetal bovine serum	Gibco Invitrogen, USA
Heparin sodium	QianHong Inp., Jiangsu Province, China
Ficoll-Paque Plus density gradient media	GE Healthcare, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma, USA
CD45-FITC, CD44-FITC, CD29-PE, CD34-PE monoclonal antibodies	BD Pharmingen, USA
Cell Counting Kit-8 (CCK-8)	BioAssay Systems, Hayward CA, USA
Computer numerical control machine (DK7740)	Taizhou Defeng CNC Machine Tools Co.，Ltd., China
ALPHA2-4 freeze dryer	Martin Christ, Germany
Scanning electron microscope (S-4800)	HITACHI, Tokyo, Japan

Methods

Preparation of scaffolds

A stainless steel cast of the veins containing valves (**Figure 1**) was designed and created using a drawing software (AutoCAD 2008, 17.1, Autodesk Company, USA) and computer numerical control machine. After the cast was assembled, it was then placed in a low-temperature freezer

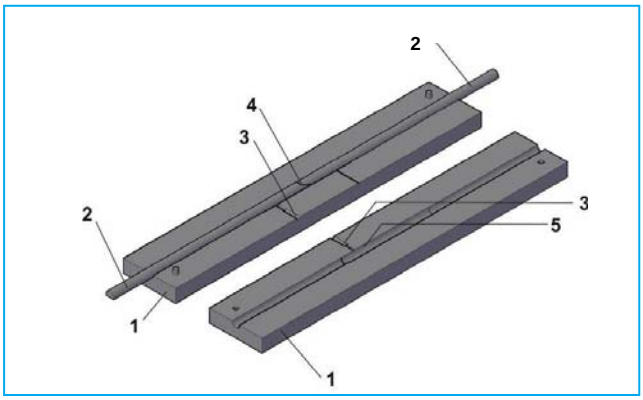

Figure 1 Schematic diagram of the cast for scaffold fabrication
Notes: The cast consisted of top and bottom halves, both of which contained cylindrical cavities that defined the outer diameter of the scaffolds. The inner cylinder (10 mm diameter) contained the valve cast which defined the inner diameter of scaffolds, which perpendicularly intersected through the center of the cylindrical cavity. 1: top and bottom halves; 2: inner cylinder; 3: injection port; 4: valve cast; 5: cylindrical cavity.

at −40 °C to ensure that the temperature of the cast was homogenous with that of the external surroundings. At an initial cast temperature of −40 °C, a 6% (w/v) PLGA solution in 1,4-dioxane was injected into the cold cast using a syringe. The injection press was maintained until the polymer solution in the injection port of the syringe was completely frozen, which occured before complete solidification of the polymer solution in the injection port. This cast was stored in a low-temperature freezer for an additional 2 hours. The scaffold was then separated from the cast, and lyophilized under 0.940 mbar at −20 °C for 72 hours. Following lyophilization, the scaffolds were stored in a dry environment until use.

Isolation and culture of bone marrow mesenchymal stem cells

Bone marrow was aspirated using a 12-gauge bone marrow aspiration needle from the posterior iliac spine of each dog was anaesthetized with 3% sodium pentobarbital (0.5 mL/kg). The aspirated bone marrow was mixed with heparin sodium at a dose of 100 IU/mL. 10 mL of bone marrow were transferred into a 15-mL centrifuge tube, and centrifuged at 1 100 r/min at room temperature for 5 minutes. The supernatant was discarded and the cell layer was diluted with 5 mL of PBS. The mixture was then slowly loaded onto a Ficoll-Paque Plus density gradient media and centrifuged at 1 500 r/min at room temperature for 30 minutes. The mononuclear cell layer at the interface was removed and washed twice in PBS by centrifugation at 1 100 r/min for 5 minutes. Mononuclear cells were resuspended in LG-DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/L streptomycin. The resuspended cells were plated in 75 cm^2 flask and incubated at 37 ℃ in a humidified atmosphere of 5% CO_2.

After 72 hours, the nonadherent cells were discarded and adherent cells were further incubated for 7−10 days. When the cells reached 80%−90% confluence, they were trypsinized with 0.25% trypsin-0.02% EDTA and passaged at a 1:3 ratio. Bone marrow mesenchymal stem cells at the third passage were used in the present study.

Cell characterization

Following isolation, the purity of the cells was examined using flow cytometry. Fluorescence-activated cell sorting analyses was performed on bone marrow mesenchymal stem cells at the third passage. Cells were detached from flasks by incubation in 0.25% trypsin-0.02% EDTA at room temperature and suspended in PBS. Monoclonal antibodies, such as CD45-FITC, CD44-FITC, CD29-PE, CD34-PE were utilized. Isotype IgG negative controls were also used.

Confirmation of phenotype of bone marrow mesenchymal stem cells

Since bone marrow mesenchymal stem cells are commonly defined by their ability to differentiate into osteogenic and adipogenic lineages, the phenotype of these BMSC were further confirmed[9].

Osteogenic induction: 3×10^4 bone marrow mesenchymal stem cells were seeded in 2 mL LG-DMEM per well of 6-well plates and allowed to adhere to the culture surface for 24 hours prior to replacement of the LG-DMEM with the osteogenesis induction media. The media used for induction of bone marrow mesenchymal stem cells were changed every 3−4 days for 3 weeks by completely replacing the media with fresh osteogenesis induction media. For the non-induced control bone marrow mesenchymal stem cells, the frequency of the LG-DMEM media was changed as previously mentioned. Alizarin Bordeaux staining (for mineralization nodule-formation) was utilized to examine osteogenesis in the cell culture. Mineralized bone nodules were identified by labeling for Alizarin Bordeaux staining and observed under an optical microscope (CKX41, Olympus, Japan).

Adipogenesis induction: 2×10^5 bone marrow mesenchymal stem cells were seeded in 2 mL LG-DMEM medium per well of a 6-well plate and fed every 3 days until the culture reached confluence. When the cells were confluent, three cycles of induction/maintenance treatments were performed to stimulate optimal adipogenic differentiation. Each cycle consisted of feeding the cells with adipogenesis induction media for 3 days followed by 1 day culture in adipogenic maintenance media. Non-induced control cells were fed with LG-DMEM only based on the same schedule. Following three complete cycles of induction/maintenance, the extent of adipogenesis was examined under the microscope by fixation with 10% neutral buffer formalin and stained with oil red O for lipid formation.

Scaffold cytotoxicity assessment using CCK-8

Circular samples (5 mm in diameter) of PLGA scaffolds were prepared and placed in 96-well tissue culture polystyrene plates. Scaffolds were sterilized in 75% alcohol,

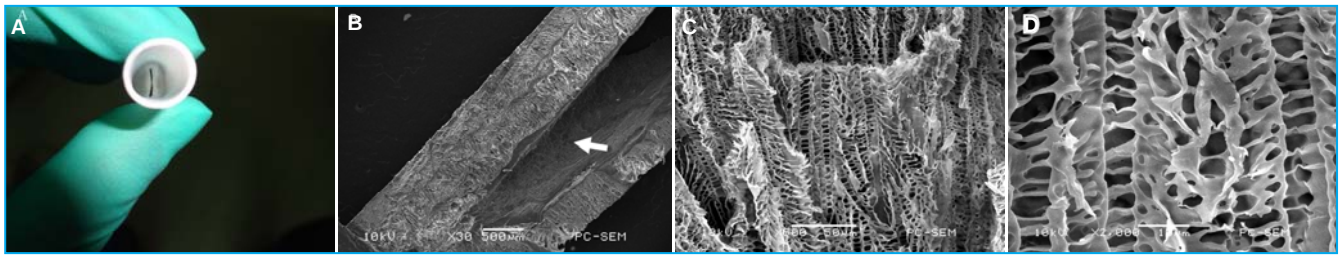

Figure 2 Characteristics of the poly(lactic-co-glycolic acid) (PLGA) scaffold

Notes: A: Photograph of the PLGA scaffold as seen from the proximal end

B: Scanning electron microscopic picture depicting the longitudinal section of the scaffold, shown the area of valve sinuses (arrow; ×30)

C: Scanning electron microscopic picture of the scaffold demonstrating the presence of regular ladder-like porous structures (×600)

D: Another scanning electron microscopic picture taken at a higher magnification (×2 000)

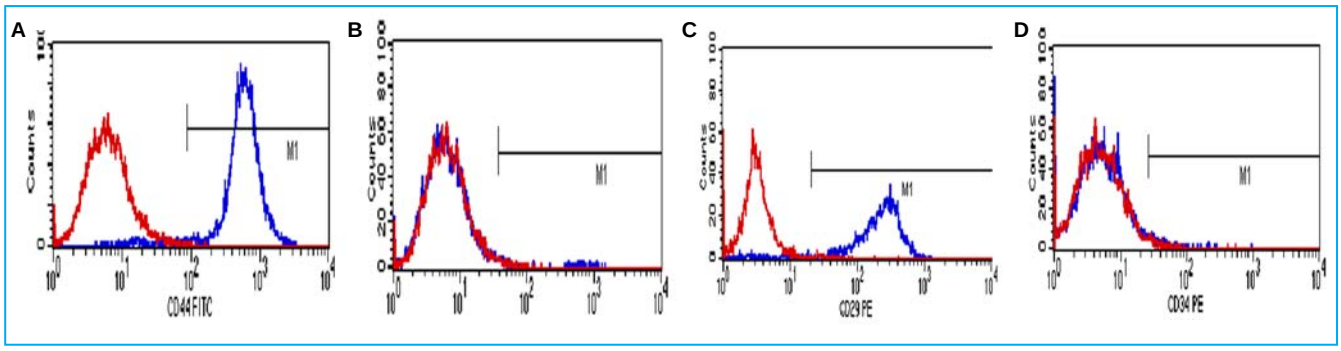

Figure 3 Flow cytometry analyses of bone marrow mesenchymal stem cells

Notes: Bone marrow mesenchymal stem cells express CD44 and CD29, but not CD34 and CD45 at the same time point.

irradiated with ultraviolet light for 30 minutes, and then rinsed extensively with sterile PBS. Subsequently, 100 μL media of cell suspension at a density of 1×10^4 cells/mL was added to each well and maintained in a humidified atmosphere with 5% CO_2 at 37 ℃. In the control group, cell suspension with the same density was seeded in empty tissue culture polystyrene plates and maintained under the same conditions as the experimental groups. Cells were allowed to proliferate for 7 days. The number of attached cells was determined every day using CCK-8 kit according to the manufacturer's instructions. Absorbance was detected in the micro-plate reader at 450 nm wavelength. 10 μL of CCK-8 kit solution was added to each well and incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 4 hours. Six parallel experiments for each sample were used to assess cell viability.

Determination of optimal cell number for seeding

Optimal cell number for seeding was determined, and in addition, the examination of the extent and morphology of bone marrow mesenchymal stem cells adhering to PLGA surfaces was also performed. The scaffolds were cut longitudinally and small discs/scaffold pieces with a surface area of 1.5 cm^2 were fitted into 24-well cell culture plates and sterilized in 75% alcohol. These were then irradiated with ultraviolet light for 30 minutes, and rinsed extensively with sterile PBS. The scaffolds were incubated in 1 mL of LG-DMEM at 37 ℃ in a humidified atmosphere of 5% CO_2 overnight before the cells were seeded in order to improve cell attachment. Following detachment, the cell number of bone marrow mesenchymal stem cells was adjusted to 1×10^4, 1×10^5 and 1×10^6 respectively. Cells were suspended in 1 mL of LG-DMEM, placed on top of the scaffolds and cultured for 1, 3, 5 and 7 days. The medium was changed every 3 days.

Scanning electron microscope images were taken to assess cell adhesion and confluence. In brief, cellular scaffolds were harvested, washed with PBS, and fixed with 4% glutaraldehyde. After rinsing in triplicate with PBS, the samples were dehydrated and lyophilized. Dry cellular scaffolds were sputter-coated with gold and observed under the scanning electron microscope at an accelerating voltage of 10 kV.

Cell seeding and culture on scaffolds

Prior to cell seeding, the scaffolds were sterilized in 75% alcohol and irradiated with ultraviolet light for 30 minutes. The scaffolds were then immersed in PBS for 3 hours to remove residual ethanol. This step was repeated in triplicate. The scaffolds were then placed in a vacuum while submerged in LG-DMEM, and were immersed and incubated in LG-DMEM at 37 ℃ in a humidified atmosphere of 5% CO_2 overnight prior to cell seeding to improve cell attachment.

Bone marrow mesenchymal stem cells cultured at the third passage were centrifuged at 1 100 r/min for 5 minutes and re-suspended to a concentration of 1×10^6 cells/mL in culture media. 1×10^6 cells/mL of the cell suspension was gently pipetted into the lumen of the scaffold. Following seeding, the cellular constructs were placed, static in a Petri dish. After 2 hours, the scaffolds were rotated 90° clockwise. Another fresh cell suspension (1×10^6 cells/mL) was pipetted into the lumen for a second seeding to ensure that sufficient cells were seeded onto the

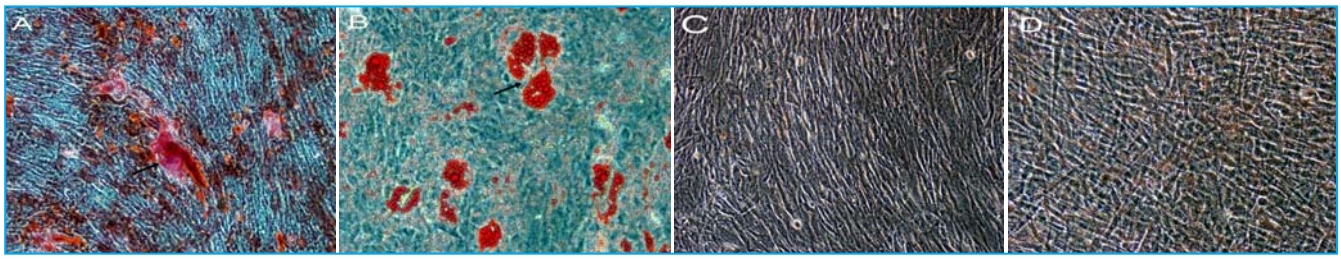

Figure 4 Osteogenic and adipogenic induction of bone marrow mesenchymal stem cells culture (×200)

Notes: A: Osteogenesis was examined three weeks following treatment in induced cultures using Alizarin Bordeaux staining (arrow)

B: After three complete cycles of induction and maintenance, the extent of adipogenesis was examined using oil red O staining in induced cultures (arrow)

C: Osteogenesis was examined three weeks following treatment in control cultures using Alizarin Bordeaux staining

D: After three complete cycles of induction and maintenance, the extent of adipogenesis was examined using oil red O staining in control cultures

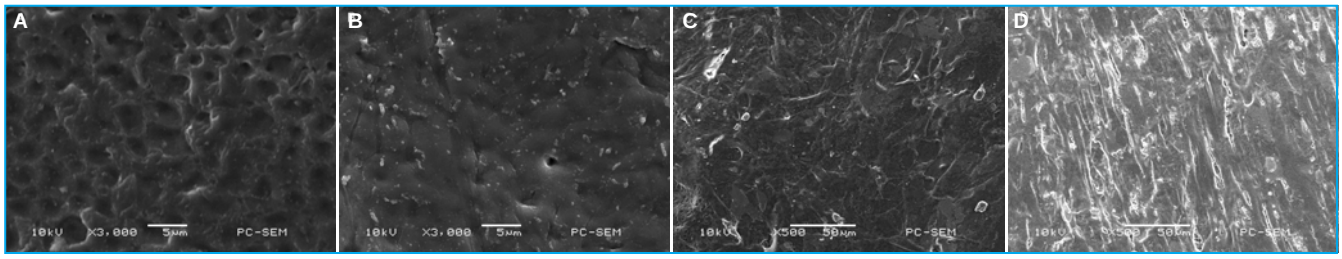

Figure 5 Scanning electron microscopic images of bone marrow mesenchymal stem cells growing on scaffolds at various cell concentrations to assess cell adhesion, seeding density and confluence

Notes: A: Cell concentrations of 1×10^4/mL attached onto the surface of the scaffold at 1 day (×3 000)

B: Cell concentrations of 1×10^6/mL attached onto the surface of the scaffold at 1 day (×3 000)

C: Cells at a density of 1×10^4/mL proliferated on the surfaces of the scaffold for 7 days (×500)

D: Cells at a density of 1×10^6/mL proliferated on the surfaces of the scaffold for 7 days (×500)

scaffold. This was repeated in duplicate and cell seeding was completed. The cellular constructs were incubated at 37 ℃ in a humidified atmosphere with 5% CO_2 in static culture overnight. The cellular constructs were then incubated in dynamic cultures, and maintained in a humidified atmosphere with 5% CO_2 at 37 ℃. Following two weeks, the cellular constructs were analyzed using scanning electron microscope and hematoxylin and eosin staining. Vein valve function was demonstrated using PBS perfusion and the synchronous opening and closing of the vein valve was observed when rinsing with PBS or treating with closed forceps.

Main outcome measures

The potential to differentiation of bone marrow mesenchymal stem cells, the morphological structure and the mechanics characteristic of the cellular constructs.

Statistical analyses

The SPSS 11.0 for Windows was used. Data are expressed as median (QL, QU). To test the significance of observed differences between the two groups of cells in scaffolds cytotoxicity assessment, a non-parametric test (Mann-Whitney *U* test) was applied. $P < 0.05$ was considered to be statistically significant.

RESULTS

Characterization of the PLGA scaffold

This was prepared using low temperature injection and S-L separation methods. The vein scaffold containing valves was fabricated without any suture materials or additional stent and resembled the natural complex anatomical structure of the human femoral vein. As shown in **Figures 2A, B**, the scaffold had an inner diameter of 9 mm with a thickness of 0.9 mm. The thickness of the valves was (0.32±0.04) mm.

Scanning electron microscopic micrographs demonstrated the presence of regular ladder-like porous structures (**Figures 2C, D**). The pores exhibited well interconnectivity, and the average pore size and porosity of scaffolds were 10–20 μm and 90% respectively.

Characterization of bone marrow mesenchymal stem cells

Adherent bone marrow mesenchymal stem cells began to exhibit a spindle-shaped morphology by 3 days in culture and expanded rapidly thereafter without contact inhibition. The surface marker expression of the isolated mesenchymal stem cells from the bone marrow was analyzed using flow cytometry (**Figure 3**).

The bone marrow mesenchymal stem cells did not express CD34 and CD45, which are known markers for hematopoietic progenitor cells[10], and differentiated into non-erythrocyte hematopoietic cells respectively. However, these cells were positive for mesenchymal cell markers, CD44 and CD29.

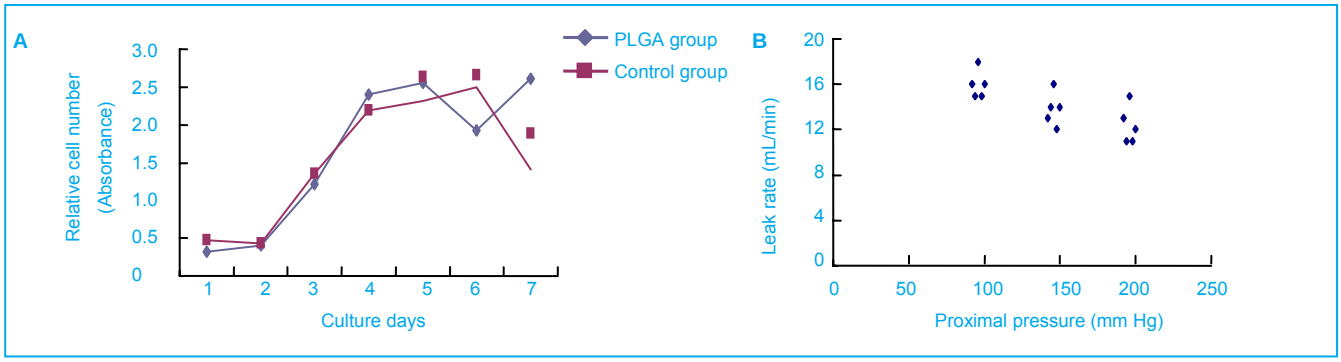

Figure 6 Cytotoxicity assessment of the scaffolds and *in vitro* reflux leak rate to assess vein valve function

Notes: A: Cytotoxicity assessment of the scaffolds: relative cell number/proliferation of bone marrow mesenchymal stem cells was analyzed at days 1–7 in poly(lactic-co-glycolic acid) (PLGA) and control groups (repeated six times).

B: *In vitro* reflux leak rate to assess vein valve function: the analyses indicated that the greater the proximal pressure, the smaller the leak rate.

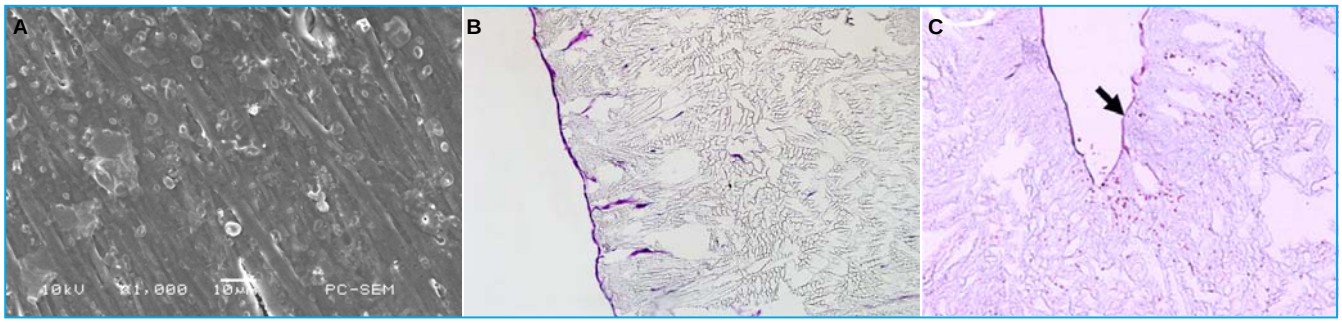

Figure 7 The morphological characteristic of the cellular constructs

Notes: A: Scanning electron microscopic analyses showed a confluent cell monolayer grown on the luminal surface of the scaffold (×1 000)

B: Hematoxylin-eosin staining of the hemispherical constructs following 14 days in culture (×200)

C: Hematoxylin-eosin staining of the hemispherical constructs following 14 days in culture, showed the area of valve (arrows) and valve sinuses (×200)

Phenotype of bone marrow mesenchymal stem cells was further confirmed by induction towards the osteogenic and adipogenic lineages. Osteogenesis was examined using Alizarin Bordeaux staining, following three weeks of induction. Alizarin Bordeaux-positive areas appeared red in color in osteogenic cultures (**Figure 4A**). In contrast, no mineralized bone nodules were found in the control culture (**Figure 4C**). After three cycles of adipogenic induction and maintenance, lipid-containing cells became very prominent, which were positively stained red by oil red o staining (**Figure 4B**). In contrast, no oil red o positive cells were found in the cell control culture (**Figure 4D**). These studies, in combination with the fluorescence-activated cell sorting data, confirmed the mesenchymal stem cell identity of the starting cellular material.

Determination of the optimal cell number for seeding

Morphology of the cells and their attachment onto the PLGA scaffolds were evaluated using scanning electron microscope. Scanning electron microscopic assessment showed that the cells attained spherical morphology at the 1st day of culture. Following 3 days in culture, the cells exhibited a spindle-shaped morphology. After a week of culture, the cells had grown and covered almost all the surface of the scaffolds. It was clear that the cells adhered more firmly and formed confluent layer earlier in the group with a cell density of 1×10^6 cells/mL (**Figure 5**).

Scaffold cytotoxicity assessment

Cells were cultured as previously mentioned in PLGA and control groups for 1–7 days. The viable cell numbers in each group were determined using the CCK-8 kit method (**Figure 6A**). No significant differences were observed between the two groups ($P > 0.05$). The relative cell number at day 7 was reduced in the control group since the cells had reached confluence and the metabolic activity of the cells was inhibited. However, relative cell number was increased in the PLGA group since the scaffold provided more space for the growth of bone marrow mesenchymal stem cells. This implied that the scaffold material was non-toxic to the cells and was favorable for the growth and migration of BMSCs.

Cell seeding and culture on scaffolds

The luminal surface was smooth, glossy and white. The average opening pressure was (3.8±0.7) mm Hg. The valve had an acceptable reflux leakage (**Figure 6B**).

Following 2 weeks of culture, the lumen of the cellular constructs appeared smooth, with flattened cells covering the entire lumen (**Figure 7A**). Hematoxylin-eosin staining showed that a confluent monolayer of bone marrow mesenchymal stem cells was formed on the luminal surface of the cellular constructs in all of the tissue engineered veins containing valves (**Figures 7B, C**). These results showed the feasibility of

producing a complete tissue engineered vein containing valves from bone marrow mesenchymal stem cells.

DISCUSSION

Apart from superficial venous insufficiency, the major factor causing the occurrence and the development of clinical symptoms in chronic venous insufficiency is valve reflux in the deep venous system[11]. Effective treatment of valve reflux requires replacement of the diseased valves. Autologous vein containing valves transplantation is the main treatment for deep venous valve replacement. Although transplantation is successful in correcting reflux and is associated with ulcer healing and the relief of symptoms, vein containing valves transplant can cause significant trauma to the patient and long-term valve competency rates and symptomatic control are poor[12]. Problems arise prior to surgery since it is difficult to find a suitable donor valve. This is evidenced by the fact that 30%–40%[13-14] of axillary vein valves, which are often used for superficial femoral venous valve replacement, are found to be incompetent prior to harvesting.

To overcome these limitations, artificial creation of implantable complex structures like vein valves has been pursued, and reported with encouraging results. However, the replacement has not been successfully tested in the long term and in addition; these are thrombogenic and require life-long anti-coagulation therapy[11, 15-16]. Alternatively, tissue engineered vein containing valves can overcome these disadvantages. It has been reported that using tissue engineering, a tissue engineered vein containing valves constructed with self-derived endothelial cells and allogeneic acellular matrices, can provide the complex physiological valve structure and mechanical stability, but this elicited an immunogenic response[5].

Due to the above limitations, a search for an ideal vein containing valves replacement continues. The major goal of our research group is to create a viable and functional vein containing valves, which has the advantages of the natural living tissue, including its resistance to thrombus formation and infection, as well as the ability to grow and remodel. In the present experiment, we have successfully produced a three-dimensional, porous scaffold from a thermoplastic PLGA that could then be used for tissue engineered venous valve structures. Scanning electron microscopic images and hematoxylin-eosin straining revealed that bone marrow mesenchymal stem cells which attached to the PLGA scaffold formed a near confluent layer. Tests showed that valve leaflets were movable when rinsed with PBS.Among a variety of issues, a major focus in tissue engineering is to design a suitable three-dimensional scaffold structure that can be a template for supporting cell growth, migration, differentiation and secretion of extracellular matrix proteins, as well as for directing the formation of new tissues during the tissue regeneration process[17-18]. Ideally, scaffolds will be slowly resorbed in culture or following implantation, leaving only the tissue generated by the cells[19]. Porosity and pore size of the scaffolds are also key factors[20], since a scaffold that has high porosity and large interconnected pores can enable mass transport, infiltration of cells, and interstitial flow fluid[21-22]. As a popular biodegradable medical material, PLGA has been extensively investigated due to its good biocompatibility, controllable biodegradability, and good process ability[23-26]. In this study, we successfully fabricated a porous PLGA scaffold. Tests have shown that PLGA could induce cell adhesion and proliferation, indicating the biocompatibility of the polymer. In addition, it is important that its degradation rate can be easily controlled by varying the copolymer ratio of lactic to glycolic acid[27]. Since the valve and vein walls play different roles in venous return, it is expected that PLGA degradation and tissue invasion in valve and vein walls can proceed differently, which will be further studied in the future.

Although endothelial and smooth muscle cells have been considered to be the main seeding cells for tissue engineered vascular graft, they are harvested from intact vascular structures of the donor. Moreover, harvesting methods are traumatic; the harvested cells are limited in quantity and proliferation capacity. The limited proliferation potential of harvested cells makes it impossible to obtain large amounts of cells from a small vessel biopsy. Due to the fact that bone marrow mesenchymal stem cells have great potential in differentiating towards multiple cell lineages, they are widely utilized as an alternative cell source for tissue engineering. Bone marrow mesenchymal stem cells have been reported to be capable of differentiating into endothelial and vascular smooth muscle cells[9-10, 28-29]. Bone marrow mesenchymal stem cells and endothelial cells performed similarly against platelet adhesion[30]. These results strongly suggest that bone marrow mesenchymal stem cells can be used as an alternative cell source to endothelial cells for the prevention of thrombogenesis in vascular reconstruction. Some studies have found that bone marrow mesenchymal stem cells in the grafts could differentiate into vascular smooth muscle cells and endothelial cells *in vivo*[31-32]. In the present study, we have shown that bone marrow mesenchymal stem cells have a great capacity for differentiating into multiple cell lineages, and PLGA can promote cell proliferation and differentiation. We have directly seeded bone marrow mesenchymal stem cells onto the scaffold, in order to differentiate into cells similar to native vessels *in vitro* and *in vivo*, and *in vitro* test have showed durability of cell adhesion is good.

Cell seeding is the first step in tissue engineering approaches that incorporate cells into or onto scaffolds for culture or implantation. Since PLGA is hydrophobic, the porous scaffolds fabricated with these polymers float on the cell culture media and the scaffold does not absorb the culture media. Some approaches have been performed to improve the hydrophilicity of biodegradable polymer scaffolds and thus to ensure uniform and dense cell seeding. In our study, other than treating the scaffolds by pre-wetting with ethanol, we have customized a vacuum device. Prior to cell seeding, the scaffolds were placed in a vacuum flask, to

allow its pores to be filled with culture media, and to increase seeding efficiency and reduce cell seeding time.

In summary, tissue engineered veins containing valves have been successfully constructed using a three-dimensional PLGA scaffold and bone marrow mesenchymal stem cells *in vitro*. Results of this study suggest that tissue engineered veins containing valves are potentially useful for the substitution and regeneration of vein valves, although further studies are warranted. Issues such as scaffold characteristics, patency, thrombosis, biodegradation speed and valve function should be assessed in long-term animal studies.

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骨髓间充质干细胞联合可降解支架体外构建组织工程带瓣静脉

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文章亮点：

1 文章特点在于应用自制带瓣静脉的不锈钢模具和热致相分离法制备了具有类似人体带瓣静脉段三维结构的组织工程带瓣静脉支架。

2 文章创新性利用三维聚(乳酸-乙醇酸)共聚物支架和骨髓间充质干细胞在体外成功构建了具有一定瓣膜功能的组织工程带瓣静脉。

关键词：

生物材料；材料相容性；可降解吸收材料；静脉瓣膜；骨髓间充质干细胞；聚(乳酸-乙醇酸)共聚物；慢性静脉功能不全；组织构建；干细胞；国家自然科学基金

主题词：

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摘要

背景：临床上治疗慢性静脉功能不全的主要方法是静脉瓣膜修复及带瓣静脉段移植，但这些方法创伤较大，且带瓣静脉来源有限。组织工程学和再生医学在修复病变血管方面取得的进步，而以自体来源的内皮细胞为种子细胞的组织工程带瓣静脉也见于了报道，但存在排出反应。

目的：构建一个有可自我更新、修复、类似天然瓣膜结构并具有功能的带瓣静脉。

方法：麻醉取 Beagle 犬的骨髓获取骨髓间充质干细胞，采用密度梯度离心和贴壁法获取骨髓间充质干细胞，并进行细胞的传代、冻存复苏、流式细胞仪检测和定向诱导分化。采用热致相分离技术，以聚(乳酸-乙醇酸)共聚物为基材，利用自制带瓣静脉模具制备三维组织工程带瓣静脉支架，制备组织工程带瓣静脉支架，并研究其形态结构。将骨髓间充质干细胞种植在支架上构建可降解的带瓣静脉，在体外培养 2 周。

结果与结论：扫描电镜观察显示支架孔隙率高。培养的细胞符合骨髓间充质干细胞的形态特征，培养的细胞大部分表达 CD29 和 CD44，不表达 CD34 和 CD45。细胞毒性实验显示支架无毒性，有利于细胞增殖和迁移。将细胞种植在支架表面上培养后可形成单层细胞层。体外实验验证细胞支架复合物的瓣膜有一定的开闭功能。利用三维聚(乳酸-乙醇酸)共聚物支架和骨髓间充质干细胞可成功构建组织工程带瓣静脉，组织工程带瓣静脉将有可能作为静脉瓣膜的的替代物治疗静脉瓣膜疾病。

作者贡献：实验设计为刘池拽、殷恒讳、吕伟明、王文见，实验实施、资料收集均为第一作者主要完成，组织工程支架实验部分曾晨光、全大萍参与设计及实施，细胞实验部分刘畅、项鹏参与设计及实施，第一作者成文并对全文负责，通讯作者审校。

利益冲突：文章及内容不涉及相关利益冲突。

伦理要求：实验过程中对动物的处置应符合 2009 年《Ethical issues in animal experimentation》相关动物伦理学标准的条例。

学术术语：静脉功能不全—是静脉疾病的总称，主要表现为浅静脉扩张或曲张；腿部沉重、乏力、胀痛或疼痛，下肢浮肿，皮肤损害及已愈合或活动性溃疡等。

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