Physiological Performance of a Detergent Decellularized Heart Valve Implanted for 15 Months in Vietnamese Pigs: Surgical Procedure, Follow-up, and Explant Inspection

*1Michele Gallo, *1Filippo Naso, †1Helen Poser, ‡Antonio Rossi, †Paolo Franci, §Roberto Bianco, §Matteo Micciolo, §Fabio Zanella, ¶Umberto Cucchini, **Luca Aresu, *Edward Buratto, †Roberto Busetto, ††Michele Spina, *Alessandro Gandaglia, and *Gino Gerosa

Departments of *Cardiac, Thoracic, and Vascular Science; †Clinical Veterinary Science; **Public Health, Comparative Pathologies, and Veterinary Hygiene; and ††Experimental Biomedical Sciences, University of Padova; ‡Department of Biochemistry, University of Pavia; and §Cardiac Surgery Center and ¶Clinical Cardiology Center, Hospital of Padova, Padova, Italy

Abstract: This study features the longest experimental follow-up for decellularized heart valves implanted in an animal model. Porcine aortic heart valves were decellularized according to a disclosed standardized method in which TRITON X-100 and sodium cholate (TRICOL) are used in succession, followed by a further treatment with the endonuclease Benzonase to completely remove the nucleic acid remnants. Experimental animals (n = 17), represented by Vietnamese pigs (VPs), received a decellularized aortic allograft as a substitute for the replacement of their right ventricular outflow tract. The surgical implantation of the TRICOL-treated aortic valve conduit was successful in 11 VPs, while perioperative or postoperative complications occurred in the remaining six animals. In the sham-operated group (n = 4), the native pulmonary root was excised and immediately reimplanted orthotopically in the same animal. Echocardiography demonstrated a satisfactory hemodynamic performance of the TRICOL-treated valves during follow-up as well as the absence of relevant leaflet alterations concerning thickness and motility or valve insufficiency. At explantation, macroscopic inspection of tissue-engineered heart valve conduits did not evidence calcifications and showed a decreased wall thickness, comparable to that of the reimplanted native pulmonary roots. Noteworthy, extended functional performance, recovery of DNA content, and active extracellular matrix precursor incorporation are apparently compatible with the properties of a living self-supporting substitute. **Keys Words: Translational research—Tissue engineering—Heart valves—Decellularization—Swine—Animal model.

Heart valve diseases represent a rising, global cause of mortality, responsible each year for more than 15 000 deaths in the USA (American Heart Association report) (1). The incidence of death from aortic valve pathology is almost 12 500 per year and it is listed as a contributory factor in an additional 26 300 cases. Every year, almost 300 000 cardiac valve interventions are performed worldwide in order to repair the affected valves or replace them with prostheses.

Presently, mechanical and biological valves (bioprostheses and homografts) are commercially available for clinical practice, but they are subject to limitations. Patients receiving a mechanical valve require lifelong anticoagulation therapy due to the risk of thromboembolic events, reported at a rate of up to 5% per patient in the first year (2). However, bioprostheses progressively degenerate due to mechanical stress and calcification phenomena; homografts, despite optimal hemodynamics, minimal thromboembolic complications, and resistance
to infection, are subject to chronic inflammation and long-term cusp retraction. Moreover, both mechanical and bioprosthetic valves are unable to integrate into the host due to their inability to grow and be remodeled, especially in pediatric patients who must undergo multiple surgical interventions in order to overcome a progressive downsizing of the implanted device (3).

Over the last 20 years, tissue-engineered heart valves (TEHVs), such as the Synergraff (4), have not succeeded in delivering a fully compatible and biomimetic graft for clinical use, especially in pediatric patients (5). This is also the case for synthetic polymers and biological scaffolds of nonvalvular origin (6) that have been used to generate a tridimensional valve-like environment able to promote cell colonization either in vitro, in dynamic bioreactors, or in vivo using different animal models (7). Therefore, considering that a replacement valve should function properly while withstanding physiological pressures and biomechanical stresses, decellularized aortic and pulmonary valves, of xenogeneic or allogeneic origin, are widely considered good candidates to be used as scaffolds for the production of clinically implantable TEHVs (8). Of particular note, a major impediment to the use of human allografts is the paucity of donors, while xenografts must be carefully screened for the possible presence of endogenous retroviruses especially after decellularization treatments, where traces of nucleic acids are detected in the resulting tissue (9). Moreover, in order to avoid a possible host immunogenic reaction, incubation of xenogeneic decellularized tissue biopsies with human plasma should be performed prior to clinical implantation to identify any potential adsorption of IgG (10).

In this study, we specifically analyzed the hemodynamic performance of a detergent-based decellularized (TRICOL) allograft, the maintenance of its biomechanical strength, and its eligibility to be implanted in an animal model over the longest follow-up performed to date. Other studies indicated that, even when implanted for shorter periods, decellularized heart valve scaffolds are capable of spontaneous cell repopulation (11–14). The propensity for this to occur is related to both the protocols adopted to obtain a cell-free prosthetic scaffold and the physiology of the experimental living model used.

As matter of fact, the choice of an appropriate animal for TEHV implantation largely determines the ability to achieve reproducible results, relevant to human application. Prior to the adoption of sheep, calves (15) and dogs (16) had been used with questionable results. The use of the ovine species has provided important data (17–20) and represents to date the standard model recommended by the Food and Drug Administration. However, despite advantages such as a high propensity for calcifying implanted tissue and good tolerance to invasive cardiothoracic surgery, sheep also have several shortcomings as reported recently by Butcher et al. (20). In fact, unlike in humans and pigs, sheep feature a spontaneous endothelialization of the cardiovascular grafts, which may artificially improve the implanted devices performance.

Swine share many anatomical and physiological similarities to humans such as cardiac output and stroke volume (21), mean arterial pressure (22), and heart rate, with a nearly identical coronary blood flow (23,24), and similar platelet–material interactions (25), while, in contrast to sheep, they do not overestimate the tissue remodeling potential of the graft (20).

Unlike common breeding pigs and other miniaturized porcine models like Göettingen, Yorkshire, Yucatan, and Hanford, the Vietnamese pig (VP), used in this study, is cost-effective, does not present experimental variability due to technical challenges, and features a stable standardized weight and organ dimensions closer to humans, eventually matching the average adult human terminal body weight (26,27).

**MATERIALS AND METHODS**

**Vietnamese pigs**

Animals were provided by CISRA Institute (120TO025—ASL 3 Collegno, Torino, Italy). VPs used were 12 months old with weights of $54.3 \pm 2.1$ kg for adult females and $65.1 \pm 2.5$ kg for adult males, respectively. All animals were screened for swine vesicular disease and vaccinated for Aujeszky’s disease parvovirosis, and swine erysipelas. The University of Padova was authorized to use VPs for experimental purposes (art. n°12 D.Lgs.116 27.01.1992) under project registration number 27/08 C16, authorized by the Italian Ministry of Health, according to the principles given by ISO 10993-2 (28).

**Study design**

Animals were divided into two groups: A—experimental: represented by VPs ($n = 11$) receiving the TRICOL-decellularized aortic allograft in substitution of their right ventricular outflow tract (RVOT) (6-month follow-up, $n = 5$; 15-month follow-up, $n = 6$); B—control: sham-operated group represented by VPs ($n = 4$) that had their autologous RVOT excised and immediately re-anastomosed in order to study the consequences of the surgery itself.
(6-month follow-up, \( n = 2 \); 15-month follow-up, \( n = 2 \)). Additional aortic valves, both native (\( n = 3 \)) and decellularized (\( n = 3 \)) allografts, were used for routine histological stainings (hematoxylin and eosin and Movat’s pentachrome) and SEM and TEM observation, as described in a preliminary report (29) (Fig. 1).

Heart valve scaffold fabrication

The aortic valves of young common breeding piglets (3–4 months old) were used as allografts. Hearts were collected at a local abattoir, transported to the laboratory in sterile cold saline in the presence of a full complement of antibiotics (penicillin 100 U/mL, streptomycin 100 mg/mL, and amphotericin B 250 \( \mu \)g/mL). Aortic roots were isolated from hearts and decellularized using the TRICOL method as previously described (30). Following the detergent protocol, nucleic acids (DNA/RNA) were removed according to previously described endonuclease Benzonase protocol (Merck Chemicals LTD, Nottingham, UK) for two successive 24-h periods at 37°C under constant stirring (29). Extensive washings with phosphate-buffered saline were thereafter carried out at 4°C.

DNA content determination

The evaluation of the original amount of DNA in untreated aortic and pulmonary roots (\( n = 2 \), animals X and Y) and the effectiveness in DNA removal in TRICOL-treated aortic allograft were carried out through a double cross-check comprising the determination with both the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and by fluorescence analysis. This latter was carried out on paraformaldehyde (4%)-fixed slices, stained with 4,6-diamidino-2-phenylindole (DAPI; 1 \( \mu \)g/mL) for 5 min. Tissue sections were observed using a Leica DM4000 microscope. Total DNA content was also determined in both the original pulmonary root and the TRICOL aortic allograft in the two animals called K and W explanted after 12 and 15 months, respectively.

Anesthesiology

VPs were sedated during echocardiography and surgery with midazolam (0.3 mg/kg), medetomidine (15 \( \mu \)g/kg), and ketamine (10 mg/kg), administered into the neck muscles. Anesthesia was induced with isoflurane in oxygen and maintained via a circular breathing system. A loading dose of alfentanil (1–1.5 \( \mu \)g/kg/min) was followed by a constant rate infusion (CRI). A long-standing 16G central venous catheter was positioned into the jugular vein using an ultrasound-guided technique. After the initiation of the extracorporeal circulation (ECC), ventilation was suspended as well as isoflurane administration. Propofol, 1 mg/kg, was infused for 5 min, followed by a lower dose of 0.2 mg/kg/min at CRI.

Surgery

Animals underwent surgery at the Department of Clinical Veterinary Science at the University of Padova. In order to ensure an adequate supply of blood for transfusion, a further set of adult breeding pigs acted as blood donors. The blood compatibility was assessed by cross-match assays (31). A xypho-
jugular incision, followed by median longitudinal sternotomy represented the surgical approach. Systemic heparinization was achieved with an activated coagulation time >400 s.

A pediatric aortic cannula was then inserted, and venous drainage was obtained via separate cannulae in both venae cavae. Normothermic ECC was turned on at 2.6 L/min/m along with ventilation suspension. VPs diuresis was stimulated with infusion of 18% mannitol in the ECC prime and with furosemide at an initial dosage of 1.5 mg/kg.

The native pulmonary artery root was removed and, in the A—experimental group, the RVOT was then reconstructed using TRICOL aortic roots with the coronary ostia trimmed (Fig. 2). After completion of the procedure, VPs were weaned from ECC with restoration of the mechanical lung ventilation.

Postoperative and long-term care
Postoperative analgesic therapy (morphine 0.1 mg/kg bid and then buprenorphine 10 mg/kg bid) was administered intravenously (IV) for 4 days after surgery. Anticoagulation (100 mg lysine acetylsalicylate bid), antibiotic (cefazolin 25 mg/kg bid), and anti-inflammatory therapies (phenylbutazone 4 mg/kg daily) were administered IV for 15 days after surgery.

Hemocultures were performed for each animal 1 month after surgery and at sacrifice to verify early and chronic blood infection, respectively.

During follow-up, VPs received a specific low-fat maintenance diet (Mucedola PF1663, Settimo Milanese, Italy), provided dry ad libitum in order to avoid the increase in weight due to fat drift.

Echocardiography
Transthoracic echocardiography examinations were performed using an ultrasound device (Zonare Zone Ultra, Mountain View, CA, USA) equipped with a phased array sector transducer (1–4 MHz). Thickness of graft wall and cusps, as well as leaflet motility, maximal transvalvular gradients (peak transvalvular velocity), and the degree of valve regurgitation were investigated. The diameter of the conduit was measured before surgery and during follow-up by echocardiography and at explantation by means of Hegar’s dilators. Echocardiography was performed prior to surgery and throughout the follow-up at definite intervals (15 days, 1, 3, 6, 9, 12, 15 months).

Macroscopic inspection
Animals were euthanized either at 6 or 12–15 months after surgery in order to assess the morphological integrity of the whole heart valves and specifically the presence of calcification, infiltration, and tearing of the graft cusps. The general condition of swine’s internal organs (cardiac apex, lung, spleen, liver, and kidney) was also assessed.

Metabolic labeling of valve explants with $^{[35S]}$ sulfate
Allograft explants in A—experimental group ($n = 2$), recovered under sterile conditions, were comprehensive of the distal anastomosis (between the implanted aortic root wall and the VP pulmonary artery). Each explanted segment was divided in three sectors by longitudinal cutting at the level of the commissures and each sector comprised the whole cusp, the above Valsalva sinus, the allograft wall, and the distal anastomosis. Two animals of the same age did not undergo any surgical treatments and acted as control group (C—untreated); the explanted RVOT of this C—untreated group comprised the whole pulmonary valve cusp, the above sinus, and the corresponding pulmonary artery wall.

In turn, each sector of the explanted segments was further divided into two halves longitudinally (Fig. 3): one half used for metabolic labeling and the other for DNA content determination, respectively. From each half sector, the graft or the pulmonary
leaflets, the sinuses, and the arterial walls were separated and kept apart. Tissues for DNA analysis were immediately frozen in dry ice, while specimens for metabolic labeling were washed in serum-free Dulbecco's Modified Eagle's Medium (sfDMEM) and then incubated in sfDMEM containing \( 125 \mu \text{Ci/mL} \) \( \text{Na}_2\text{(35S)O}_4 \) (Perkin Elmer) under 5% CO\(_2\) atmosphere at 37°C for 24 h. At the end of the metabolic labeling, medium was harvested and protease inhibitors were added; in turn, the tissues were digested with 30 U of papain in 0.1 M sodium acetate, pH 5.6, 5 mM cysteine, and 5 mM ethylenediaminetetraacetic acid (EDTA) at 65°C for 24 h. At the end of the digestion, papain was inhibited by denaturation at 100°C for 10 min; samples were then centrifuged to remove undigested material. Supernatants were exhaustively dialyzed against 50 mM sodium acetate, pH 6.0, containing 8 M urea, 10 mM EDTA, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride, 5 mM N-ethylmaleimide, and 0.15 M NaCl. The medium was dialyzed against the same buffer. At the end of the dialysis, \(^{35}\text{S}\) incorporation was measured by liquid scintillation counting and normalized to the DNA content of the parent samples.

**Statistics**

Data are presented as mean ± standard deviation. Significant differences between data populations were tested using the Student's \( t \)-test via SAS 9.1 software package (SAS Institute Inc., Cary, NC, USA). Difference is considered to be significant for \( P \) values lower than 0.05. Peak velocity values are plotted in a box-plot. The box stretches from the lower hinge (defined as the 25th percentile) to the upper hinge (the 75th percentile) and therefore contains an internal square that represents the median.

**FIG 3.** Schematic model of two sectors of an aortic explanted root; the sector dividing line is indicated by white longitudinal dashed line (A). Schematic image regarding the further longitudinal cut in the middle of each sector: one half is used for the metabolic labeling and the other for DNA content determination (B). Two sectors of an aortic explanted TRICOL graft (the third sector has been previously cut away and it is not shown) (C).
RESULTS

Surgical and postoperative results

Compared to humans, the VP’s heart is more sensitive to anemia, hypokalemia, and hypothermia; for this reason, prior to ECC, normothermia (36–37°C) stability was maintained until anesthesia induction. Potassium level remained unaffected at 5 mEq/L during surgery (preoperative potassium value was 3–3.5 mEq/L).

During surgery, two animals died due to ventricular fibrillation, and, during ECC, arterial embolization provoked paraplegia in one animal after intervention. Iatrogenic complications occurred in one VP caused by the oversewing of the noncoronary graft cusp during proximal anastomosis. At day 25 and 34 after surgery, respectively, two animals died due to endocarditis that led to TEHV stenosis and severe right ventricular failure (Fig. 4A,B). The autopsy of these VPs revealed congestive organ damage, especially of the liver (Fig. 4C), lungs, and right side of the heart. All these animals were excluded from data collection.

In all remaining 15 VPs, belonging to group A—experimental (n = 11) and B—control (n = 4), surgery and postoperative follow-up were uneventful. Blood sample analysis, before and after intervention, revealed that VPs featured a consistent number (0.55–4.96 \( \times 10^9 \)/L) of circulating monocytes corresponding to 3–13% of blood count for nucleated cells, a higher number compared to common pigs (0–1% of blood count) but very similar to humans (4–11% of blood count).

Two animals in group A and one in group B presented a positive hemoculture within the first month after surgery and were immediately treated with antibiotics. At sacrifice, no sign of blood infection was revealed.

Echocardiography

Echocardiographic analysis of TRICOL substitutes, according to UNI EN ISO 5840 and ISO 10993-1 (32,33), provided evidence of preserved biomechanical and good physiological performance up to 15 months follow-up with no signs of valve dysfunction. Leaflet pliability and motility were preserved in all animals, and no valve calcification or degeneration was identified. The mean diameter of the native pulmonary annulus, consistent for each VP, was 1.8 ± 0.2 mm, while the mean diameter of the implanted grafts was 1.5 ± 0.5 mm (Table 1), showing a slight mismatch.

At explantation after 6 months, no change occurred in the internal diameter of the TRICOL allograft compared with its dimension prior to implantation. On the contrary, a trivial increase was found for the grafts explanted 15 months after surgery, although it was nonsignificant (\( P > 0.05 \)). Leaflet motility of the control pulmonary repositioned autograft in group B performed better, at

<table>
<thead>
<tr>
<th>Group</th>
<th>Before surgery</th>
<th>6 months after surgery</th>
<th>15 months after surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (n = 11)</td>
<td>1.53 ± 0.51 cm</td>
<td>1.52 ± 0.54 cm</td>
<td>1.55 ± 0.52 cm</td>
</tr>
<tr>
<td>B (n = 4)</td>
<td>1.82 ± 0.17 cm</td>
<td>1.83 ± 0.15 cm</td>
<td>1.84 ± 0.18 cm</td>
</tr>
</tbody>
</table>

FIG 4. Explanted TRICOL allograft showing massive thrombotic-septic vegetation in right ventricular outflow tract (A); endocarditis at the level of the graft cusps (B); VP’s liver congestion (asterisk) in an animal subjected to TRICOL graft implantation after 15 months follow-up (C).
15 months, compared to TRICOL valves (group A), showing a normal value and a mild reduction, respectively. Hyperechogenic areas, corresponding to the zone of proximal and distal anastomosis, were also evident (Fig. 5). The peak velocity across the valve in group A increased significantly compared to group B at 15 days post-surgery and remained unaltered at 6 and 12–15 months follow-up (Fig. 6). A trivial valve insufficiency was present equally in A and B groups from 15 days post-surgery (Table 2) and remained unchanged during the entire follow-up.

**Histology of the decellularized allograft and assessment of DNA removal**

According to H&E and Movat’s Pentachrome stainings (Fig. 1), TRICOL aortic preparations are completely cell free and exhibited preserved extracellular matrix (ECM) structures as indicated by previous characterization of collagen and elastin fibers by thermal analysis in the solid state (34). In accordance with the previous biochemical report (29), complete removal of DNA from TRICOL preparations was confirmed by the herein reported DAPI imaging (Fig. 7). The DNA content in the wall and cusps of the TEHV aortic allografts, after 12 and 15 months follow-up, were comparable to that found in the original aortic root as well as that in the pulmonary root replaced by the graft (Table 5).

**Macroscopic analysis**

Moderate difficulty was encountered during the isolation of the graft from the aortic root due to the

---

**FIG 5.** RVOT before surgery (A); RVOT of the TRICOL allograft 15 months after surgery with hyperechogenic subvalvular areas at the level of surgical anastomosis (B).

**FIG 6.** Box-plot graph shows the peak velocity pattern of TRICOL TEHV (A—experimental) and native pulmonary autograft (B—control), after surgery at different time points compared to values before surgery.
mild degree of formation of new tissue adhesions. Before implantation, the TEHV wall (Fig. 8A) was significantly thicker ($P < 0.05$) compared to the VP’s native pulmonary root. After 15 months, the explanted TEHV displayed a decrease in wall thickness that, although not significant, is likely indicative of graft adaptation to the dimension of the original conduit during in vivo performance (Table 3). The luminal surface of both the cusps and walls of the TRICOL grafted roots was smooth, with the same morphological characteristics as the native aortic valves, without signs of gross calcification or general deterioration such as the presence of thrombi (Fig. 8B,C) or inflammatory infiltrates. Presence of possible microscopic calcification is under investigation with appropriate methodologies.

**Sulfated glycosaminoglycans neosynthesis by $^{35}$S incorporation**

To explore the ECM components production in the implanted grafts, the synthesis of sulfated glycosaminoglycans (sGAG) was investigated by in vitro metabolic labeling with $^{35}$S (35,36) and compared to the activity found in VPs’ original untreated pulmonary and aortic roots.

**TABLE 2. Echocardiographic evaluations of implanted: TRICOL TEHV (A—experimental group); and native pulmonary autograft valves (B—experimental group) at 12–15 months**

<table>
<thead>
<tr>
<th></th>
<th>A—experimental ($n = 11$)</th>
<th>B—control ($n = 4$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean transvalvular gradient (mm Hg)</td>
<td>18.3 ± 9.5</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Pulmonary regurgitation</td>
<td>1.0 ± 0.5</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Thickness of leaflets</td>
<td>1.5 ± 0.5</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Motility of leaflets</td>
<td>2.5 ± 0.5</td>
<td>3.0 ± 0.5</td>
</tr>
<tr>
<td>Calcification in leaflets or conduit wall</td>
<td>Absent</td>
<td>Absent</td>
</tr>
</tbody>
</table>

Pulmonary regurgitation: 0, absent; 1, trivial; 2, mild; 3, moderate; 4, severe. Thickness of leaflets: 1, normal; 2, mild increase; 3, severe increase. Motility of the leaflets: 3, normal; 2, mild reduction; 1, severe reduction; 0, absent. All data are reported as mean ± standard deviation.

**FIG 7.** DAPI (4,6-diamidino-2-phenylindole, 1 µg/mL) fluorescence microscopy of the aortic leaflet before (A, 10× magn.) and after TRICOL + Benzonase treatment (B, 20× magn.); in B, matrix autofluorescence background is shown.

**FIG 8.** TEHV macroscopy before implantation (A); distal view of TRICOL TEHV from the outflow tract, 15 months from in vivo implantation (B); focus on the TRICOL allograft cusps 15 months after implantation: no macroscopic evidences of valve deterioration (C).
In control animals, the incorporation of $^{35}$Si was found to be higher with respect to the corresponding artery walls (Table 4). The same experiment, carried out ex vivo in two explanted TRICOL-treated grafts (after 12 and 15 months follow-up, respectively), showed a similar pattern of active incorporation of $^{35}$S. Particularly, like in the controls, the incorporation of $^{35}$S was higher in the leaflet with respect to the wall (Table 5).

**DISCUSSION**

Implantation of valve substitutes in an animal model is mandatory for developing devices aimed for clinical use while, in turn, the animal model is expected to simulate human physiology as best as possible. This study reports a 15-month follow-up for an in vivo implanted TEHV, the longest presented in the literature in an animal model, with the VPs still healthy and preserved cardiac function at sacrifice.

In the period immediately subsequent to implantation, from day 1 to day 15, grafts are more susceptible to infection due to the presence of the central venous catheter used to administer antibiotic and anti-inflammatory drugs. The catheter represents a potential portal of entry for infectious agents, and, therefore, VP blood analysis was performed by hemoculture 1 month after surgery, in order to minimize the risk of using silently infected swine during follow-up.

Before moving to in vivo implantation of TRICOL-treated valves, their hydrodynamic performance was assessed in vitro in a Sheffield pulse duplicator (37) showing significantly lower transvalvular values than the fresh intact aortic porcine roots, regardless of cardiac output, as well as lower valvular

**TABLE 3.** Wall thickness evaluation of implanted TRICOL TEHV (A—experimental group); and native pulmonary autograft valves (B—experimental group)

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>Wall thickness at surgical time</th>
<th>Wall thickness at explantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRICOL aortic roots</td>
<td>11</td>
<td>$2.2 \pm 0.16 \text{ mm}$</td>
<td>$1.8 \pm 0.28 \text{ mm}$</td>
</tr>
<tr>
<td>Control pulmonary root</td>
<td>4</td>
<td>$1.8 \pm 0.22 \text{ mm}$</td>
<td>$1.7 \pm 0.27 \text{ mm}$</td>
</tr>
</tbody>
</table>

$P < 0.05^*$

$P > 0.05$

$P > 0.05$

*$Significant difference in wall thickness between the two groups.

**TABLE 4.** $^{35}$S macromolecular radioactivity after metabolic labeling for 24 h with $^{35}$S-sulfate of the aortic (AW) and pulmonary artery walls (PW) and of the aortic (AL) and pulmonary leaflets (PL) of two normal untreated VP (X and Y)

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Animal X</th>
<th>Animal Y</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm x ($\mu$g DNA)$^{-1}$ x (biopsy weight [mg])$^{-1}$</td>
<td>cpm x ($\mu$g DNA)$^{-1}$ x (biopsy weight [mg])$^{-1}$</td>
</tr>
<tr>
<td>AW</td>
<td>542</td>
<td>2394</td>
</tr>
<tr>
<td>PW</td>
<td>1031</td>
<td>2290</td>
</tr>
<tr>
<td>AL-1 NC</td>
<td>16 865</td>
<td>16 335</td>
</tr>
<tr>
<td>AL-2 RCC</td>
<td>16 257</td>
<td>22 651</td>
</tr>
<tr>
<td>AL-3 LCC</td>
<td>17 513</td>
<td>23 790</td>
</tr>
<tr>
<td>PL-1</td>
<td>7 238</td>
<td>12 475</td>
</tr>
<tr>
<td>PL-2</td>
<td>14 953</td>
<td>13 168</td>
</tr>
<tr>
<td>PL-3</td>
<td>17 897</td>
<td>13 400</td>
</tr>
</tbody>
</table>

$^{sGAG}$ precursors synthesis measured by $^{35}$S-sulfate uptake by the tissue, expressed as scintillation counting per minute (cpm).

RCC, right coronary cusp; LCC, left coronary cusp; NC, no coronary cusp.

Of note, all specimens from the implanted graft exhibited a metabolic activity higher than that exhibited by the equivalent tissues from control animals.

**TABLE 5.** $^{35}$S radioactivity after metabolic labeling for 24 h with $^{35}$S-sulfate and DNA content of the original aortic (OAW) and original pulmonary artery walls (OPW), the original aortic leaflet (OAL), the implanted pulmonary wall (IPW), and the implanted pulmonary leaflet (IPL) of two VPs (K after 12 months and W after 15 months of follow-up)

<table>
<thead>
<tr>
<th>Biopsy</th>
<th>Animal K</th>
<th>Animal W</th>
<th>cpm x ($\mu$g DNA)$^{-1}$ x (biopsy weight [mg])$^{-1}$</th>
<th>($\mu$g DNA) x (biopsy weight [mg])$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>OAW</td>
<td>3 834</td>
<td>6 376</td>
<td>0.79</td>
<td>0.83</td>
</tr>
<tr>
<td>OPW</td>
<td>4 561</td>
<td>9 651</td>
<td>0.75</td>
<td>0.80</td>
</tr>
<tr>
<td>OAL</td>
<td>8 490</td>
<td>113 181</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>IPW</td>
<td>5 126</td>
<td>13 023</td>
<td>0.77</td>
<td>0.80</td>
</tr>
<tr>
<td>IPL</td>
<td>17 897</td>
<td>27 460</td>
<td>0.76</td>
<td>0.76</td>
</tr>
</tbody>
</table>

*$Significant difference in wall thickness between the two groups.
resistance. The in vivo transvalvular gradient greatly increased after surgery compared to the pulmonary autograft used as a control in group B, but it remained unaltered throughout the follow-up showing a comparable performance and noninferiority to human stentless bioprostheses like the Medtronic Freestyle (38). Moreover, the slight mismatch between the TRICOL graft and the substituted RVOT could be a further explanation of the increased transvalvular gradients exhibited. During diastole, TRICOL aortic grafts presented a prolonged phase of the leaflet coaptation in vitro (37), and these results correlate with our in vivo studies showing a trivial regurgitation and insufficiency even if a normal coaptation area was preserved (Fig. 8B).

Previous reports concerning aortic bioprostheses used to reconstruct the RVOT indicated a range of regurgitation from very low (38) to mild (39) mainly related to the different intrinsic geometry between aortic and pulmonary roots. Unlike glutaraldehyde treated bioprostheses, TRICOL-treated grafts retain the potential to be repopulated and possibly remodeled by the host’s cells even if evidence of remodeling events as yet has not been produced. The wall thickness of the TEHV at 15 months (Table 2) was reduced even if not significantly compared to the thickness of the original conduit. These findings indicate a dimensional adaptation possibly related to the change in the blood pressure to which the aortic TRICOL graft has been subjected after implantation and concomitant activity of new cells in the graft.

Hyperechogenic areas in subvalvular muscle tissue, close to the proximal anastomosis, were also evident in almost all the grafts and are probably due to structural impairment between the aortic TRICOL graft and the VP’s RVOT. Steinhoff et al. (40) described for the first time calcifications in subvalvular muscle tissue, in all grafts they used, within 3 months post-surgery. Likewise, Butcher et al. (20) reported areas of calcium deposition in the distal suture line and speculated that surgical injury was pro-inflammatory itself.

Also, 7 days after intervention, Navarro et al. (41) detected an acute inflammatory infiltrate in the adventitial layer around the anastomosis, consisting mainly of polymorphonuclear cells (neutrophils). This initial and transient inflammatory infiltrate did not result in degeneration of the graft and is thought to be important in triggering a chemotactic stimulus for repopulating cells, as suggested also by Elkins et al. (42).

The mean diameter of TRICOL conduits showed a trivial increase during the 12–15 months from surgery (Table 1), although not significant. An analogous increase in the diameter of a decellularized homograft (trypsin-EDTA), used for the RVOT reconstruction in a child and seeded in vitro with autologous endothelial cells prior to in vivo implantation, has also been demonstrated during 36 months follow-up (43). The grafts maintained low gradients and valve competence, suggesting an arrangement similar to that of our TRICOL-treated valve. As VPs presented a consistent number of circulating elements belonging to the monocyte lineage in contrast to other porcine species but overlapping with normal human features both before surgery and during follow-up, it has been hypothesized that these cells may play an important role in the possible recolonization of implanted tissue-engineered scaffolds (44). In fact, the TRICOL scaffolds have already displayed capacity for in vitro static cell homing (45) preserving the 3-D collagen and elastin integrity (34) while devoid of original cells and DNA “debris.” Moreover, the TRICOL protocol is able to fully remove xenogeneic epitopes, such as alpha-Gal, responsible for hyperacute human rejection when xenogeneic tissues are used for clinical purposes (46).

In order to point out the encouraging and novel results obtained in this study, data should be compared to that achieved in the literature using the sheep as an accepted animal model. In 2009, Baraki et al. (47) disclosed the results for a TEHV implanted in an ovine model with 9 months follow-up using an aortic valve decellularized by a different detergent system and positioned orthotopically in the left ventricular outflow tract of a lamb. Notwithstanding the encouraging physiological performance of the TEHVs, it is important to point out that sheep feature quite different parameters regarding the cardiac output, stroke volume, and heart rate as compared to humans. As already reported, sheep, and particularly lambs, unlike humans, are more inclined to spontaneous reendothelialization and repopulation of the valve cusps' matrix, whether biological or biopolymeric (20,48). However, similarly to common pigs, VPs exhibit a coagulation mechanism comparable to that of humans and a similar inflammatory response (49). In the ovine model, instead, platelet activity is reduced (25), thus masking potential thrombotic events as happened for the Medtronic Parallel mechanical valve (50).

The decellularized allogeneic valves, implanted in the RVOT of juvenile sheep for 12 months by Hopkins et al. (19), showed a satisfactory hemodynamic performance similar to that retained after 15 months permanence by TRICOL TEHVs in VPs featuring a mild regurgitation and a good mean gradient.
However, calcific degeneration occurred in two of the five lambs that received a graft which did not undergo a glycerol treatment storage (−80°C) before the implantation.

Recently, Quinn et al. reported a 4.6-month follow-up for decellularized pulmonary allografts implanted in sheep (51). Their hemodynamic performance was comparable with that exhibited by TRICOL grafts at 6 months follow-up even if, at explantation, leaflets revealed the presence of intracuspal hematomas leading possibly to eventual foci of calcium deposits (52). On the contrary, TRICOL leaflets did not exhibit such alterations.

Finally, the VP was also chosen for specific features, like the dolichomorphic structure and the long thorax featuring a scarce quantity of adipose tissue that made it ideal for heart valve substitution and echocardiographic analysis. Intubation of the animal, performed before the surgical intervention, was facilitated by the VP’s large trachea and the short soft palate, in contrast to other described swine (53,54), thus avoiding frequent lung embolization phenomena and damage to the respiratory system.

Histological, ultrastructural, and gene expression studies are in progress in order to fully evaluate in detail the possible occurrence and eventual extent of host-cell homing and engraftment. High synthetic rate of sGAG after labeling with 35S-sulfate is consistent with the presence of metabolic active cells in the TRICOL substitute. Furthermore, the presence and extent of DNA content in explants at 12 and 15 months, comparable to that of the original body parts, appears compatible with graft recellularization events, even if it does not indicate the effective cell spatial distribution in the tissue and the types of cells involved. Otherwise, the endothelialization of a TRICOL graft in VPs has already been the object of a communication and matches reports concerning endothelialization of detergent-treated acellular grafts in sheep model (55). DNA content and metabolic activity data here reported confirm the presence of active cells in the explanted grafts even if growth and remodeling phenomena must still be proven.

However, following the recent statement by Schenke-Layland that much hype and hope resulted in unrealistic expectations from tissue engineering ushering in new methodologies to overcome current clinical shortcomings (56), the here reported indication of in vivo cell recolonization as well as the preliminary report concerning the presence of the endothelium-like cells monolayer on both aortic valve sides and the evidence of several CD34+, alpha-SMA+, and fibroblast-like cells within the valve (57), as presented at the XXXV Annual Meeting of European Society for Artificial Organ (ESAO 2008, Geneva, Switzerland), indicate that a step forward has been made in exploring the potentiality of a cell removal procedure producing scaffolds already able to be repopulated in vitro (29,30).

CONCLUSION

The overall results of this study are likely to validate the Vietnamese pig as a novel alternative to the sheep animal model for the preclinical investigation of tissue-engineered substitutes. Moreover, the TRICOL-decellularized valved conduit appeared to retain a physiological behavior up to 15 months, the longest follow-up reported in the literature for an animal model, although an even longer implantation time would be advisable for clinical purposes.

Ex vivo studies are being carried out to fully evaluate the potential of these preliminary results indicative of in vivo host-cell engraftment; noteworthy, the presence of DNA and of specific metabolic activity in two grafts explanted after 12 and 15 months is compatible with the occurrence of cell colonization and some kind of active structural adaptation.

Accordingly, this investigation indicates that TRICOL-decellularized substitutes behave oppositely to the cytotoxic glutaraldehyde-fixed bioprosthetic valves not allowing for cell homing. This is also in accordance with the recent report of Cebotari et al. (58) on fresh decellularized valve allografts in humans, thus highlighting the differences between decellularized grafts and bioprostheses: analyses of the former revealed almost complete repopulation of the arterial wall, partial repopulation of the cusps, and absence of any signs of calcification.

Acknowledgments: The authors wish to thank Dr. Ludovico Scenna for the legislative support in the management of animal welfare. We are also thankful to Dr. Roberto Barbieri, Dr. Alessandro Carlotto, and Dr. Franco Salvatore (Veterinarian ULSS 15, Alta Padovana), Dr. Massimo Marchesan, Mazzon Bros.’ stud farm (Piazzola s/B, Padova, Italy), Zanato’s stud farm (Ponso, Padova, Italy) and Guerriero Bros.’ slaughterhouse (villafranca Padovana, Padova, Italy) for the generous supply of swine hearts. This research was supported by the Italian grants Azione Biotech III and RSF 286/08 from the Regione Veneto. All authors do not have any conflicts of interests to disclose.

Author Responsibilities: Gallo, Michele: drafting article; Naso, Filippo: drafting article; Poser, Helen:
data analysis and interpretation; Rossi, Antonio; data analysis and interpretation; Franci, Paolo; data collection; Bianco, Roberto; protocols used in the research; Matteo Miccilo: data analysis and interpretation; Umberto Cucchin: data analysis and interpretation; Zanella, Fabio: protocols used in the research; Aresu, Umberto Cucchini: concept and design of this study; Geradiglia, Alessandro: approval of article.

REFERENCES


