

# Histological Evaluation of Tissue-Engineered Heart Valves Implanted in the Juvenile Sheep Model: Is There a Need for In-Vitro Seeding?

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**Background and aim of the study:** A new-generation heart valve was developed to improve long-term durability. The study aim was to evaluate the need for in-vitro autologous endothelial cell seeding of a decellularized valve scaffold.

**Methods:** A seeded (group I, n = 6) or non-seeded (group II, n = 6) decellularized valve was implanted into the right ventricular outflow tract of 12 juvenile sheep. In group I, a jugular vein was harvested to characterize and expand endothelial cells (ECs). The scaffold was seeded in vitro, using a sedimentation technique. In group II, non-seeded decellularized scaffolds were implanted and explantation was performed at three and six months. Valves were evaluated by echocardiography, gross pathology, X-radiography, histology, and immunohistochemistry.

**Results:** In group I, the EC seeding density was  $1.06 \pm$

$0.01 \times 10^5$  cells/cm<sup>2</sup>, with a cell viability of  $95.7 \pm 1.4\%$ . No regurgitation was visualized by echocardiography. Gross pathology showed smooth leaflets without retraction; calcification was absent at X-radiography, and this was confirmed by von Kossa staining. Histologically, group I valves showed a persistence of ECs, whereas a monolayer of host ECs was seen at six months in group II valves. Host fibroblasts were available in both groups, and numbers increased over time. Differences were identified in the recellularization density of in-vitro-seeded and non-seeded valves for up to three months, but no such difference was seen after six months.

**Conclusion:** Based on results of studies in a sheep model, there appears to be no need for in-vitro cell seeding of decellularized scaffolds.

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Right ventricular outflow tract (RVOT) reconstruction remains a demanding issue in congenital heart surgery. Although patch repair is initially well tolerated (1), in time severe pulmonary valve insufficiency will lead to right ventricular failure, increased tricuspid valve regurgitation and ventricular arrhythmias (2). Usually, pulmonary homografts are used at this stage to recompensate right ventricular failure, but these heart valves show tissue degeneration, most likely due to immunologic reactions (3). The results of experimental studies have shown that tissue-engineered heart valves are able to improve the quality of

tissue valves, with an absence of calcification and without compromising hemodynamic behavior (4,5). The main disadvantages of the in-vitro seeding of heart valves include the complexity of the seeding process and the transportation of viable heart valves; an additional problem is that there is a waiting period of at least four to six weeks before implantation.

The study aim was to investigate the need for in-vitro seeding of a decellularized valve scaffolds, using a juvenile sheep model.

## Materials and methods

### Animal studies

Twelve juvenile sheep were utilized in these studies. Six animals (group I) received an in-vitro-seeded valve scaffold, and another seven (group II) a non-seeded decellularized valve scaffold. One animal in group II subsequently died from endocarditis and the valve matrix was explanted; thus, this valve was rejected from the study as it could not be fixed within an appropriate time before cell lysis.

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All animal experiments were performed in accordance with the *Principles of Laboratory Animal Care*, prepared by the National Society of Medical Research, and the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH publication 85-23, revised 1985). The study was approved by the ethical committee of the Pontificia Universidade Catolica de Parana.

### **Valve assembly**

#### ***Production of the decellularized valve matrix***

Details of this procedure have been reported previously (6). In brief, porcine pulmonary heart valves were prepared at the slaughterhouse in order to reduce the warm ischemic time, which was less than 2 h. After preparation, the valves were placed in an antibiotic solution to commence decellularization, ensuring that the cold ischemic time was also <2 h. The valves were then decellularized with 0.1% deoxycholic acid (Sigma Chemical Co., St. Louis, MO, USA) at 37°C and rinsed extensively with physiological saline solution. The decellularized matrices were examined for fenestration, atheroma, or other pathological changes.

#### ***In-vitro seeding of a decellularized valve matrix***

Details of this procedure have also been reported previously (4,7,8). In brief, at four weeks before implantation of the tissue-engineered heart valve of group I, a piece of vein was harvested from each juvenile sheep. The veins were rinsed with physiological saline solution, stored in Delbecco's modified Eagle's Medium (DMEM, Sigma Chemical Co.) containing antibiotics (penicillin 100 U/ml, streptomycin 100 µg/ml, amphotericin B 250 ng/ml; all Sigma Chemical Co.), and transported to the cell culture laboratory. Endothelial cells (ECs) were harvested and separated using collagenase P 0.1% (Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CN, USA) for 15 min in a humidified incubator (37°C, 5% CO<sub>2</sub>, 98% air saturation). The cultivation of ECs was performed in DMEM with 20% autologous sheep serum, 5 µg/ml basic fibroblast growth factor (Boehringer Ingelheim Pharmaceuticals) and antibiotics (penicillin, 100 U/ml; streptomycin, 100 µg/ml; amphotericin B, 250 ng/ml). Before seeding, the acellular matrices were treated for 3 h with fibronectin (Boehringer Ingelheim Pharmaceuticals) to increase the ECs binding capacity. The seeding process was maintained during a 4-h process, during which time the ECs were allowed to sediment both on the matrix wall and the leaflets. The final tissue-engineered heart valves were stored in a humidified incubator until sterility was proven.

### **Surgical technique**

Details of the implantation technique have been

reported previously (9). In brief, the sheep was sterile-draped and a left mini-thoracotomy performed at the third intercostal space. The pericardium was vertically incised to expose the heart; the main pulmonary artery was then isolated, taking great care to preserve the left main coronary artery. The descending aorta was dissected free and cannulated with a 21-Fr aortic tube. A 34-Fr cannula was used for venous return of the right atrium. Normothermic cardiopulmonary bypass (CPB) was performed with a Biomedica ECO-01 (Biomedica Ind. Com. E. Repres. Ltda, Sao Jose do Rio Preto, Brasil). After starting CPB, the truncus pulmonaris was transected and the native valve leaflets were resected. Either the seeded valves of group I or the non-seeded valves of group II were implanted using two running 5-0 polypropylene suture lines with a no-touch technique. CPB was discontinued and the chest closed in layers as soon as hemostasis was completed. Postoperative care was similar in both groups, without anticoagulation therapy.

### **Transthoracic echocardiography**

Transthoracic echocardiography was performed using a Hewlett Packard Sonos 5500 (Sunnyvale, CA, USA) with a 7.5-MHz probe. Pulmonary insufficiency was graded with color Doppler flow during the examination. The length and width of the regurgitation jet into the RVOT was graded as 0 (none), 1+ (trace), 2+ (mild), 3+ (moderate), and 4+ (severe). The mean flow velocity at the pulmonary valve was measured with continuous-wave Doppler.

### **Explantation and analysis**

Three valves in both groups were explanted at three months after implantation, and the other three at six months. The left mini-thoracotomy was reopened and intravenous 3 mg/kg heparin applied to prevent blood clotting at the inner surface of the heart valve. After exsanguination, the implanted valves were excised together with the proximal and distal parts of the native pulmonary artery.

### **Gross pathology**

The explanted valves were inspected and photographed. Leaflets were especially inspected for fenestrations, retraction, thrombotic material, and atheroma or calcification. Each graft was transected longitudinally through the commissures, including a short segment of the sheep's native pulmonary artery at both ends.

### **X-radiography**

X-radiography was performed under mammographic conditions to identify macroscopic calcification at the explanted valves.

### Histological examination

Histology was performed on longitudinal sections of the specimen through the middle of the left pulmonary cusp, which was embedded in paraffin. Sections (4  $\mu\text{m}$  thickness) were routinely stained with hematoxylin and eosin (H&E) and von Kossa staining.

### Immunohistochemistry

Immunohistochemical staining was performed with von Willebrandt factor antigen (DAKO, Hamburg, Germany).

### Statistical analysis

Quantitative data were expressed as mean  $\pm$  SD. Between-group comparisons were made using a paired *t*-test. A *p*-value  $<0.05$  was considered to be statistically significant. All data management and statistical analyses were performed using SSPS software 11.5 (SSPS Inc., Chicago, USA).

## Results

### Echocardiography

Transthoracic echocardiography demonstrated an absence of valve regurgitation in both groups; neither was any trivial regurgitation identified. At three and six months after implantation, the mean flow velocities at the seeded valve (group I) were  $0.6 \pm 0.2$  m/s and  $0.7 \pm 0.2$  m/s respectively, and  $0.8 \pm 0.1$  m/s and  $1.0 \pm 0.1$  m/s respectively at the non-seeded valve (group II) (*p* = NS, group I versus group II).

### Valve assembly

The median annulus diameter of the decellularized porcine scaffold (*n* = 12) was 21 mm (range: 19 to 24 mm). In group I, after four weeks the median available EC value was  $50 \times 10^6$  cells (range:  $24 \times 10^6$  to  $104 \times 10^6$  cells). Each of the cell cultures was von Willebrandt factor-positive, and there was no interstitial cell contamination. The total number of ECs used to seed the matrix ranged from  $4.0 \times 10^6$  to  $5.0 \times 10^6$ , depending on the valve diameter. The mean EC-coverage density after seeding was  $1.1 \pm 0.1 \times 10^5$  cells/cm<sup>2</sup> (range:  $1.0$  to  $1.2 \times 10^6$  cells/cm<sup>2</sup>), with a mean cell viability of  $95.7 \pm 1.4\%$  (range: 93.4 to 97.7%).

### Gross pathology

No hematomas, vegetations or thrombotic material were identified after three and six months in all explanted heart valves of both groups. Moreover, the leaflets showed no signs of tearing, perforation, fibrous tissue overgrowth, cusp deformation, retraction, or hardness. There was no evidence of embolic infarction in the lungs after three and six months' follow up in either group.

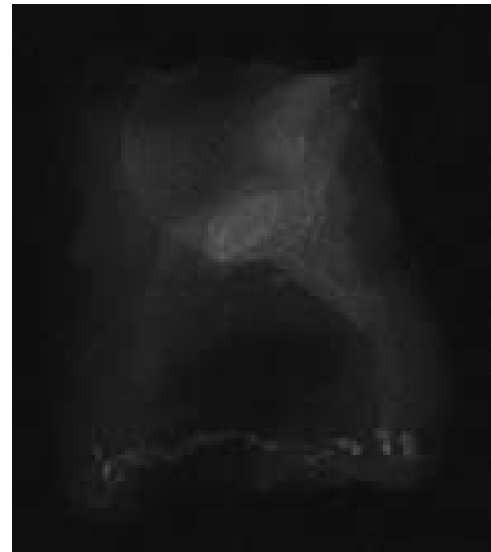


Figure 1: X-radiography demonstrating an absence of calcification in both group at six months. a) A seeded decellularized valve shows only a suture line at the proximal anastomosis. b) A non-seeded decellularized valve.

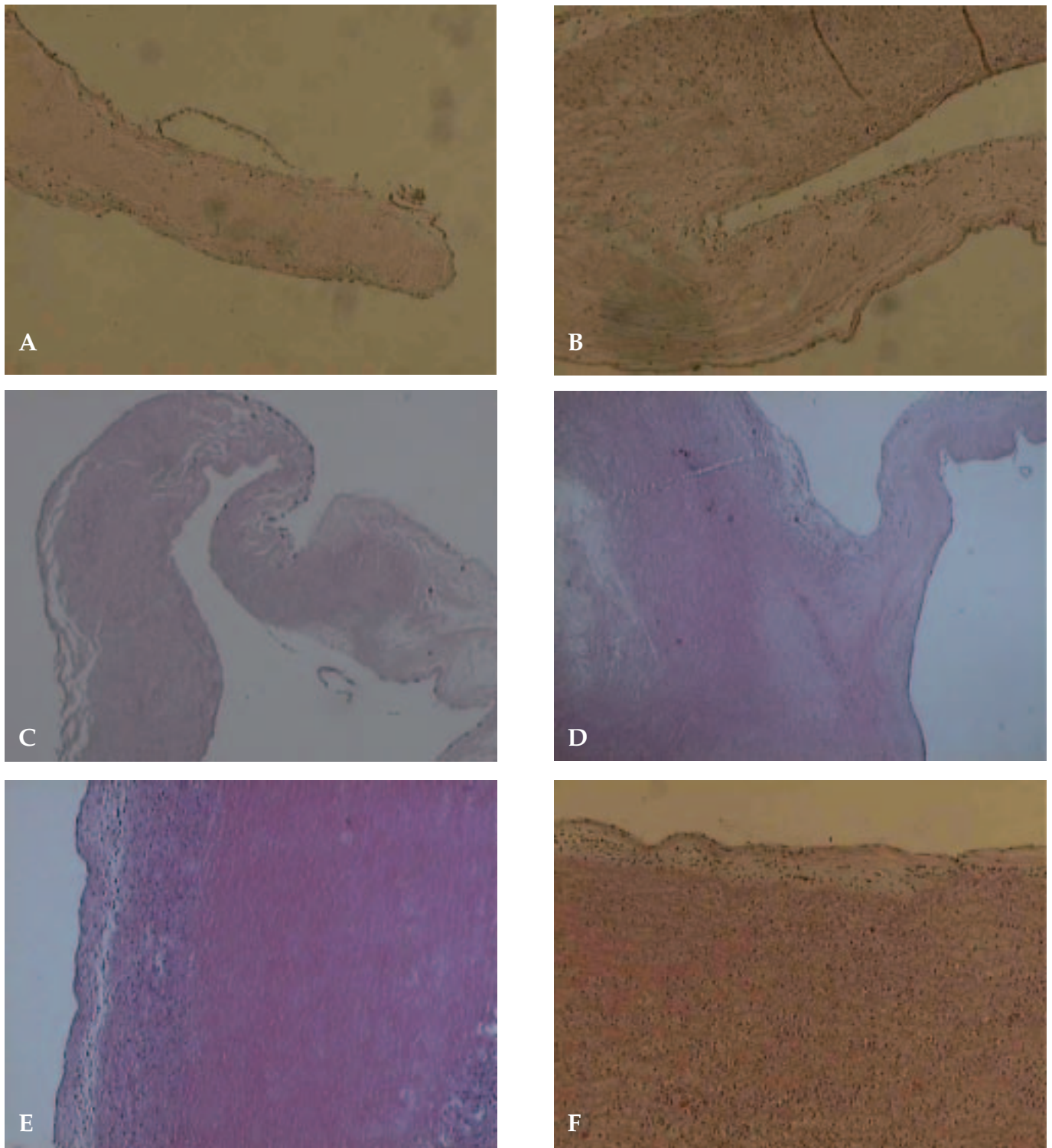


Figure 2: H&E staining. a) A monolayer of endothelial-like cells at the free edge of the leaflets of an in-vitro-seeded decellularized heart valve (original magnification,  $\times 20$ ). b) Endothelial cells and an almost complete recellularization of the layers of the in-vitro endothelial cell-seeded decellularized matrix of the valve wall, as well as the leaflets (original magnification,  $\times 20$ ). c) Islands of endothelial-like cells at the leaflets of an in-vitro-non-seeded decellularized valve (original magnification,  $\times 40$ ). d) A complete valvular wall and leaflet re-endothelialization of a decellularized valve at six months (original magnification,  $\times 10$ ). e) Endothelial-like cells at the inner surface of the valve wall, with ingrowth of interstitial cells in the deeper layers of the scaffold. At three months the media of the valve wall is still acellular (original magnification,  $\times 20$ ). f) A representative sample of the valve wall, which has been completely recellularized (original magnification,  $\times 20$ ).

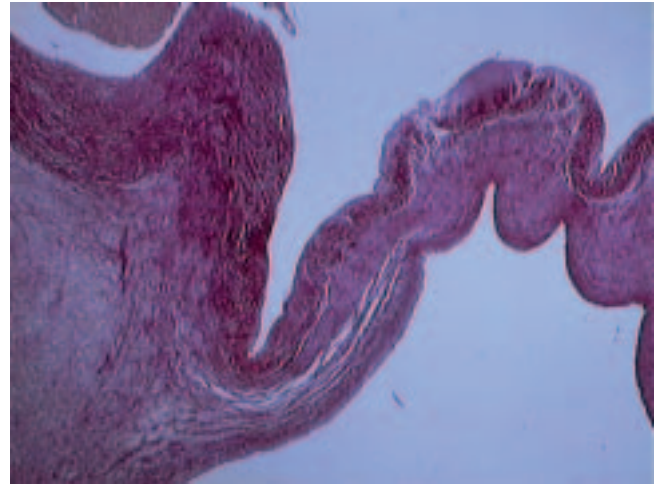
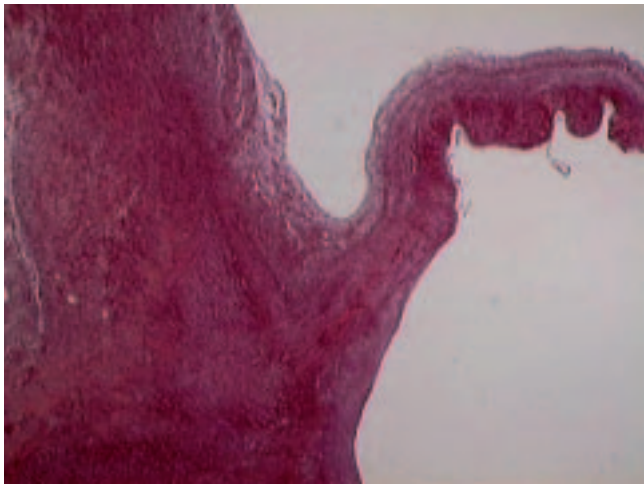


Figure 3: von Kossa staining. a) Absence of calcification in an *in-vitro*-seeded decellularized valve (original magnification,  $\times 20$ ). b) Absence of calcification at six months in a decellularized valve (original magnification,  $\times 10$ ).

### X-radiography

On X-radiographic examination, no calcification was evident after three and six months of implantation in either group (Fig. 1a,b).

### Histology

H&E staining demonstrated a monolayer of endothelial-like cells at the inner surface of the valve wall and leaflets of group I at three and six months after implantation (Fig. 2a,b). In group II, islands of endothelial-like cells were seen at the inner surface as early as three months after implantation (Fig. 2c), but there was no confluent monolayer at this time. At six months after implantation, the valves were completely covered by endothelial-like cells (Fig. 2d).

Fibroblast ingrowth appeared to be influenced by the availability of ECs, as the valve scaffolds of group I were completely recellularized by host cells at an earlier stage than those of group II (Fig. 2e). After six months of implantation, however, there were no intergroup differences (Fig. 2f). Independent of the *in-vitro* seeding process, no thrombotic material was found on any of the implanted heart valves.

von Kossa staining demonstrated an absence of calcification in both groups, with neither the valve leaflets nor wall being calcified at three and six months after implantation (Fig. 3a,b).

### Immunohistochemistry

Immunohistochemical staining with von Willebrandt factor demonstrated that the cells covering the inner surface of the valves were ECs in both groups (Fig. 4a,b). Initially, valves in group I showed a confluent monolayer of ECs; these appeared to resist the shear stress of the blood flow as they remained unchanged after three and six months of implantation. A monolayer of ECs was not apparent after three

months on the non-seeded valves (group II), but after six months complete re-endothelialization was visible.

In both groups there was evidence of the development of a new vasa vasorum, commencing at the tunica adventitia of the valve wall and penetrating from that point. In the medial part of the wall, the medial vasa were seen to be composed of thin-walled endothelial 'channels' (Fig. 4c).

### Discussion

Tissue engineering provides benefits for the creation of new-generation heart valves that possess a number of advantages over the present, commercially available heart valve prostheses. These tissue-engineered heart valves are not only viable but also have the potential for regeneration, remodeling, and growth. The key region of any tissue-engineered heart valve is the scaffold, which should provide an optimal environment allowing autologous interstitial cells and ECs to bind with persistent and unique functions. As several potential groups of scaffolds are available today in the field of tissue engineering, the present authors preferred to use a decellularized xenogeneic scaffold. This has a natural configuration and thus the potential to allow excellent hemodynamic behavior, without losing optimal strength, as demonstrated previously (9). Essentially, the aim of decellularization treatment is to eliminate completely any interstitial cells and ECs, while preserving the extracellular scaffold, including the micro-environment. Recently, Leyh et al. (10) showed that, when using a trypsin/EDTA decellularization treatment, heavy calcification could be found in the decellularized scaffolds only three months after surgery. However, the decellularization method used by Leyh et al. seemed to be extremely aggressive, such that not only were the interstitial cells removed but the

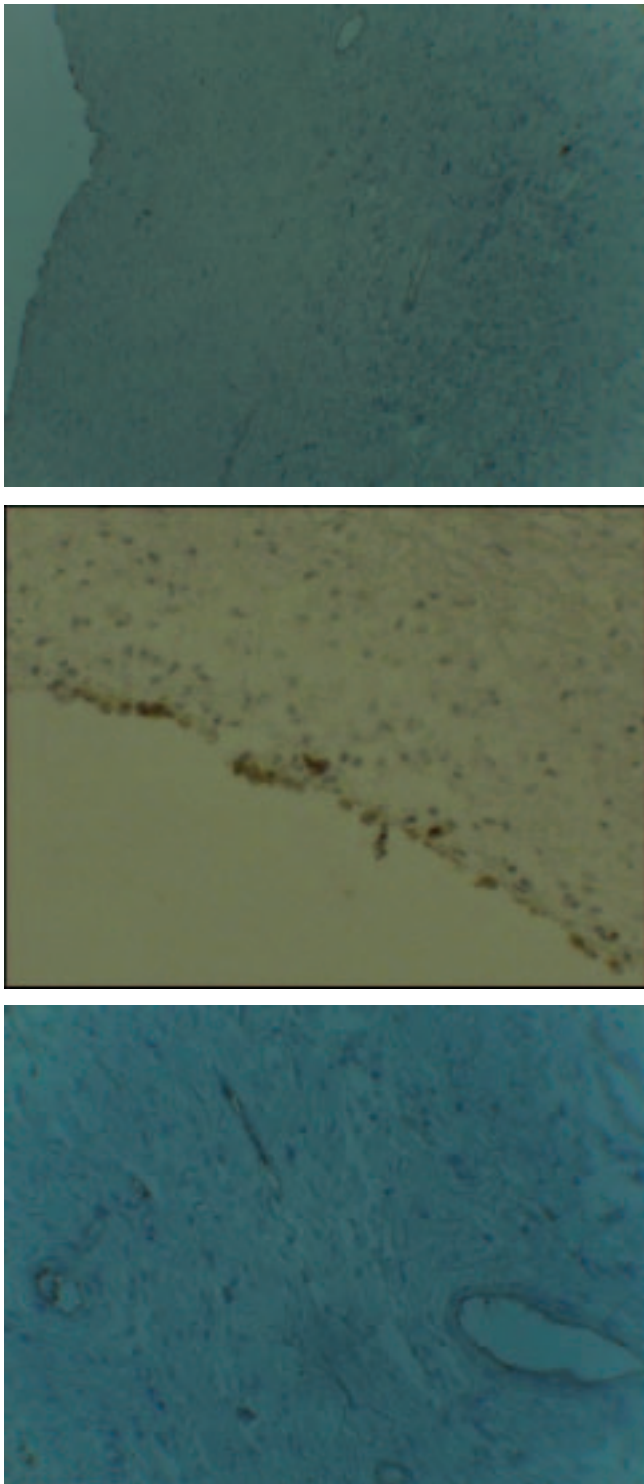


Figure 4: Von Willebrandt factor staining. a) The cells at the inner surface of the heart valve wall were endothelial cells (original magnification,  $\times 4$ ). b) The monolayer of endothelial cells at the inner surface of the valve wall in close connection with the decellularized valve scaffold (original magnification,  $\times 40$ ). c) The newly developed vasa vasorum; this detailed area represents the medial part of the wall, composed of thin-walled endothelial 'channels' (original magnification,  $\times 40$ ).

extracellular scaffold was also destroyed. Moreover, the hemodynamic behavior of these valves was found to be less optimal than that of glutaraldehyde-treated bioprostheses.

After having produced sufficient scaffold, the present authors were able to demonstrate - both experimentally and clinically - the use of these heart valves. Neither calcification nor degeneration were demonstrated, and the valve provided an impressive hemodynamic performance. Unfortunately, new logistic problems may arise with the use of this type of tissue-engineered heart valve (4,7). First, the heart valve is a living structure, which can lose viability during long-distance transportation, and consequently new - and perhaps very complicated - methods must be developed for their transportation. The production process is also very demanding and complicated, and may introduce several risk factors during assembly. These problems can be largely overcome by standardizing the procedure as far as possible, and installing a high-quality control management system. The final problem is that of the time delay of implantation, as this treatment would be available only to those patients undergoing elective valve surgery.

Thus, it was decided to evaluate the possibility that, as a scaffold could be recellularized in vitro by host interstitial cells, it could also be implanted and complete recellularization allowed in vivo. The potential problems of such an approach may be a higher risk of endocarditis, thrombogenicity and antigenicity of the material, and hence these points required evaluation.

In the present study, one animal died from endocarditis. Previously, Costerton et al. (11) described a technique by which a bacterial biofilm could be produced in the absence of ECs. Due to natural healing mechanisms, phagocytosis would commence and phagocytic enzymes be released, causing damage to the surrounding tissue. Among the present animals, no further endocarditic changes were identified, and at three months after implantation the valve walls showed an EC layer in the non-seeded heart valves, which was completed after six months and extended to the free edge of the leaflets.

Other groups have also reported endocarditis in valve implantation in a right-sided sheep model. Herijgers et al. (12) explained this complication by the need for multiple injection both perioperatively and postoperatively, as well as difficulties associated with wound draping in the non-anesthetized animal. Bernal et al. (13) reported that endocarditis could be due to poor preparation of the surgical field, which was hampered by the animal's thick woolen coat. Both groups demonstrated a higher incidence of endocarditis as compared to previous experiences. It is possible that the endocarditis which caused one of the present animals to die was caused by contamination contracted either during surgery or postop-

eratively; such speculation was based on the fact that the animal died within the first month of follow up.

One other advantage of the in-vitro EC seeding of tissue-engineered heart valves might be protection against early platelet aggregation. Andrews and Berndt (14) demonstrated the potential stimulation of blood vessel wall injury to create activate platelet aggregates or thrombus acceleration due to glycoprotein availability in collagen. These adhered, activated platelets might also interact with inflammatory leukocytes and facilitate platelet-leukocyte endothelial cell adhesion. At gross pathological examination, no pannus formation was seen at the inner surface of the non-seeded heart valves. In fact, the neointima - which normally can be seen in cryopreserved allografts (15) could not be seen - and therefore no leaflet retraction could be detected in both groups of heart valves.

One final possible disadvantage of the non-seeded heart valves might be the potential antigenicity of the xenogeneic collagen. Lynn et al. (16) reported the potential immunological responses of bovine collagen, with allergy developing in 1 to 8% of those patients treated with a bovine derma. However, Nakamura (17) could not demonstrate any adverse reaction with porcine collagen, and it appears unnecessary to cover the porcine collagen tissue in vitro with autologous cells to prevent this potential problem.

*In conclusion*, no evidence was found for the in-vitro seeding of decellularized heart valves, as the hemodynamic behavior and valve remodeling potential was comparable in both the seeded and non-seeded groups. The only difference was that, during the first three postoperative months, the valve leaflets were not confluent covered by ECs in the non-seeded group, but in the seeded group there was a full covering of EC due as a result of the seeding process. At six months of follow up, however, there were no longer any intergroup differences.

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