Is Transcatheter Aortic Valve Implantation of Living Tissue-Engineered Valves Feasible?
An In Vitro Evaluation Utilizing a Decellularized and Reseeded Biohybrid Valve


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Abstract: Transcatheter aortic valve implantation (TAVI) is a fast-growing, exciting field of invasive therapy. During the last years many innovations significantly improved this technique. However, the prostheses are still associated with drawbacks. The aim of this study was to create cell-seeded biohybrid aortic valves (BAVs) as an ideal implant by combination of assets of biological and artificial materials. Furthermore, the influence of TAVI procedure on tissue-engineered BAV was investigated. BAV (n=6) were designed with decellularized homograft cusps and polyurethane walls. They were seeded with fibroblasts and endothelial cells isolated from saphenous veins. Consecutively, BAV were conditioned under low pulsatile flow (500 mL/min) for 5 days in a specialized bioreactor. After conditioning, TAVI-simulation was performed. The procedure was concluded with re-perfusion of the BAV for 2 days at an increased pulsatile flow (1100 mL/min). Functionality was assessed by video-documentation. Samples were taken after each processing step and evaluated by scanning electron microscopy (SEM), immunohistochemical staining (IHC), and Live/Dead-assays. The designed BAV were fully functioning and displayed physiologic behavior. After cell seeding, static cultivation and first conditioning, confluent cell layers were observed in SEM. Additionally, IHC indicated the presence of endothelial cells and fibroblasts. A significant construction of extracellular matrix was detected after the conditioning phase. However, a large number of lethal cells were observed after crimping by Live/Dead staining. Analysis revealed that the cells while still being present directly after crimping were removed in subsequent perfusion. Extensive regions of damaged cell-layers were detected by SEM-analysis substantiating these findings. Furthermore, increased ICAM expression was detected after re-perfusion as manifestation of inflammatory reaction. The approach to generate biohybrid valves is promising. However, damages inflicted during the crimping process seem not to be immediately detectable. Due to severe impacts on seeded cells, the strategy of living TE valves for TAVI should be reconsidered. Key Words: Cardiovascular tissue engineering—Aortic valve—Decellularization—Transcatheter aortic valve implantation—Homografts—Bioreactor.
valve implantation (TAVI) was developed as a less-invasive treatment alternative. Having started in 2002 with the first-in-man-implantation (4), TAVI has quickly evolved to become a standard operating procedure in high risk patient populations (5). High success rates from an average of >93% are reported currently. The therapy shows low mortality rates after 30 days (<13%) as well as after 1 year of follow-up (<24%) (6). The TAVI procedure does not necessitate sternotomy, cardioplegia, and consequently the usage of a heart lung machine. This renders TAVI to be the most important alternative for otherwise inoperable patients.

Transcatheter aortic valve prostheses (TAVP) are mostly composed of fixated xenogenic materials sewn into a metal frame. Their design shows excellent hemodynamic behavior with only minor thrombogenic complications after implantation (7). However, these biological materials have a few drawbacks as well. Their fixation with glutaraldehyde might lead to tissue deterioration and limited durability due to calcification (8). Moreover, the crimping procedure might also accelerate the structural change and degeneration of TAVP (9). Additionally, the lack of long-term results on durability of TAVP after implantation and confirmed guidelines for postoperative anticoagulation therapy are currently critical problems (10, 11). To overcome those shortcomings, current prostheses are constantly improving in design.

The application of tissue engineering methods for TAVP production may provide solutions for these problems. The combination of various scaffolds and different cell types can yield an ideal tissue-engineered prosthesis (12). Such prostheses show a high capability to overcome the drawbacks of conventional ones. Their low thrombogenicity, resistance against infection and potential of self repair after implantation, represent the most important benefits (13). Currently, many research groups are attempting to develop such a new type of prosthesis using various materials such as decellularized biological materials, biodegradable, and nondegradable scaffolds. For cellularization of the scaffold, different types of cells were used, like human vascular cells, umbilical cord, and bone marrow stromal cells (14). The usage of pulsatile bioreactors allows the colonized cells to gradually adapt to the physiological shear stress. Thus, stable cell layers on the scaffold and a resilient valve can be achieved (15).

In this study, we designed and manufactured a new decellularized and reseeded biohybrid heart valve prosthesis for TAVI application. After pulsatile low-flow conditioning (500 mL/min) we examined the influence of TAVI simulation in an in vitro setting. During a subsequent reperfusion period (1100 mL/min) further effects were investigated and the functional performance of the valves was assessed.

**MATERIALS AND METHODS**

**Cell isolation and cultivation**

Endothelial cells (EC) and fibroblasts (FB) were isolated from human saphenous vein segments. These vein segments, approximately 5 cm in length, were obtained from cardiac bypass operations and were only used anonymously with the donors written consent. Additionally, the utilization of the veins, respectively the cells, was approved by the Ethic Committee of Ludwig-Maximilians University Munich. The grafted vein segments were stored for up to 2 days until further processing at 4°C in M199 medium (Biochrom AG, Berlin, Germany) supplemented with 0.2% penicillin-streptomycin (Pen/Strep, Sigma Aldrich Chemie GmbH, Taufkirchen, Germany).

Prior to the cell isolation, the vein segments were cannulated and rinsed with M199 supplemented with 1% gentamycin (Invitrogen GmbH, Karlsruhe, Germany) and 0.2% heparin 5000 I.E. (B. Braun Melsungen AG, Melsungen, Germany). To isolate the EC, the vein segments were filled with a collagenase solution, consisting of 14 mg collagenase type II (Worthington Biochemical Corporation/Cell Systems GmbH, St. Katharinen, Germany) in 10 mL human serum albumin (HSA, ZLB Behring, Bern, Switzerland) and incubated at 37°C and 5% CO2 for 15 min. Afterwards, the obtained cell solution was centrifuged for 5 min at 500 rpm. The obtained cells were resuspended and initially cultivated in 12.5 cm2 cell culture flasks (Becton Dickinson GmbH, Heidelberg, Germany) with endothelial cell growth medium (ECGM, Promocell GmbH, Heidelberg, Germany) supplemented with 5% fetal calf serum (FCS, Biochrom AG) and 0.2% Pen/Strep. By repeating the isolation procedure with the same veins, a second EC fraction was retrieved, which was likewise cultivated.

After the EC isolation, as described above, the veins were further processed to retrieve the FB. For this purpose, the vein segments were cut lengthwise and sliced into square pieces of approximately 1 mm × 1 mm. These pieces were placed in a culture petri dish (Nunc GmbH & Co. KG, Langenselbold, Germany) and covered by a glass plate (Zefa-Laborservice GmbH, Harthausen, Germany), which was fixed with silicone dots positioned at the corner points of this plate. The FB were cultivated with fibroblast growth medium (FGM, Promocell GmbH),...
supplemented with 10% FCS and 0.2% Pen/Step. The medium was replaced at an interval of 2–3 days. At confluence the cells were passaged.

**Manufacturing of biohybrid aortic valve scaffold**

Each of the investigated biohybrid aortic valve scaffolds (BAV; n = 6) were composed of a biological and a synthetic component. Flawless aortic valve homografts with an average diameter of 25.5 ± 0.76 mm provided the cusps for the BAV. The walls of the BAV were made of a polyurethane (PU) patch (DITV, Denkendorf, Germany). As described previously, the cryopreserved homografts were thawed in Ringer’s solution at 56°C and washed in M199 for two hours at room temperature (16,17). The thawed homografts were cut lengthwise and carefully spread open. The cusps were cut out, while preserving their original form and were temporarily stored in phosphate buffered saline (PBS; Biochrom GmbH). In a next step, the cusps were sutured with 5-0 Prolene (Ethicon, Johnson & Johnson Medical GmbH, Norderstedt, Germany) to the PU. By suturing the vertical edges of the flat patches together, cylindrically shaped BAV were formed, resembling the structure of a heart valve. The BAV were inserted into a CoCr-stent with a diameter of 20 mm and fixed with single stitches. Finally, the BAV were fixed to a Teflon mounting for secure handling and usage during the different processing steps (Fig. 1A).

**Decellularization**

The BAV were stored in 400 mL of decellularization solution for 24 h as described previously (17,18). The solution consisted of PBS supplemented with 0.5% sodium deoxycholate and 0.5% sodium dodecyl sulfate. The BAV were placed on a three-dimensional (3D) rotating table (Froebel Labortechnik GmbH, Lindau, Germany) at RT to achieve a multiplanar agitation and a homogeneous distribution of the solution. After decellularization, the remaining detergents were removed by six washing cycles (PBS supplemented with 1% Pen/Strep, 24 h). The BAV were then stored in 200 mL of M199 at a temperature of 4°C.

**BAV seeding and preconditioning**

The calculated average surface area of BAV was 60.28 ± 2.05 cm². Cell seeding procedure was performed as previously described (19). Briefly, the decellularized BAV were first seeded with FB (45.23 ± 1.55 × 10⁶ cells) using a 3D rotating seeding device for 24 h at 37°C and 5% CO₂. The seeding device periodically rotates for 3 min to spread the cells over the surface of the BAV and then stops 30 min for cell adhesion. Thereafter, the BAV were transferred into a glass bin containing 400 mL of fresh FGM, and cultured statically in an incubator for 6 days. Subsequently the EC (38.24 ± 14.91 × 10⁶ cells) were seeded likewise. After the dynamic seeding the BAV were stored in ECGM, which was changed once in 3 days.

Furthermore, the BAV were conditioned in a pulsatile bioreactor containing 400 mL of ECGM at 37°C and 5% CO₂ (Fig. 1B,C) (20). The conditioning was performed for 5 days at pulsatile low flow conditions (500 mL/min). To regulate the temperature and CO₂, the bioreactor was placed in a standard incubator during the whole conditioning phase.
TAVI simulation and pulsatile perfusion

After low flow conditioning, the BAV were removed from the conditioning bioreactor and the Teflon mounting. First, the BAV were crimped in a conventional crimping device (Edwards Lifesciences, S.A., Irvine, CA, USA) to a diameter of 10 mm and stored for ten minutes in ECGM. Subsequently the BAV were dilated by a conventional balloon catheter, \( d = 20 \text{ mm Edwards Lifesciences} \). After TAVI simulation, the BAV were placed inside the pulsatile conditioning bioreactor again and were perfused with pulsatile flow (1100 mL/min) for 2 days.

Evaluation procedures of BAV

The BAV were evaluated using video analysis, scanning electron microscopy (SEM), immunocytochemistry/immunohistochemistry (ICC/IHC) and Live/Dead Assays, which are separately described in the following paragraphs. For this purpose, samples of 0.5 cm \( \times \) 2 cm were taken from each BAV prior conditioning as well as before and after TAVI simulation. After perfusion, the samples were taken from four different areas of the BAV: supravalvular, valvular, subvalvular and cusps area.

Scanning electron microscopy

Samples were fixed in a solution containing 456 mL aqua bidest (Ampuwa, Fresenius Kabi Deutschland GmbH, Bad Homburg v. d. H., Germany), 43.5 mL glutaraldehyde (Sigma-Aldrich Chemie GmbH), 0.75 mL 1 N hydrochloric acid (Titrisol, Merck KGaA, Darmstadt, Germany) and 5.65 g sodium cocodylate trihydrate (Sigma-Aldrich Chemie GmbH) for a minimum of 48 h at 4°C. Afterward, the fixed samples were dehydrated by an ascending ethanol series, distilled water and PBS. Subsequently the cell membranes of the specimens were permeabilized with Triton-X (Sigma Aldrich Chemie GmbH) and gold sputtering (28 mA; 570 V) for 180 s at 10\(^{-5}\) mbar (Sputter Coater, SCD 50, Bal-Tec GmbH, Schalksmühle, Germany) and placed in Roti-Histol (Carl Roth GmbH, Karlsruhe, Germany). The samples were then embedded in paraffin and sectioned in 10 \( \mu \text{m} \) thick slices. The paraffin was rinsed by Roti-Histol (Carl Roth GmbH, Karlsruhe, Germany). The samples were then embedded in paraffin and sectioned in 10 \( \mu \text{m} \) thick slices. The paraffin was rinsed by Roti-Histol (Carl Roth GmbH, Karlsruhe, Germany) and AEC-peroxidase Substrate as described previously in the following paragraphs.

Immunohistochemistry

Samples were fixed in 4% formalin solution (Microcos GmbH, Garching, Germany) for a minimum of 48 h at 4°C. Thereafter, they were stained in Mayer’s Hemalaun solution (Merck KGaA). For assessment and documentation of the stained samples bright field microscopy was used.

For ICC staining FB and EC were cultivated with 0.5 mL medium in eight-well culture slides (BD Falcon, Erembodegem, Belgium) until confluency was achieved. Thereafter, the cells were fixed in precooled 96% ethanol and stored at \(-80^\circ\)C. After fixation, the specimens were rinsed with PBS and endogenous peroxidase was blocked using a solution of 30% \( \text{H}_2\text{O}_2 \) (0.15% Sigma Aldrich Chemie, GmbH) in PBS. Subsequently cells were incubated with primary antibodies (AB) for 30 min at RT. For visualization, samples were incubated afterwards with HRP Streptavidin label (horseradish peroxidase, Biozol GmbH, Eching, Germany) and AEC-peroxidase substrate (3-amo-no-9-ethylcarbazole-peroxidase, Vecto Laboratories, In. Burlingame, CA, USA) for 10 min. The following primary AB were used: \( \alpha \)-Actin (0.44 \( \mu \text{g/mL} \); Dako Deutschland GmbH, Hamburg, Germany) and Connexin-43 (1 \( \mu \text{g/mL} \); Millipore GmbH, Schwalbach/Ts., Germany) were selected as indicators for generation of cytoskeleton and intercellular connections. CD-31 (Cluster of differentiation 31, 205 \( \mu \text{g/mL} \); Dako Deutschland GmbH) and VE-Cadherin (Vascular endothelial cadherin, 0.2 \( \mu \text{g/mL} \); Beckmann Coulter Inc., Marseille, France) was used for staining of ECs. SMC-Myosin (Smooth muscle cell myosin, 954 \( \mu \text{g/mL} \); Dako Deutschland GmbH) and TE-7 (Thymic epithelial cells 0.1 \( \mu \text{g/mL} \); Millipore GmbH) stainings were performed to identify SMC and FB, respectively. Collagen IV (5.4 \( \mu \text{g/mL} \); Sigma Aldrich Chemie GmbH) and fibronectin (0.6 \( \mu \text{g/mL} \); Sigma Aldrich Chemie GmbH) expression were used as criterion for extracellular matrix (ECM) buildup. ICAM (intercellular adhesion molecule, 50 \( \mu \text{g/mL} \); Millipore GmbH) was used as indicator for the stresses induced. For nuclear counterstaining, cells were stained using a 25% Mayer’s Hemalaun solution (Merck KGaA). For ICC staining FB and EC were cultivated with 0.5 mL medium in eight-well culture slides (BD Falcon, Erembodegem, Belgium) until confluency was achieved. Thereafter, the cells were fixed in precooled 96% ethanol and stored at \(-80^\circ\)C. After fixation, the specimens were rinsed with PBS and endogenous peroxidase was blocked using a solution of 30% \( \text{H}_2\text{O}_2 \) (0.15% Sigma Aldrich Chemie, GmbH) in PBS. Subsequently cells were incubated with primary antibodies (AB) for 30 min at RT. For visualization, samples were incubated afterwards with HRP Streptavidin label (horseradish peroxidase, Biozol GmbH, Eching, Germany) and AEC-peroxidase substrate (3-amo-no-9-ethylcarbazole-peroxidase, Vecto Laboratories, In. Burlingame, CA, USA) for 10 min. The following primary AB were used: \( \alpha \)-Actin (0.44 \( \mu \text{g/mL} \); Dako Deutschland GmbH, Hamburg, Germany) and Connexin-43 (1 \( \mu \text{g/mL} \); Millipore GmbH, Schwalbach/Ts., Germany) were selected as indicators for generation of cytoskeleton and intercellular connections. CD-31 (Cluster of differentiation 31, 205 \( \mu \text{g/mL} \); Dako Deutschland GmbH) and VE-Cadherin (Vascular endothelial cadherin, 0.2 \( \mu \text{g/mL} \); Beckmann Coulter Inc., Marseille, France) was used for staining of ECs. SMC-Myosin (Smooth muscle cell myosin, 954 \( \mu \text{g/mL} \); Dako Deutschland GmbH) and TE-7 (Thymic epithelial cells 0.1 \( \mu \text{g/mL} \); Millipore GmbH) stainings were performed to identify SMC and FB, respectively. Collagen IV (5.4 \( \mu \text{g/mL} \); Sigma Aldrich Chemie GmbH) and fibronectin (0.6 \( \mu \text{g/mL} \); Sigma Aldrich Chemie GmbH) expression were used as criterion for extracellular matrix (ECM) buildup. ICAM (intercellular adhesion molecule, 50 \( \mu \text{g/mL} \); Millipore GmbH) was used as indicator for the stresses induced. For nuclear counterstaining, cells were stained using a 25% Mayer’s Hemalaun solution (Merck KGaA). For assessment and documentation of the stained samples bright field microscopy was used.
Hemalaun was used for nuclear counterstaining and images were taken by bright field microscopy. To allow a quantitative evaluation of IHC staining, the images were postprocessed using image processing software (Adobe CS 6, Adobe Systems Inc., San José, CA, USA). The stained areas were automatically identified and converted into vector geometries. A script was developed that automatically calculates the stained area, image height and the average staining thickness. This was performed for all CD31 and TE-7 stains of all valves. Thus, a percental value of cell loss due to crimping injuries could be calculated.

**Live/Dead Assay**

Cell vitality was assessed after crimping and after the perfusion period using fluorescence microscopy. The samples were briefly stored in 500 µL of ECGM. Subsequently 1 µL propidium iodide (Sigma-Aldrich GmbH) and 0.5 µL Syto 9 (Life Technologies GmbH, Darmstadt, Germany) were added to the ECGM. A Zeiss Axio Observer (Zeiss MicroImaging GmbH, Jena, Germany) was used for fluorescence analysis. Dead cells were marked in red using a wavelength of 485 nm, whereas living cells were marked green using a wavelength of 540 nm.

**Video analysis**

To evaluate the performance of the created valves, the aperture ratio of the valves was calculated. Therefore, the derived images of the video analysis were edited using the software package Adobe Creative Suite 6. By increasing contrast and adapting the gradation curve, the distinct opening surface of the valves was isolated. Afterward, this surface was transformed into a vector shape to calculate the surface area. Based on that, a quantitative opening ratio of the valves could be calculated and compared with physiological conditions.

**RESULTS**

**Efficiency of cell seeding and conditioning**

On samples of native PU patches, randomly sprayed fibers were visible in the SEM inspection. Fiber diameters between 0.2 and 2 µm (A). A confluent and uniform cell layer was documented after cellularization with FBs and ECs (B). After 5 days low-flow conditioning a confluent and homogenous cell layer was still detectable. No damages or gaps were found in the cellular coverage (C). Damages of the cell layer after TAVI simulation were identifiable by parallel fissures and gaps in the cellular coverage. Morphologically, the damages resembled bruises and lacerations (D). After subsequent reperfusion extensive areas showed cell detachment. The areas seemed to correspond to the lacerated gaps and fissures after TAVI simulation. In these areas, the underlying PU-scaffold was visible and exposed to the lumen (E). Evaluation of the crimped and reperfused cusps showed extensive damages as well, mostly fissure-shaped with parallel orientation (F). On cell-seeded PU, Live/Dead Assays indicated almost exclusively living cells by green fluorescence (G). Up to 50.5 +/- 17.7% dead cells (red stained) were observed after the TAVI procedure. Dead cells were not homogeneously allocated to the scaffolds surface and in some areas a significant cumulation was detectable (H). After reperfusion, the fraction of dead cells remarkably decreased. Furthermore, an unnatural and pathologic morphology of green stained living cells was partially observed (I).

![Image of Topographical SEM-analysis and Life/Dead assay of the BAV.](image-url)
After colonization of the patches with FB and EC, confluent and uniform cell layers were detected across the complete surfaces. Neither specific cell orientation nor detached cells were found (Fig. 2B).

Confluent and uniform cell layers were also observed on all samples after the 5d low-flow conditioning period. No delaminations of the cell layer or detached cells were detected (Fig. 2C). The samples of the homograft’s cusp showed a smooth surface, as well as a cobblestone pattern. Based on that, it can be concluded that the cusps have been coated successfully with FB and EC (data not shown).

The efficiency of FB and EC colonization was further verified by IHC-staining, using different antibodies. Positive staining against TE-7 proved the presence of FB (Fig. 3A1). EC were stained by CD31 (Fig. 3B1) and VE-cadherin. Positive staining was achieved in all samples after FB- and EC-seeding. It can be seen that the low flow conditioning led to a strong increase of FB and a thickening of the FB layer (Fig. 3A2), while preserving an intact layer of EC (Fig. 3B2).

The development of ECM was monitored by staining against fibronectin and collagen IV. Prior to conditioning, respectively, 2 weeks after FB colonization, ECM was observed in all samples. A strong layer of collagen IV (Fig. 3C1) as well as a confluent layer of fibronectin (Fig. 3D1) could be distinguished. The expression of both proteins increased after the conditioning phase (Fig. 3C2,D2), indicating an ECM buildup. Staining against α-actin and connexin-43 was used to evaluate the presence of a cytoskeleton and intercellular connections. After the seeding procedure, all samples showed a weak positive staining for connexin-43 (Fig. 3E1) as well as for α-actin (Fig. 3F1). However, after the first conditioning process, a significant increase of both proteins was detected (Fig. 3E2,F2).

To analyze the induced stress to the cells during the procedure, the inflammatory response was indicated using ICAM staining. A light inflammatory response was observed directly after crimping (Fig. 3A3,B3). However, a strong inflammatory reaction is indicated by the high levels of ICAM expression (Fig. 3G3). After perfusion, a reduced layer of FBs was observed (Fig. 3A4,B4). Only few remaining ECs could be detected by CD31 staining (Fig. 3B4). According to Collagen IV expression, less ECM is existent as well (Fig. 3C4), while no major change in α-Actin expression could be detected (Fig. 3F4). The inflammatory reaction even intensified according to ICAM expression (Fig. 3G4).
response was observed after FB- and EC-seeding (Fig. 3G1). This expression decreased to some extent after conditioning (Fig. 3G2). The development of the IHC stainings over the process procedure is shown in Table 1.

**Influence of TAVI procedure**

After the conditioning process, TAVI was simulated with all BAV, followed by pulsatile flow perfusion. Subsequent to the perfusion, the valves were reanalyzed following the same methods. In this way, the influence of the TAVI procedure, in particular the crimping process, was evaluated.

It was observed that directly after the crimping process, the FB layer was still present. However, the expression of TE-7 was reduced compared with the samples taken before crimping and damaged areas are visible (Fig. 3A3). The number of life and dead cells was determined by examination of the Live/Dead Assay images of all valves. After the crimping procedure, approximately 50.5 ± 17.7% of cells were indicated as dead. After perfusion, only a thin layer of FB remains. Especially if compared with the images taken before crimping, the difference is substantial. Cell counting revealed in all valves that the cell number is reduced by 58.1 ± 8.2%. Directly after crimping, the EC layer is intact and confluent as well (Fig. 3B3). Yet after perfusion, severe damages and gaps on the colonized surface can be detected. In some cases, only sporadic and insular ECs were found (Fig. 3B4).

Staining against collagen IV (Fig. 3C3) as well as fibronectin (Fig. 3D3) showed a slight but not significant decrease in layer thickness after crimping. However, this layer remained stable and no deterioration could be identified after perfusion (Fig. 3C4,D4). The crimping procedure lead to a decrease in connexin-43 expression (Fig. 3E3), while having nearly no effect on α-actin expression (Fig. 3F3). Both layers remained stable throughout perfusion (Fig. 3E4,F4).

The inflammatory reaction to the crimping procedure were assessed by ICAM staining. A strongly intensified expression was revealed after crimping (Fig. 3G3). During perfusion, a further intensified expression was detected, indicating a strong inflammatory reaction (Fig. 3G4).

Directly after TAVI simulation, damages of the cell layer were identified by SEM analysis. They mainly appeared in form of parallel fissures and

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<th>Table 1. IHC staining results</th>
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-, indicates no visible staining; +, light staining; ++, moderate staining; ++++, strong staining; EC, endothelial cells; FB, fibroblasts; N.D., not done.

**FIG. 4.** Live imaging of the BAVs and vector-based analysis. Snapshots from video recordings of the movement of a BAV exposed to pulsatile flow, displaying the opening process from the completely closed valve (A) to the fully opened state (D). Image processing and vector-based analysis allowed the calculation of the respective aperture areas (E–H). The dotted line in represents the maximal possible aperture of the valves (E).
gaps in the cellular coverage (Fig. 2D). After perfusion, the cellular layer was further impaired. Native PU fibers were found in wide areas of the patches (Fig. 2E). Crimping and reperfusion further increased the damage. Extensive areas with ruptures and fissures in the cellular layers were detected (Fig. 2F).

To allow a quantitative analysis of loss in cell number, the calculated average layer thickness was taken into account. For EC an average reduction of 54.8 ± 16.4% was calculated. For FB an even higher reduction of 67.2 ± 14.3% was determined.

Live/Dead staining revealed a significant number of dead cells (approximately 50%) directly after the crimping process (Fig. 2H). After perfusion, only a few remaining dead cells were detected. Additionally, a part of them appeared morphologically pathological (Fig. 2I).

**Functionality of the scaffolds**

The opening and closing performance was evaluated using video recordings. An insufficient closing performance of all valves was observed at low flow conditions (500 mL/min). At higher flow conditions (1100 mL/min), a proper opening and closing behavior was recorded from all six BAV. Image analysis showed a maximal aperture of 49.1 ± 10.7% (1.55 cm²). The minimal aperture was at 0.17 ± 0.33% (0.54 mm²), indicating zero insufficiency during perfusion (Fig. 4).

**DISCUSSION**

According to Eveborn et al., with the prevalence of an aging population, the incidence of aortic valve diseases increases annually (21). In addition, a constant growth of the percentage of percutaneous aortic valve implantations, in relation to the conventional treatments, was observed during the last years. As a result, it is predictable that the total number of TAVI procedures will reach a tremendous quantity. As mentioned previously, the biological prostheses currently used for TAVI have certain disadvantages, such as their progressive calcification, as well as limited self-repairing and remodeling. The manual fabrication process and the crimping procedure intensify the progressive calcification (8). TAVI started as a solution for patients rated unsuitable for surgery. Thus, this collective consisted mainly of patients 75 years of age and older. Consequently, problems concerning long-term behavior were considered as issues with low priority. However, as the indication for TAVI is progressively extending, these issues become more and more in focus. Especially when young patients or children are concerned, these drawbacks are of utmost importance. TAVI is far from being the preferred solution for young patients. Nonetheless, even today clinical indications exist—such as patients facing reoperation after Ross procedure—where a minimal invasive solution with granted long-term function would be requested. The application of tissue engineering to heart valve prostheses has the potential to overcome these limitations and might present a possible solution. In this study, we attempted to fabricate an ideal scaffold for tissue-engineered heart valves by combining two different materials: decellularized homografts and sprayed PU patches, which differ in their physical, mechanical, and biological properties. They have been combined in a way to compensate the lack of the other. After the decellularization treatment, homografts promise to be effective scaffolds. Additionally, they show a better durability and provoke less immunogenic reactions compared with xenogenic materials. Xenografts have to be fixed with glutaraldehyde solution or decellularized (22). Yet it has been proven that even after decellularization, inflammatory reactions can be stimulated if xenografts are used (23). Consequently, we decided on homografts as main material. However, the large amount of tissue in the wall of homografts is inappropriate for TAVI. Thus, only the cusps of the homografts were utilized and the wall was substituted by a polyurethane patch. PU has a high resilience and can maintain its structure against systolic pressure and shear stress (24,25). This way thin wall thicknesses could be achieved while maintaining the necessary stability to withstand physiological pressures. Furthermore, PU is known for its high rate of biocompatibility. Hence the direct attachment of the cells onto the porous PU is enabled, which effectively induces proliferation of cells (26). In previous research, it was shown that tissue engineered homografts, as well as PU patches, are effective scaffolds (17, 27). It was further confirmed in this study, that the BAV showed a high aptitude for cell seeding. The functionality of BAV was proven by video monitoring during perfusion (1100 mL/min). The BAV demonstrated a good closing performance, and no insufficiency could be detected. The calculated opening ratio of 49% would not be sufficient for human implantation. However, it can be postulated, that at higher flow rates, the aperture would increase accordingly. Furthermore, the image generation has to be improved to allow clear images even at maximal systole. This will be subject to further analysis.

To improve the durability of BAV and reduce the thrombogenic reaction, as well as inflammatory and immunogenic responses, BAV were seeded...
with two types of vascular cells: FB and EC. Scaffolds which are preseeded with FB provide better conditions for attachment of EC (28). Due to this serial seeding procedure, the cellular layers of tissue engineered heart valves resemble human cardiac valves (29). It has been shown by Butcher et al., that cells of aortic valvular origin are a potent cell source for aortic tissue engineering (30). While this is reasonable, the application of autologous cells from this origin seems unpractical for the described process. However, Schnell et al. evaluated the suitability of venous FB and EC for cardiovascular tissue engineering. Their ability to construct ECM and intercellular connections after seeding on scaffolds is highlighted in particular. Furthermore, their high availability renders them ideal for tissue engineering applications. Consequently, utilizing venous cell sources seems a promising alternative for the generation of BAV (31). According to Sierad et al., ECM formation and buildup of intercellular connections can be induced by exposure to physiologic pulsatile flow conditions (32). Using a customized bioreactor, BAV were physiologically conditioned at low pulsatile flow (500 mL/min) for 5 days. This led to an improved formation of ECM. It enables the scaffold to preserve tissue morphogenesis and to reconstruct itself, following injury in association with constructive tissue remodeling (33). Presence of ECM was indicated in IHC-analysis using antibodies against collagen IV and fibronectin (Fig. 3; Table 1). It was observed that the ECM of all BAV was further developed and increased after flow conditioning. Vital cells seeded onto a scaffold show structural stability, due to cytoskeletal and intercellular molecules. This was substantiated by staining against α-Actin and Connexin-43. The results showed strong positive reactions after conditioning and perfusion. SEM analysis proved that the surface integrity was not compromised by the conditioning process (Fig. 2).

Crimping is the designation of the compression of TAVPs to decrease their outer diameter. It is a necessary step in the TAVI procedure, regardless of the stent material used (34). According to the current tendency to produce small sized aortic valve prostheses, as well as to develop implants for younger patients (35,36), we fabricated the BAV in a diameter of 20 mm. Additionally, in comparison to commercial TAVP, which are crimped to 6 mm or 8 mm (37), the BAV were compressed to a diameter of 10 mm to verify the integrity at lower levels of stress.

Immunohistochemistry as well as SEM analysis showed intact layers of EC and FB directly after crimping. Additionally, samples were stained against ICAM, to allow an interpretation of the cells immunoreaction. Yang et al. proved that elevated levels of ICAM-1 promotes transcellular transendothelial migration of leukocytes. Thus, it can be utilized as an indicator for inflammation, injury, and immune reactions (38). While no significant expression of ICAM was detectable after seeding and conditioning, the crimping process seemed to induce a strong upregulation (Table 1). The strong expression of ICAM indicated an intense inflammatory reaction (Fig. 3G3). This was substantiated by fluorescence analysis. The Live/Dead Assay showed a high percentage of dead cells. These cells are still present on the valves after crimping. Therefore, they can be detected by immunohistochemistry and SEM analysis. However, staining against TE-7 and CD31 does not allow to draw conclusions in regard to the vitality of EC and FB.

After perfusion, immunohistochemical analysis only showed imperfect and fragmented layers of EC. The fibroblast layer was strongly reduced and sometimes even exhibited lacerations as well. SEM images showed similar results, revealing lacerated spots and gaps in the cellular layers. Live/Dead analysis after perfusion exhibited only a few dead cells and a large amount of vital cells. Their morphology suggests them to be mainly FB, which was confirmed by staining against TE-7 (Fig. 3A4). Most certainly, the majority of the endothelial layer was damaged or lost its mechanical integrity during the crimping process (Fig. 3B3,B4). Thus it can be postulated that if exposed to physiologic flow conditions, the damaged cells would have been detached and carried off by the flow. A further evaluation after perfusion for a far longer time would be necessary to evaluate the long-term damages inflicted by crimping.

This analysis leads to the postulation that the damages inflicted during the crimping process seem not to be immediately detectable using only immunologic detection methods. The level of expressed ICAM can be taken as indicator of induced stress. Additional methods, for example, Live/Dead staining, and long-term evaluations are required, to correctly evaluate the induced damages during crimping.

Our aim was to evaluate the possibility of living tissue-engineered valves for minimally invasive application. In prior studies, we already evaluated PU valves as well as homografts. Both solutions had advantages and drawbacks. PU valves can be crimped very well, but we faced issues regarding the leaflet integrity. Homografts conversely provide excellent leaflets. However, they are unsuitable for crimping, due to the structure and thickness of the
walls. In this study, we combined those materials to achieve an ideal prosthesis for minimally invasive implantation. We achieved excellent results in regard to colonization of the valve and ECM expression. The exposure to pulsatile flow conditions stimulated the construction of ECM and intercellular connections. Hence, it can be derived that the process of consecutive FB- and EC-seeding, followed by a conditioning phase, leads to a strong ECM expression and stable cell layers. However, analysis showed that the damages inflicted during the crimping process, especially to the endothelial coverage, are severe. This research demonstrated the intensity of the long-term effects of crimping. Consequently, the approach of crimping living tissue-engineered products should be reconsidered.

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REFERENCES


