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In vitro re-endothelialization of detergent decellularized heart valves under simulated physiological dynamic conditions

Artur Lichtenberg[a,*,1], Igor Tudorache[a,1], Serghei Cebotari[a], Stefanie Ringes-Lichtenberg[a], Gerrit Sturz[a], Klaus Hoeffler[a], Christof Hurscheler[b], Gudrun Brandes[c], Andres Hilfiker[a], Axel Haverich[a]

[a]*Division of Thoracic and Cardiovascular Surgery, Hannover Medical School, Carl-Neuberg-Str. 1, 30625 Hannover, Germany*
[b]*Laboratory for Biomechanics and Biomaterials, Orthopedics Department, Hannover Medical School, Germany*
[c]*Laboratory for Cell Biology and Electron Microscopy, Hannover Medical School, Germany*

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Abstract

The production of viable biological heart valves is of central interest in tissue engineering (TE). The aim of this study was to generate decellularized heart valves with an intact ultra-structure and to repopulate these with endothelial cells (EC) under simulated physiological conditions. Decellularization of ovine pulmonary valve conduits was performed under agitation in detergents followed by six wash cycles. Viability of EC cultures exposed to washing solution served to prove efficiency of washing. Resulting scaffolds were free of cells with preserved extracellular matrix. Biomechanical standard tension tests demonstrated comparable parameters to native tissue. Luminal surfaces of decellularized valvular grafts were seeded with ovine jugular vein EC in dynamic bioreactors. After rolling culture for 48 h, pulsatile medium circulation with a flow of 0.1 L/min was started. The flow was incremented 0.3 L/min/day up to 2.0 L/min (cycle rate: 60 beats/min), while pH, pO_2, pCO_2, lactate and glucose were maintained at constant physiological levels. After 7 days, a monolayer of cells covered the inner valve surface, which expressed vWF, indicating an endothelial origin. A complete endothelialization of detergent decellularized scaffold can be achieved under simulated physiological circulation conditions using a dynamic bioreactor system, which allows continuous control of the culture environment.

Keywords: Cardiac tissue engineering; Extracellular matrix; Endothelialization

1. Introduction

Cardiac valves replacement is a common surgical procedure for patients with severe valve dysfunction. The use of appropriate cardiac valve prosthesis in each particular case can be efficient in decreasing of patients' morbidity and mortality and may lead to satisfactory long-term results. However, mechanical and biological prostheses still have several limitations [1,2]. The viable cryo-preserved allograft valves induce a high immunological response especially in young patients, causing valve degeneration [3]. The inability of currently available valvular prostheses to grow and remodel represents another particular disadvantage leading to repeated operation in pediatric patients [4].

Tissue engineering (TE) of valve prostheses using autologous cells might provide promising solutions to overcome these limitations. An ideal bioengineered cardiac valve should mimic the natural heart valve. The anatomical and morphological structure of TE valves must be similar to the native tissue. Moreover, the viable artificial valve tissue should have the capacity to self-remodel and regenerate along with the ability to grow. Furthermore, life-long constant hemodynamic stress requires an adequate mechanical stability of bioengineered valve prosthesis.

*Corresponding author. Tel.: +49 511 532 2189; fax: +49 511 532 5404.
E-mail address: lichtenberg.artur@mh-hannover.de (A. Lichtenberg).
[1]Both the authors contributed equally to this work.

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Tissue engineered valves are constructed using either synthetic or biological matrices [5–8]. Biological matrices are based on xeno- or allogeneic tissue, which primarily undergo a decellularization process [5,6,9,10]. Afterwards, these scaffolds are repopulated with autologous cells prior to implantation [5,6,8,10]. Optimization and standardization of all these bio-technological processes as well as the use of specially designed pulsatile bioreactor mimicking the physiological circulation environment could improve the heart valve TE outcome.

The aim of this study was to obtain decellularized scaffold with mechanical and morphological properties similar to native tissue and to dynamically repopulate this scaffold with endothelial cells (EC) in a novel pulsatile bioreactor, with continuous adjustment of all technological processes to provide near physiological conditions.

2. Material and methods

2.1. Ovine pulmonary valves

All animal experiments and surgical procedures were performed in compliance with the *Guide for the Care and Use of Laboratory Animals* as published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the local animal care committees. Pulmonary valve conduits (PV) including a short subvalvular myocardial cuff, valve annulus, valve leaflets, and pulmonary artery wall (3 cm length) were harvested from juvenile sheep (15–20 kg) under sterile conditions for the production of allogeneic scaffolds. After removal of adherent fat, the PV were stored in phosphate-buffered solution (PBS) at 4 °C.

2.2. Decellularization protocol

Decellularization was performed as described previously [10]. Briefly, PV ($n = 16$) were suspended in a solution of 0.5% sodium deoxycholate (Sigma) and 0.5% sodium-dodecylsulfate (SDS; Carl Roth) for 24 h. Six wash cycles (12 h each) with PBS supplemented with penicillin and streptomycin (100 μg/mL, P/S, Biochrom) served for removal of residual detergents and cell debris.

2.3. DNA isolation and quantification

Decellularized PV were treated by a deoxyribonuclease digestion using 17 IU/mL DNase I (Sigma) for 4 h at 37 °C to remove free residual DNA. Since nuclear DNA in intact cells is protected from DNase I, DNA isolated from tissue after digestion is indicative for the presence of residual cells. Therefore, DNA was isolated from 25 μg decellularized valve tissue (cusps and wall, respectively) using the DNeasy Tissue Kit (Qiagen) according to the recommended protocol. Potentially extracted DNA was subjected to spectrometric analysis.

2.4. Histology and immunohistochemistry

Formalin-fixed paraffin-embedded valvular tissue sections were stained by standard Hematoxylin–Eosin (H&E), and Movat pentachrome protocols. Immunohistochemical staining of cryo-sections was performed by use of the avidin-biotinperoxidase technique. The integrity of collagen IV, as a main protein of the basement membrane, was evaluated by mouse monoclonal anti-collagen IV (clone CIV 22, Dako). EC were characterized by the presence of von Willebrand factor (vWF) using a mouse monoclonal anti-vWF antibody (clone 8/86, Dako) as previously described [11]. Native ovine pulmonary valve tissue samples served as positive controls.

2.5. Scanning electron microscopy (SEM)

Samples of PV (cusps and wall) were fixed in 2.5% glutaraldehyde (Polyscience) in 0.1 M sodium cacodylate buffer (Merck) at 4 °C, for a minimum of 4 h followed by dehydration in an ascending concentration series of ethanol. Samples were critical point-dried (Balzers CPD 030) and sputtered with an ultra-thin gold layer (Polaron SEM Coating System). Intraluminal surface and sharp cut cross-section areas of the specimens were examined by SEM (Phillips SEM-505).

2.6. Biomechanical tests

Wall specimens (size: 15 mm × 10 mm) of native and decellularized PV were mounted in clamps so that the unloaded reference length of the specimens hanging under their own weight was 10 mm. Cross-sectional area was measured using a non-contact laser micrometer (LDM-303H-SP, Takikawa Engineering). Specimens were prepared for testing in the longitudinal and circumferential directions and kept moist throughout testing procedure (PBS). The specimens were preloaded to 0.01 N, and subsequently elongated until macroscopic failure at 0.1 mm/s in a materials testing machine (Model 1445, Zwick). Load–elongation and stress–strain curves were generated and the ultimate load, structural stiffness, ultimate stress, ultimate strain, and elastic modulus (Young's modulus) were determined. The ultimate load was taken as the point at which the first significant reduction in load occurred. The structural stiffness and elastic modulus were determined from the linear portion of the load–elongation and stress–strain curves, respectively.

2.7. Cell source and culture

For cell isolation, jugular veins were harvested from juvenile sheep under sterile conditions. These individuals were different from those that served as scaffold donors. EC were digested from the vessel wall with 2% collagenase A (Roche Diagnostics) in M199 (Gibco) and resuspended in culture medium (CM) composed from Endothelial Cell Basal Medium-2 (Clonetics), supplemented with SingleQuot Kit (Clonetics), 10% FCS (Biochrom), 100 μg/mL P/S (Biochrom) and finally seeded into culture flask. A suitable number of cells (1.2×10^7, 2nd or 3rd passage) were used for reseeding for each valve conduit. Additionally, EC were seeded into 24-well plates in order to determine the toxic effect of PBS washing solution (WS) on cell viability. The expected endothelial phenotype of cultured cells was checked microscopically (cobblestone growth pattern typical for EC) and immunohistochemically for expression of vWF.

2.8. Dynamic bioreactor system

The cell-seeding experiments were performed using a specially developed dynamic bioreactor system (filled with 500 mL CM, kept in a conventional cell incubator at 37 °C (MCO-20AIC, Sanyo)), which allowed pulsatile circulation (Fig. 1). The system is able to mimic the physiological condition of pulmonary circulation. Flow rate and pulsation were continuously measured (Flowmeter, Medi-Stim) and adjusted. The pressure and temperature were constantly maintained and monitored with a monitoring system (Model-64S, Hewlett-Packard). The gas exchange occurred by constant medium surface aeration inside the oxygenation/compliance chamber (Fig. 1). Fresh gas (average 94% air, 6% CO_2) was transported into the reservoir by a roller pump (Ismatec). The pH level in the circulating CM was adjusted by the flexible changing of CO_2 supply.

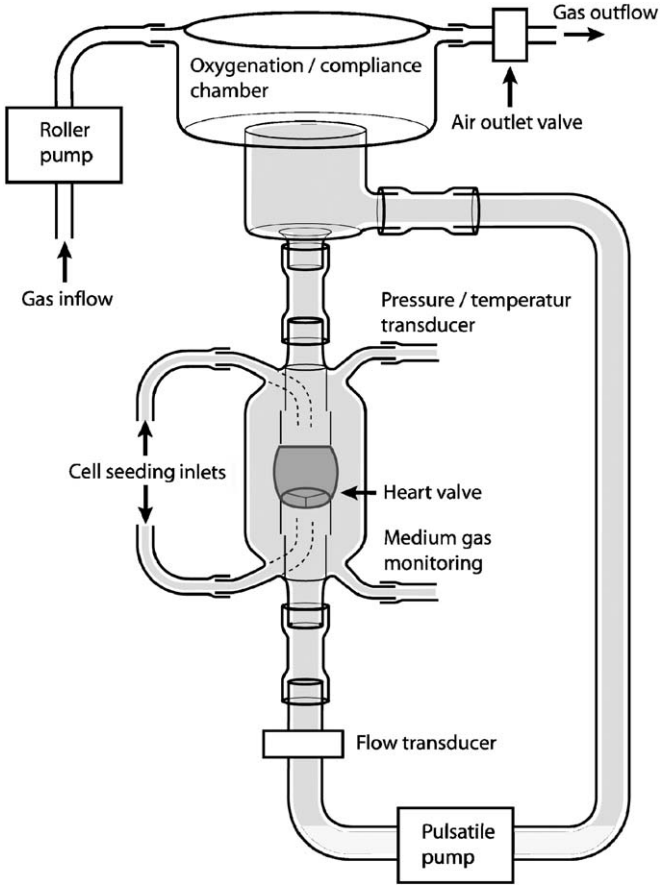

Fig. 1. Schematic drawing of the novel dynamic bioreactor system for tissue engineering of heart valves.

2.9. EC repopulation and dynamic culture

Under rotation conditions: Decellularized PV ($n = 11$) were inserted into bioreactors after pre-incubation in CM for 24 h. Cultured cells were inspected morphologically (cobblestone pattern) and immunohistochemically (vWF) and were accepted for reseeding when expressing predominantly an endothelial character. In three rounds, EC (0.4×10^7 cells each) were injected precisely in the valve lumen through specially designed cell-seeding inlets (Fig. 1). Each seeding step was followed by a 12 h period of slow rotation of the bioreactor (0.1 rotation/min), exposing the entire valve surface to achieve optimal attachment conditions. After completed reseeding three PV were analyzed for morphology and viability of cells. *Under dynamic conditions:* Following the reseeding, eight bioreactors were attached to a pulsatile pump. The pulsatile circulation was started with 0.1 L/min. The initial flow rate was increased by 0.15 L/min twice a day until a maximal flow of 2.0 L/min (mimicking physiological conditions: 60 beats/min) was reached. Mean system pressure was maintained at 25 ± 4 mm/Hg during the entire duration of dynamic cultivation. At day 3, half of the CM volume was exchanged. The morphology of reseeded valves and its metabolic activity (see below) were analyzed at flow rates of 1.0 L/min (3 PV) and 2.0 L/min (5 PV).

2.10. Lactate, glucose, pO_2, pCO_2, and pH measurements

During dynamic culture, lactate, glucose, pO_2, pCO_2, and pH levels were repeatedly measured. Three samples of 1 mL CM each were examined on a blood-gas analyzer daily (Radiometer, ABL 300). During the measurements, samples were hermetically sealed in blood-gas syringes which prevented exposure to the atmosphere. Results of the measurement after launching the perfusion were used as baseline data.

2.11. Metabolic activity test (MTS assay)

CellTiter 96® AQ_{ueous} One Solution Cell Proliferation (MTS) Assay (Promega) was used to asses the metabolic activity of re-endothelialized valve tissue as well as to monitor the success of detergent removal from the decellularized PV during washing according to manufacturer's protocol (Promega). Briefly, this test is based on an MTS tetrazolium compound (Owen's reagent), which is bioreduced by cells into a colored formazan product soluble in CM. This conversion is accomplished by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells [12]. Decellularized valve tissue was used as negative control. For WS toxicity, ovine EC in 24-well plates (Nunclon™) were incubated with a mixture of CM (50%) and WS (50%) for 24 h. PBS instead of WS served as control. Seven hundred microliters CM and 140 μL MTS were added to each drained well and incubated at 37 °C for formazan formation. Samples of PV wall and leaflet (surface $\approx 25\,mm^2$) were incubated in wells containing 700 μL CM and 140 μL MTS at 37 °C. Wells containing CM with MTS reagent without cells or tissue samples were used as baseline. After 2 h of incubation, formazan production in a sample of 100 μL was measured photometrically at 490 nm in a 96-well plate ELISA Reader MRX (Dynatec), as previously described (6). Three replicates were read for each sample.

2.12. Statistics

All data are reported as mean ± SD. The unpaired student's *t*-test was used for analyses. Statistical significance was defined as $p < 0.05$. The SPSS statistical software package 11.0 for Windows (SPSS) was used for statistical analysis.

3. Results

3.1. Morphological and biomechanical characterization of decellularized PV

Native PV from sheep showed the following structure: Luminal surface of the conduit is covered with a monolayer of EC. The valve leaflet represents a typical 3-layered structure with mesenchymal cells in the matrix scaffold which is covered on both sides with an EC monolayer. The media of the pulmonary vessel consist of fibers populated with smooth muscle cells, whereas the adventitia is composed of connective tissue interspersed with fibroblasts (Fig. 2A, B).

Histological analysis revealed that the treatment with detergents for 24 h followed by a DNase I digestion resulted in the loss of cells in the graft to a level below a detectable threshold (Fig. 2C, D). In agreement with this result, DNA extraction from decellularized PV samples (leaflet, wall) showed less then 5% of residual DNA when compared to native tissue samples ($n = 3$ each; data not shown). In decellularized scaffolds, the collagen, elastic fibers, and glycosaminoglycans (GAG) were comparable to native tissue as observed by histological means (Fig. 3A–D). Along this line, SEM revealed an efficiently preserved three-dimensional network of ECM fibers with complete maintenance of the basement membrane all along the inner surface of the pulmonary wall as well as on both sides of the leaflet (Fig. 4A, B). This observation was confirmed by the presence of collagen IV in the basement membrane of decellularized grafts (Fig. 5A, B).

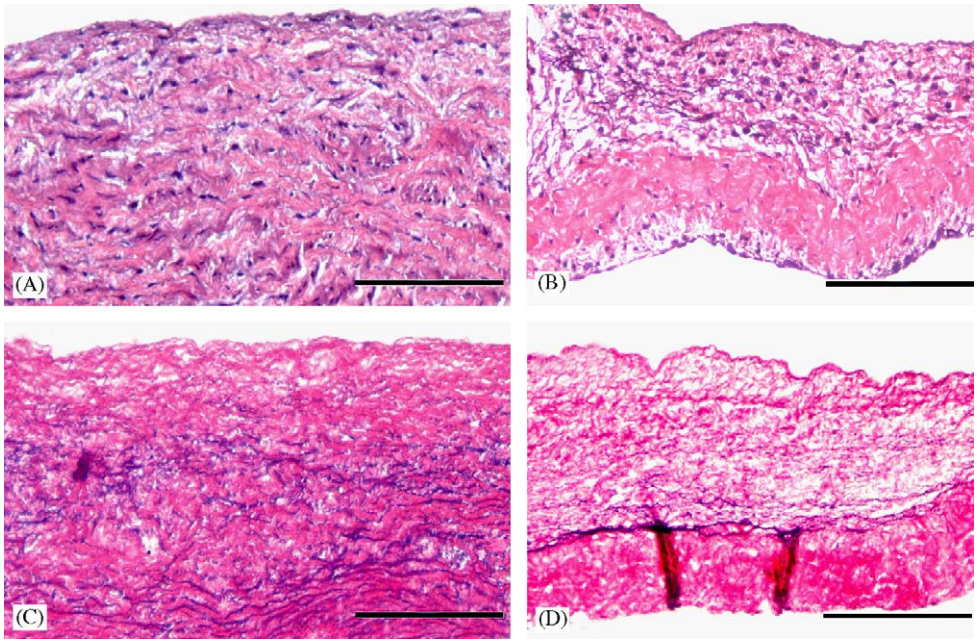

Fig. 2. Histology of native and decellularized PV. H&E staining. (A) Native pulmonary artery wall, (B) native cusp, (C) decellularized pulmonary artery wall, (D) decellularized cusp. Nuclei—blue, connective tissue—red. Bars = 100 μm.

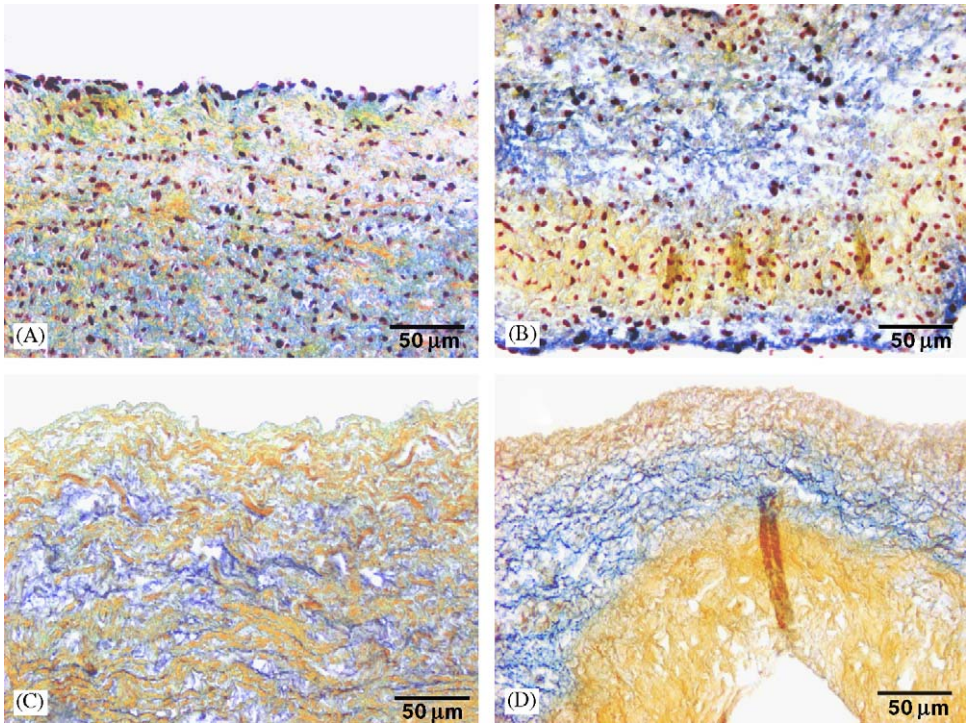

Fig. 3. Representative histological image of native and decellularized PV demonstrated an efficient preservation of matrix structures. Movat pentachrome staining of native pulmonary artery wall (A), native cusp (B), decellularized pulmonary artery wall (C), and decellularized cusp (D). Collagen—yellow, elastic fibers—red, proteoglycans—blue/green, nuclei—dark purple. Bars = 50 μm.

3.2. Biomechanics

The biomechanical parameters of native and decellularized tissue (ultimate force, structural stiffness, ultimate strain, ultimate stress, and elastic modulus) are shown in Table 1. Ovine decellularized tissue revealed biomechanical properties in both longitudinal and circumferential direction comparable to native PV. Biomechanical examination revealed a higher tissue resistance to tension in circumferential of native as well as of decellularized pulmonary conduit. Parameters as ultimate force, stiffness, ultimate strain, and ultimate stress were slightly decreased (below

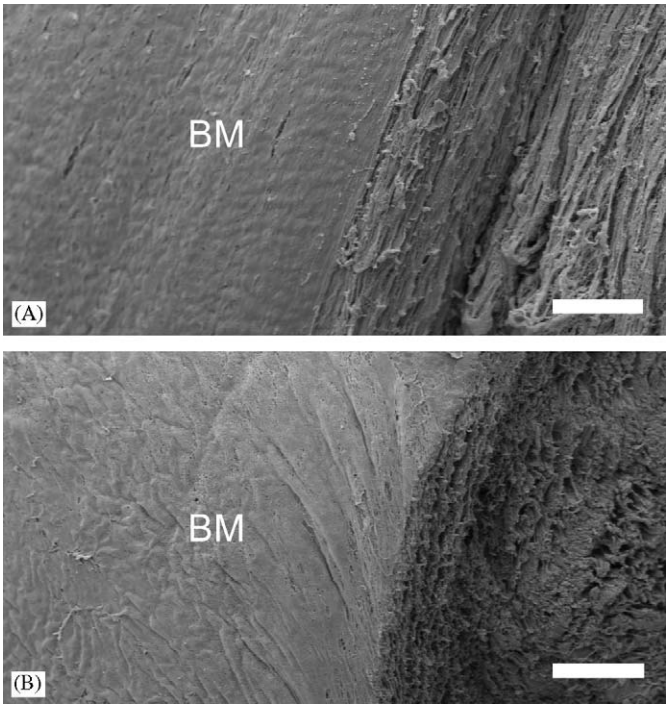

Fig. 4. Transversal section of decellularized ovine PV wall and cusp demonstrated the presence of the basement membrane (BM) on the luminal surface by SEM: (A) cusp, (B) wall. Bars = 10 μm.

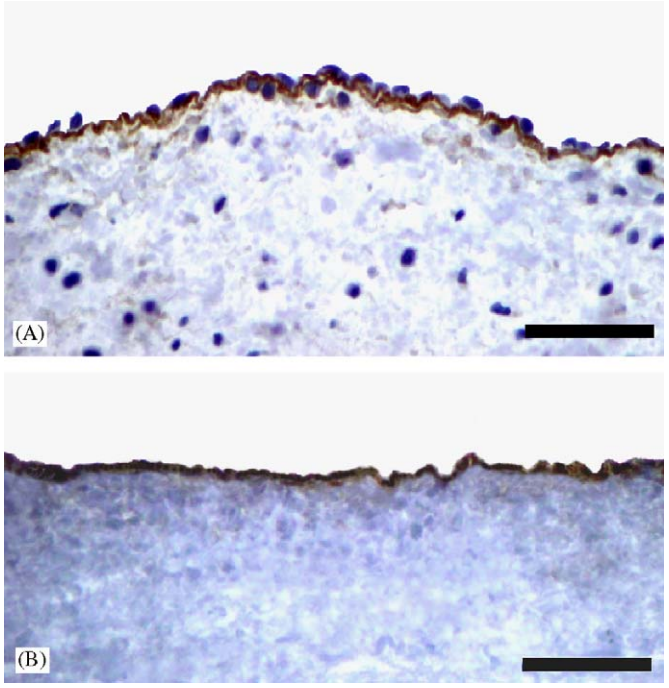

Fig. 5. Immunohistochemistry of native and decellularized PV cusps demonstrated the presence of collagen IV as main protein of the basement membrane: (A) native cusp, (B) decellularized cusp. Bars = 50 μm.

statistical significance) in decellularized PV as compared to native. Tissue decellularization caused also a slightly non-significant decrease of elastic modulus data by longitudinal and a slightly increase by circumferential tension.

3.3. Detergent removal from decellularized tissue

WS samples from the third and consecutive washing cycles proved to be non-toxic to EC as determined by metabolic activity and viability, and they were statistically comparable to the PBS control (Fig. 6). Six washing cycles over 72 h were chosen as a standard protocol for detergent removal from decellularized tissue. All valves treated according to this protocol revealed cellular attachment and formation of EC monolayer on the valvular surface after graft recellularization in a bioreactor.

3.4. Maintenance of physiological environment in bioreactors

Analysis of the gas concentration in the circulating medium showed physiological conditions of pO_2, pCO_2, and pH during culture (Fig. 7). The exact adjustment of the pH level was achieved by modulating the CO_2 supply. The optimal CO_2 concentration in the incubator was 6–7%. Glucose concentration was set to 5.5 ± 1.4 mmol/L. The low release of lactate (1.3 ± 0.2 mmol/L) implied normal aerobic cell metabolism in all cultures.

3.5. Morphological evaluation of bioengineered PV

After seeding under rotation conditions in the bioreactor for 2 days, EC were detected on the luminal surface as an incomplete monolayer (not shown). Under dynamic, pulsatile flow at a rate of 1.0 L/min, EC reached confluence and formed a complete monolayer (Fig. 8A). Immunohistological analysis revealed that cells on the scaffold expressed vWF demonstrating an endothelial origin (Fig. 8C). Even after further graduated increase of the flow rate up to 2.0 L/min, the confluent EC monolayer was also present on the whole luminal surface of the valvular scaffold (Fig. 9). Reseeded EC on the matrix surface showed a high metabolic activity as ascertained by MTS assays compared to negative control (decellularized matrix) (0.33 ± 0.12 vs. 0.03 ± 0.04 absorbance units at 490 nm; $p < 0.001$; respectively).

4. Discussion

Here we report on the development of a special bioreactor system, which permits the use of variable levels of shear stress forces on EC during the recellularization process. Accurate monitoring and continuous adjustment of cell culture parameters in our bioreactor leads to a significantly improved cell-seeding procedure. Current in vitro studies have shown that the expression of many genes in EC and activation of different signaling pathways could be regulated by shear stress-induced mechanotransduction [13,14]. In vitro development of stable cell–matrix and cell–cell connections during dynamic culture may favorably influence the biocompatibility of bioengineered

Table 1
Biomechanical properties (tension tests) of native versus decellularized PV

Value	Native PV ($n = 5$)		Decellularized PV ($n = 5$)	
	Longitudinal	Circumferential	Longitudinal	Circumferential
Area (mm^2)	29.2±5.7	29.6±6.3	31.8±6.6	29.9±1.6
Ultimate force (N)	9.5±4.3	13.9±1.5	6.6±2.1	11.4±3.9
Stiffness (N/mm)	3.0±2.6	4.3±1.6	2.7±0.7	3.9±1.3
Ultimate strain (mm/mm)	0.8±0.5	1.6±0.6	0.6±0.2	1.2±0.8
Ultimate stress (MPa)	0.32±0.15	0.50±0.18	0.22±0.11	0.39±0.14
Young's modulus (MPa)	1.04±0.94	0.86±0.57	0.90±0.38	1.18±0.55

PV—pulmonary valve conduit ($n = 5$ each). No statistically significant differences between the native and decellularized groups were found.

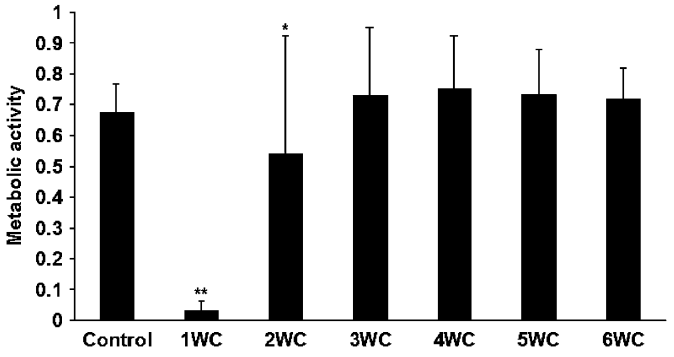

Fig. 6. Metabolic activity of sheep EC exposed to washing solution samples from six succeeding washing cycles (WC) as ascertained by MTS assay. $^{*}p<0.05$; $^{**}p<0.001$ vs. control.

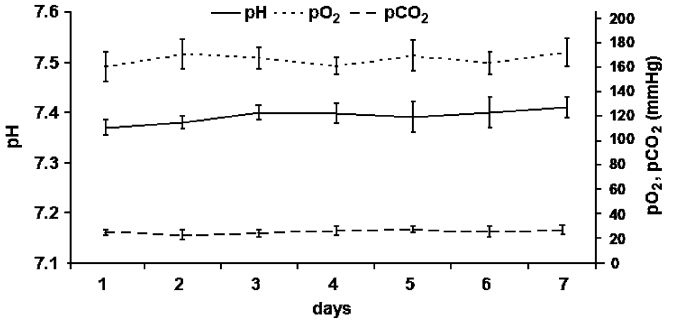

Fig. 7. pO_2, pCO_2, and pH levels in the pulsatile bioreactor during dynamic reseeding of EC onto the decellularized ovine PV.

heart valves in vivo. Experiments including functional tests in vivo are pending.

Constant levels of temperature, nutrients, pO_2, pCO_2, and pH seem to be essential for metabolism, growth, and proliferation of cultured cells [15,16]. The constant physiological balance of the medium gases could be achieved only if they are supplied in sufficient quantities into the CM. The fact that medium gases can be precisely adjusted by our bioreactor system assures constant physiological conditions.

Another important issue in heart valve TE is the maintenance of a stable EC layer under high flow rate comparable to physiological hemodynamic circulation conditions. In this respect, the abrupt transfer of reseeded EC from static conditions to high flow in a bioreactor system could disrupt the EC layer [17]. For example, the physiological high flow in a pulsatile heart valve bioreactor may cause a cell wash-off from the matrix surface, especially from distinctive turbulent areas such as valve leaflets [10]. Therefore, the slow adaptation of EC through a stepwise increase of the flow within a bioreactor system seems to be a crucial point in avoiding EC layer injury under physiological flow conditions. The flow regime in our bioreactor allowed the maintenance of a confluent layer of EC on the valve surface during the culture exposed to flows up to 2.0 L/min, which approximately corresponds to physiological pulmonary flow in a juvenile sheep.

Successful results of TE of heart valves based on a biological scaffold have previously been reported by our institution [5,6]. Tissue immunogenicity of the heart valve conduits was resolved by enzymatic treatment using trypsin/EDTA solutions for cell removal. Resulting valve scaffolds were reseeded with autologous cells in a bioreactor. Recent gains in knowledge of TE processes, together with the results of animal implantations, have encouraged us to reconsider current TE methods and to improve the biotechnological processes of cardiac valve TE.

The enzymatic treatment of the valve tissue is aggressive and leads to partial matrix injury with damage of the basement membrane [18,19]. As an alternative, we used a detergent treatment to decellularize the tissue. The use of detergents, however, necessitates a drastic washing procedure prior to reseeding since detergents are well-known cell toxins [19,20]. Nevertheless, the biomechanical properties of the decellularized matrices produced by our method, which are similar to those of the native tissue, suggest that the matrix structures of the tissue were not significantly compromised. The preservation of the mechanical stability of matrices treated in this way may result in a more adequate resistance to hemodynamic forces present in physiological circulation as implicated by our tests using high flow rates.

Implantation of decellularized allograft and xenograft valves induces tissue regeneration in vivo with efficient repopulation of the matrix by interstitial cells, but no

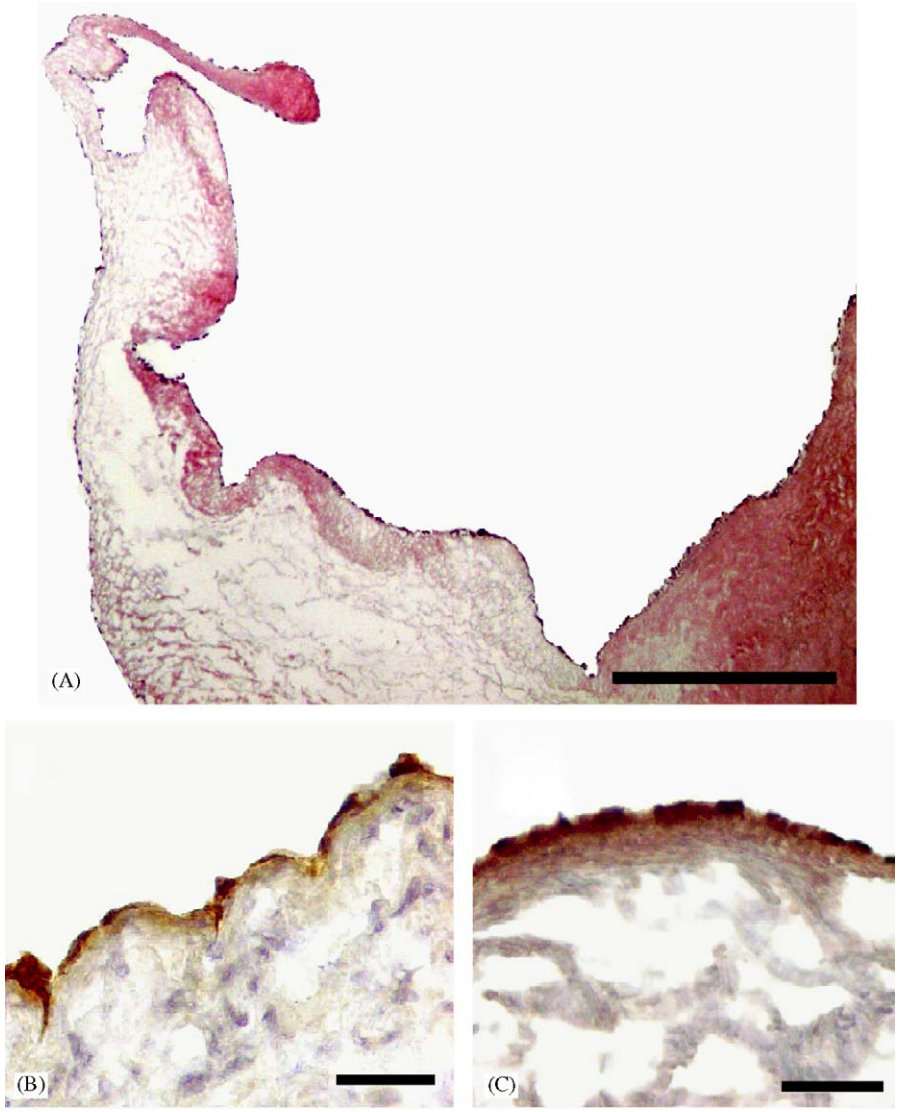

Fig. 8. Confluent EC monolayer on dynamically reseeded PV (pulsatile flow: 1.0 L/min). (A) Perfect re-endothelialization of the ventricular and pulmonary sides of cusp and wall surface (H&E staining; Bar = 1 mm). Representative immunohistochemistry showing expression of vWF (brown) in native PV cusp (B) and of reseeded cells on the decellularized PV cusp (C). Bars = 50 μm.

sufficient re-endothelialization [9,21]. The absence of a confluent endothelial layer on decellularized valves predisposes the unprotected matrix surface to thrombosis and intimal hyperplasia with subsequent graft failure [22,23]. Although the decellularized grafts have already reached clinical trials, the outcome is still controversial [24]. Therefore, our concept is based on pre-operative endothelialization of the decellularized scaffolds to facilitate tissue remodeling and to decrease the risk of graft failure.

The important point in heart valve TE is the autologous cell seeding in physiological dynamic culture environment. The cell repopulation of the decellularized scaffolds was previously performed mainly under static conditions [5,25], or under low flow circulation using non-pulsatile bioreactors [6]. The application of mechanical shear stress during in vitro culture of EC plays an important role in cell growth, orientation, and phenotypic remodeling [14,26,27].

5. Conclusion

In conclusion, we present a new method for TE of pulmonary heart valves in a dynamic bioreactor system. Using detergents, we were able to produce a non-toxic valve scaffold while simultaneously preserving the structures of the ECM and thus observed analogous biomechanical properties when compared to native tissue. The dynamic culture of EC onto biological scaffolds using a novel pulsatile bioreactor system imitating pulmonary circulation environment improves cell growth and adhesion and allows the formation and maintenance of an EC monolayer. The adaptation of reseeded EC to physiological circulation conditions by an initially low flow and gradually increasing flow in the bioreactor provides the basis for stable cell–matrix connections. In this respect, this work represents the first time that an EC monolayer on a

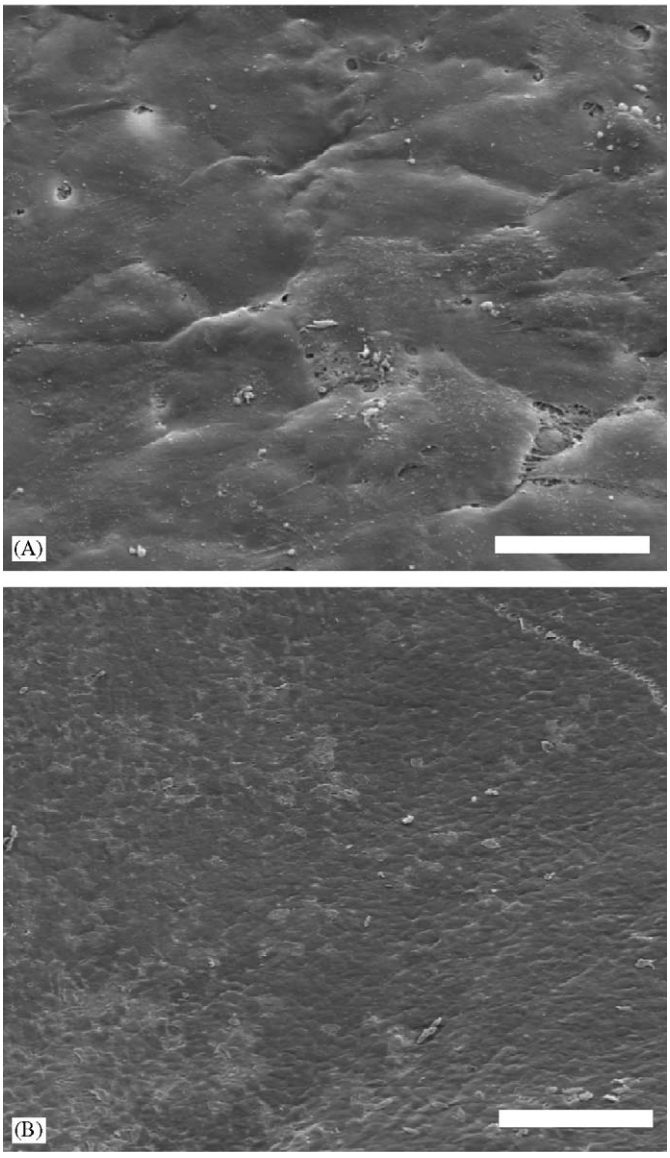

Fig. 9. Complete re-endothelialization of PV reseeded with EC exposed to pulsatile circulation (2.0 L/min flow). SEM of the cusp: (A) original magnification 1000×, bar = 10 μm; (B) original magnification 100×, bar = 100 μm.

biological scaffold exhibits sufficient adhesional stability when exposed to high pulsatile flow. Thus, permanent monitoring of biotechnological processes and continuous adjustment of the physiological conditions seem to be important in improving cardiac valve engineering outcomes.

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