Decellularization combined with enzymatic removal of N-linked glycans and residual DNA reduces inflammatory response and improves performance of porcine xenogeneic pulmonary heart valves in an ovine in vivo model

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Abstract
Background: Limited availability of decellularized allogeneic heart valve substitutes restricts the clinical application thereof. Decellularized xenogeneic valves might constitute an attractive alternative; however, increased immunological hurdles have to be overcome. This study aims for the in vivo effect in sheep of decellularized porcine pulmonary heart valves (dpPHV) enzymatically treated for N-glycan and DNA removal.

Methods: dpPHV generated by nine different decellularization methods were characterized in respect of DNA, hydroxyproline, GAGs, and SDS content. Orthotopic implantation in sheep for six months of five groups of dpPHV (n = 3 each; 3 different decellularization protocols w/o PNGase F and DNase I treatment) allowed the analysis of function and immunological reaction in the ovine host. Allogenic doPHV implantations (n = 3) from a previous study served as control.

Results: Among the decellularization procedures, Triton X-100 & SDS as well as trypsin & Triton X-100 resulted in highly efficient removal of cellular components, while the extracellular matrix remained intact. In vivo, the functional performance of dpPHV was comparable to that of allogeneic controls. Removal of N-linked glycans and DNA by enzymatic PNGase F and DNase I treatment had positive effects on the clinical performance of Triton X-100 & SDS dpPHV, whereas this treatment of trypsin & Triton X-100 dpPHV induced the lowest degree of inflammation of all tested xenogeneic implants.

Conclusion: Functional xenogeneic heart valve substitutes with a low immunologic load can be produced by decellularization combined with enzymatic removal of DNA and partial deglycosylation of dpPHV.

Keywords
decellularization, heart valves, large animal study, PNGase F, xenotransplantation
1 | INTRODUCTION

Despite great efforts to close the donation gap, shortage of human organs and tissues suitable for life-saving transplantsations is likely to prevail in the future. Therefore, research on alternatives is crucial in order to rescue many patients currently dying while waiting for a donor organ or suffering from suboptimal treatment. One possible solution for this issue could be the use of animal-derived (xenogeneic) organs and tissues. Currently, there is a considerable progress in research efforts concerning xenotransplantation of solid organs. In contrast to solid organs, xenogeneic tissues have already been used since decades for the generation of biological implants, like, for example, bioartificial heart valves. However, since then, clinically applied xenogeneic substitutes basically had been chemically cross-linked, mainly by glutaraldehyde tanning, in order to prevent strong destructive host immune responses toward the graft tissues upon implantation. Although showing very good hemodynamic properties and being the most often used heart valve replacement, chemically fixed xenogeneic valve bioprostheses feature specific disadvantages. Consequently, the quest for ideal heart valve prostheses is still open. Currently under investigation in extensive multi-centric clinical trials, decellularized allogeneic heart valves potentially provide an optimal valve substitute, exhibiting long-lasting durability, physiologic hemodynamic properties, and the ability of adaptive growth processes. Nevertheless, due to a substantial lack of human donor valves, an indefinite introduction of this innovative and promising regenerative therapeutic approach to the daily clinical routine turned out as being not possible in the foreseeable future. As a possible resolution approach, however, the simple transfer of decellularization strategies successfully applied on allogeneic heart valves to respective xenogeneic tissues approved to be unsuccessful yet, since decellularized porcine valve matrices hitherto always failed in clinical studies. The poor performance of non-fixed xenogeneic tissues considered to be acellular in the past could be partially attributed to insufficient decellularization procedures. Additionally, more recent in vitro experiments also revealed that the major-xenoantigen αGal as unique target of natural human antibodies constitutes a substantial part of porcine extracellular matrix proteins and thus cannot be removed by sole decellularization. Essentially involved in the occurrence of severe immune mechanisms even against decellularized xenogeneic tissues once engrafted a αGal-negative recipient, αGal is no longer of substantial concern, since the generation of transgenic pigs lacking the αGal-epitope could be realized. Decellularized heart valves derived from αGal-negative pigs, exhibit no differences in binding preformed human antibodies in comparison to decellularized human valves. In contrast to preformed anti-αGal antibodies, which, without further xenogenic stimulus, are present in high titer in all human, further xenoantibodies exist that exhibit variable, often low titer in healthy humans, but are rapidly elevated upon xenogeneic stimulus for, example in pig-to-baboon xenotransplantation experiments. The two known antigens inducing such xenoantibodies are Neu5Gc and the Sd(a) like antigen made by the porcine β-1,4-N-acetyl-galactosaminytransferase 2 (β4GalNT2) (reviewed in ). Using genetic engineering pigs lacking these two antigens and also lacking αGal have been developed and showed reduced binding of preformed human antibodies. Because all known xenoantigens are carbohydrates, we hypothesize that upon implantation mainly carbohydrates will be inducing new xenoantibodies. Therefore, the recently established enzymatic removal of N-linked glycans from decellularized matrices by applying PNGase F will be published elsewhere might be beneficial to achieve successful interspecies transplantation of decellularized heart valves matrices. This issue was addressed in the current manuscript by first optimizing achievable decellularization extents for porcine pulmonary heart valves, second including enzyme-mediated removal of N-linked glycans and DNA, and third investigating the in vivo-performance of and the immunologic response toward respective decellularized porcine xenogeneic pulmonary heart valve conduits (dpPHV) ortho-}

2 | MATERIALS AND METHODS

2.1 | Study design

Nine different detergent-based respectively enzymatic-supported decellularization protocols were employed to treat porcine pulmonary heart valves (3 animals per group) in order to identify the two most potent and suitable methods for the generation of structural intact, but cell-free grafts for heart valve replacement therapy. Per individual valve, each measurement (technical triplicates) was conducted at least 3 times per individual valve.

For further evaluation in vivo, the two selected protocols and the clinical applied Sodium Dodecyl Sulfate (SDS)/Sodium Deoxycholate (SD) decellularization were combined with DNase I and PNGase F treatment to remove residual nucleic acids and N-linked glycans. In total, 5 groups of dpPHV (n = 3, each, one animal died) were implanted for six months into sheep in orthotopic position: TX (TX: Triton X-100) + SDS decellularized w/o PNGase F & DNase I treatment; trypsin + TX decellularized with PNGase F & DNase I treatment; and SDS/SD w/o PNGase F & DNase I treatment. Valve function was assessed by transeosophageal echocardiographic examinations at implantation and sacrifice. After explantation, xenografts were analyzed in terms of gross appearance and cellular matrix repopulation. For comparison, previously published allogeneic ovine pulmonary heart valves (TX + SDS decellularized, n = 3) served as positive controls.

2.2 | Decellularization

In total, 49 (30 for in vitro and 19 for in vivo) fresh porcine pulmonary heart valves (pPHV) were excised from pig hearts obtained from a local abattoir. The genetic background of the animals is not defined, but based on oral information, the animals are German Landrace pigs. Because all animals have been of the same breed and originated from one farm, the genetic background of all animals used for
this study should be comparable. Housing conditions have been identical, and all pigs slaughtered have been around 6 months old and about 90 kg of weight. pPHV were transferred on ice-cool PBS solution to the laboratory, extricated from excessive fat and connective tissue, and individually transferred to 250 mL laboratory flasks. Tissue disinfection was conducted in Braunol® (B. Braun) for 5 minutes under continuous agitation, followed by a rinsing step of 20 minutes in PBS. Thereafter, pPHV were immediately subjected to decellularization (Table 1). All incubation steps were performed on an orbital shaker at 200 rpm and at room temperature.

### 2.3 PNGase F and DNase I digestion

dpPHV were treated with 150 mL of 150 U/mL DNase I (AppliChem) in PBS supplemented with 1 mmol/L MgCl₂ for 48 hours at 37°C for the removal of residual DNA. Subsequently, PHV were equilibrated to G7 buffer (50 mmol/L NaPO₄, 1% NP-40, pH 7.5) for 12 hours and afterward incubated in PNGase F solution (2000 U/mL, PNGase F, NEB) for 24 hours at 37°C to cleave off N-linked glycans from extracellular matrix proteins. Afterward, decellularized and enzyme (DNase I and PNGase F)-treated PHV were subjected to 12 wash cycles with PBS (12h each) and stored in PBS containing antibiotics (1% penicillin/streptomycin and 1% gentamycin) at 4°C until use.

### 2.4 Quantification of DNA, Hydroxyproline, GAGs, and SDS

Pulmonary artery (PA) samples were taken from the PHV to investigate DNA, hydroxyproline, GAGs, and SDS content. Matrix samples were minced with a tissue mortar after snap freezing in liquid nitrogen and resulting PA homogenates were freeze-dried. Per heart valve, at least triplicates of 2-10 mg dried matrix granulate were subjected to a complete Proteinase K (AppliChem) digestion. Conducted biochemical assays are described in detail elsewhere. In brief, DNA was quantified using Hoechst 33258, hydroxyproline quantification achieved by applying a modified HxP assay was developed by Stegemann et al., GAG components were measured according to a method established by Farndale et al. and SDS tissue contents determined using an MBAS assay.

### 2.5 Orthotopic implantation, echocardiographic examination and explantation

Animal experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the local animal care committee of lower Saxony (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), reference, 14/1527). The animal study was designed as an initial benchmark study with a small number of animals per group. Therefore, the group size (n = 3) does not allow a valid statistical analysis.

The surgical implantation was conducted as described by Theodoridis and Tudorache et al. 27 In brief, after accessing the thoracic cavity via left lateral thoracotomy and establishing cardiopulmonary bypass according to standard procedures, PA was separated from the aorta and a truncal cuff approximately 2 cm in width was excised directly above the PA root in beating heart technique. Native pulmonary valve cusps were subsequently removed, and dpPHV orthotopically implanted into supravalvular position of female sheep (average weight 39.3 ± 1.2 kg) using running sutures for proximal and distal anastomoses. Transesophageal echocardiography was performed immediately after implantation as well as before explantation 6 months post-surgery. After sacrificing the animals, implants were excised from the explanted intact hearts ex vivo, macroscopically examined and subsequently separated into relevant component specimens, in order to be finally processed for histological as well as immunofluorescence analyses.

### 2.6 Phalloidin and DAPI staining

For cell detection on the luminal side, half of one valve cusp of each explanted conduit was PFA-fixed in total and subsequently stained for cell bodies by using Phalloidin-Atto 488 (49409, Sigma-Aldrich) and nuclear stain by DAPI (Invitrogen) as previously described.

### 2.7 Histological analysis and immunofluorescence staining

Representative parts of all explanted grafts were embedded in paraffin according to standard techniques. Standard hematoxylin and eosin (H&E) stain was conducted on 2-μm tissue sections of each sample as explicitly described before. For immunofluorescence analyses, tissue slides of either paraffin (CD45, CD11b) or cryosections (CD3, CD21) were used. Cryosections of 10 μm thickness were fixed in acetone at −20°C for 8 min before staining, followed by a 1h blocking step in 1% BSA/ PBS solution (Albumin Fraction V, AppliChem). Primary antibodies (anti-CD3 (A0452, GeneTex), dilution: 1:100; anti-CD11b (SM-2627, Acris), dilution: 1:20; anti-CD21 (MCA1424GA, Bio-Rad), dilution: 1:100; and anti-CD45 (MCA2220, AbD Serotec), dilution: 1:200) and secondary antibodies (Cy2 donkey anti-rabbit (AbD Serotec), dilution: 1:300; and Cy3 donkey anti-mouse (AbD Serotec), dilution: 1:300) used for detection and staining immune cells were diluted in PBST (PBS + 0.5% Tween 20) and applied for 2 hours at RT and o. n. at 4°C, respectively. Readily stained tissue sections were afterward counterstained with DAPI (Invitrogen) and mounted using Shandon™ Immu-Mount (Thermo Fischer Scientific).

### 2.8 Quantitative evaluation of tissue sections

To evaluate the cellular repopulation and the number of inflammatory foci, at least 3 sections per heart valve specimen, obtained from
different areas of the implant, have been analyzed. To judge the repopulation, an arbitrary score with the maximum of 22 for native tissue was used.9

Cells detected by antibody staining methods were quantified surveying 11 high power fields (HPF) per slide (images taken by use of a 40× objective). For each specimen, at least two slides per staining have been analyzed. HPF distribution among the PHV components of each analyzed tissue section was chosen as follows: PA = 5 fields, valve cusp = 3 fields, and sinus area = 3 fields. Cells positive for one of the applied antibodies were counted by hand, whereas DAPI-detected cell nuclei were calculated software-assisted using ImageJ. Respective resultant data are depicted as numbers of cells identified by antibody-mediated staining per 100 DAPI-detected nuclei determined by ImageJ quantification.

2.9 | Statistical analysis

Investigation of statistical significance was performed by two-way ANOVA followed by Bonferroni post-tests. Calculated numbers are given as mean ± standard deviation. A P value of ≤0.05 is considered to be significant. P values are labeled as following: ≤0.05 (*), ≤0.01 (**) and ≤0.005 (***)..

3 | RESULTS

3.1 | Influence of decellularization on DNA reduction, extracellular matrix proteins, and tissue retention of SDS

Biochemical analysis of decellularized porcine PHV regarding content of DNA, hydroxyproline, GAGs, and SDS revealed significant differences dependent on the applied decellularization protocol. In respect to residual DNA, the clinically approved SDS/SD protocol was unable to remove DNA, while the combined use of trypsin and TX was very efficient (Figure 1A). Upon removal of cells and cellular debris, a direct impact of all types of decellularization procedures, the hydroxyproline content is increasingly measured in relation to the dry mass of the resulting cell-free matrix (Figure 1B). The analysis of GAGs shows that all decellularization processes remove GAGs, but dependent of the method applied the residual content may vary. Combining trypsin, TX, and SDS yielded the lowest values of remaining GAGs (Figure 1C). The application of detergents for decellularization may result in detergent retention in the resulting matrix. Since detergents, especially SDS, are cytotoxic, we determined the SDS content depending on the decellularization method applied (Figure 1D). Whereas, methods without SDS involved show no SDS content, and therefore, serve as negative controls, the TX and SDS method exhibited the highest concentration of matrix-related SDS, directly followed by the stand-alone use of SDS (Figure 1D).

3.2 | Hemodynamic and clinical performance of decellularized porcine heart valves in sheep

Decellularized heart valves were successfully implanted into female sheep (n = 16) in orthotopic position (Figure 2A,B). One animal died during surgery due to uncontrollable arrhythmia. All others, either with allogeneic or xenogeneic implant, were in good physical conditions and showed a normal weight development from 39.3 ± 1.2 kg to 54.1 ± 5.1 kg during the 6-month experimental time (Figure 2C).

However, in three animals, echocardiographic analysis revealed a massive dilatation of the pulmonary artery leading to graft failure (Figure 2C). As these failures could not be assigned to a specific parameter, all three animals have been excluded from further analysis (highlighted in red, Figure 2C).
Echocardiographic analysis of the remaining 12 functional valves showed very good hemodynamic performance at the beginning of the experiment. During the experiment, xenogeneic valves treated according to the TX + SDS method without further enzymatic treatment developed (Figure 2C) a significant increase in mean valvular gradient and an increased degree of stenosis (Figures 2C & 3B). Interestingly, xenogeneic TX + SDS decellularized valves, additionally treated with PNGase F + DNase I, showed very good hemodynamic performance and no increase in the mean valvular gradient, or in insufficiency (Figure 3B). The performance of all other xenogeneic heart valves was comparable to the allogeneic dophv previously published (Figures 2C & 3B).22

Macroscopically, most xenogeneic explants exhibited thin, pliable, and translucent leaflets and a shiny and smooth PA comparable to allogeneic controls (Figure 3A). Only TX + SDS-treated valves exhibited slightly reddish, thickened, and opaque cusps (Figure 3A). Furthermore, two out of three TX + SDS decellularized xenogeneic explants displayed signs of calcification, whereas no sign of calcification could be observed in any other explant.

3.3 | Histological analysis of explants

Conventional H&E stain revealed an overall intact histoarchitecture of all functional explants. Compared with all other groups, the cusps of xenogeneic TX + SDS-treated explants appear to be slightly thickened (Figure 4A). All grafts have been covered by a highly vascularized adventitial layer. Cells could be found within the pulmonary artery as well as sinus region and the cusp. However, a complete repopulation with cells could not be seen, especially the distal part of the cusps happened to be cell-free in all explants (Figure 4C). Applying an arbitrary score to judge, the cellular repopulation revealed the highest degree of repopulation for xenogeneic trypsin + TX+enzyme-treated valves (Figure 4D). These valves showed native-like repopulation of the pulmonary artery and sinus and proximal proportion of the cusp. In contrast, allogeneic as well as xenogeneic implants treated with TX + SDS with or without further enzyme showed significantly less cellular repopulation (Figure 4). In all explants, accumulations of inflammatory cells could be seen at the side of the anastomosis. Identical inflammatory foci have been found.
FIGURE 2  Setup and clinical data obtained during the in vivo study. A, Schematic drawing of the experimental setup. B, In total, 19 animals (1 died early (†)) underwent orthotopic heart valve replacement surgery receiving either decellularized ovine (allo) implants as control or decellularized with or without enzymatic deglycosilated porcine (xeno) heart valves. C, Data obtained by echocardiographic analysis at the time of implantation and sacrifice after 6 mo. At the time of sacrifice, three conduits exhibited a massive dilatation of the pulmonary artery influencing the functionality of the implants. These animals (highlighted in red) were excluded from further analysis. *Results of the allogeneic control group are taken from Goecke and Theodoridis et al.²²
within the pulmonary artery of some xenogeneic, but not allogeneic explants (Figure 4A). Quantification of these foci revealed that xenogeneic implants decellularized with TX + SDS exhibited the highest number of inflammatory foci (5.7 ± 1.2) among all experimental groups. Allogeneic TX + SDS and xenogeneic trypsin + TX + enzyme decellularized implants exhibited equally low number of inflammatory foci (1.2 ± 0.2 vs 0.9 ± 0.4), which concentrate at the side of the proximal anastomosis (Figure 4B). Although not statistically significant, xenogeneic implants decellularized and treated with PNGase F and DNase I showed less inflammatory foci as their decellularized counterparts without enzyme treatment (Figure 4B).

3.4 | Cellular repopulation of the cusp surface

Whole-mount staining with phalloidin and DAPI of half a cusp per valve revealed that the ventricular side was always better repopulated with recipient cells as the arterial side (Figure 5A). Furthermore,
differences could be seen dependent on the decellularization method, showing that trypsin + TX+enzyme decellularized implants exhibited the highest degree of surface repopulation on both sides. SDS/SD decellularized grafts showed better arterial and comparable ventricular repopulation compared with TX + SDS decellularized grafts (Figure 5B).

3.5 | Characterization of inflammatory cells found within the explants

Applying the pan-leukocyte marker, CD45 revealed that in all xenogeneic explants the proportion of CD45-positive cells was always bigger than in allogeneic explants (Figure 6A-C). Among xenogeneic implants,
FIGURE 5 Cellular repopulation of cusp surfaces in vivo. A, Phalloidin and DAPI staining of half a cusp in toto showed partial surface covering with recipient cells. The free edge of the cusp was always cell-free. B, Quantification of the covered surface revealed less repopulation of the arterial than ventricular side of the cusp. Xenogeneic trypsin + TX+ enzyme-treated valves exhibited the best arterial and ventricular repopulation among all tested groups. \( n = 2 \) or \( n = 3 \) individual animals per group have been included in the analysis.

FIGURE 6 Immunofluorescence examination of explanted heart valves. CD45-positive cells could be found within (A) allogeneic and (B) xenogeneic explants. C, Allogeneic explants exhibited the lowest number of CD45-positive cells, whereas xenogeneic (xeno) SDS/SDS-treated explants exhibited the highest number, about 40% of all invading cells. High numbers of CD11b-positive cells were present in all (D) allogeneic and (E) xenogeneic explants; however, (F) no significant differences were found. G, In allogeneic explants, CD3-positive T cells were almost absent, but in (H) xenogeneic explants, a low number of CD3-positive cells could be found. I, Enzyme-treated valves tended to contain less CD3-positive cells as untreated explants. J, In allogeneic as well (K) xenogeneic explants, (L) only few CD21-positive B-cells could be found. C, F, I, L, Every dot represents one individual animal included in the analysis. For each PHV specimen, at least two sections obtained from different areas have been analyzed, counting 11 HPFs per section. All scale bars represent 100 µm.
trypsin + TX+enzyme-treated valves exhibited the lowest number of CD45-positive cells (20 ± 7%) (Figure 6C). Although statistically not significant, the proportion of CD45-positive cells tended to be lower in xenogeneic valves treated with PNGase F and DNase I compared with decellularized only xenogeneic implants (TX + SDS 27% ± 9% vs TX + SDS+enzyme 20% ± 12% and SDS/SD 35% ± 10% vs SDS/SD + enzyme 18% ± 8%). Phagocytic cells, like macrophages and some granulocytes, could be identified in all explants by staining with a CD11b antibody (Figure 6D,E). No significant differences could be detected, although the proportion of CD11b-positive cells tend to be higher in xenogeneic compared with allogeneic explants (Figure 6F). Interestingly, in allogeneic explants, about 10% of the invaded cells were CD11b, which is in the same range as CD45-positive cells (Figure 6C,F).

Consistently, almost no CD3-positive T cells (2% ± 1%) were found in allogeneic explants (Figure 6G). On the contrary, a considerable number of CD3-positive T cells could be found in xenogeneic explants (Figure 6H). The number of T cells found in xenogeneic explants tends to be reduced by the enzyme treatment (TX + SDS 10 ± 5% vs TX + SDS+enzyme 6 ± 1% and SDS/SD 10 ± 6% vs SDS/SD + enzyme 7 ± 4%) (Figure 6I). Xenogeneic trypsin + TX+enzyme-treated valves exhibited the lowest number of CD3-positive cells (5% ± 1%) among all xenogeneic implants (Figure 6I). Only very few CD21-positive B-cells could be detected in allogeneic as well as xenogeneic explants (Figure 6J-L).

4 | DISCUSSION

In this study, we focused on the functional assessment and tolerability of xenogeneic heart valve in a long-term sheep transplantation model. We have chosen this model knowing that the immunologic hurdle in pig-to-sheep xenotransplantation is much lower than in pig-to-human xenotransplantation. Recipient (sheep) and donor (pig) share many antigens, especially the known xenotransgresses-like αGal epitopes or Neu5Gc. Therefore, hyperacute or acute rejections of the porcine implants are unlikely to happen in the pig-to-sheep xenotransplantation model. The strengths of the chosen model are the investigation of functionality, and long-term tolerability as the recipient sheep are not immunocompromised and able to mount an adaptive immune reaction. This has also value for the field of xenotransplantation because, recent studies in baboons showed that porcine hearts derived from genetically antigen-deprived pigs, lacking αGal epitopes, also do not face hyperacute or acute rejection in a long-lasting transplantation model. As we could show previously, decellularized heart valves obtained from αGal-negative pigs exhibit similar levels of antibody binding as human heart valves. This indicates, that upon transplantation into humans, hyperacute rejection of αGal-negative porcine decellularized heart valves is very unlikely. Meanwhile, it could be shown that even native pericardium derived from pigs lacking further xenografts, Neu5Gc, and Sd(a) did not bind more preformed antibodies as human tissue, which makes a hyperacute rejection in future clinical practice of xenotransplantation even more unlikely.

In this study, the clinically applied decellularization protocol SDS/SD and two protocols selected after screening a panel of decellularization protocols have been evaluated in vivo. Because the experiment was designed as an initial benchmark study, the low number of only 3 animals per group did not allow the analysis of small differences. Nevertheless, it could be observed that porcine valves treated with TX + SDS became slightly insufficient and stenotic and also showed the highest number of inflammatory foci and a high number of infiltrating CD45 and CD3-positive cells. Interestingly, TX + SDS decellularized valves treated with DNase I and PNGase F did not become stenotic or insufficient and showed lower numbers of inflammatory foci and CD3, CD11b, and CD45-positive cells within the explants. Since the enzymatic treatment of dpPHV always included the use of both, PNGase F and DNase I, the positive effect cannot be attributed to one specific enzyme. As the DNA removal by trypsin + TX as well as TX + SDS decellularization per se resulted in low DNA content of the dpPHV, it is unlikely that the DNase I treatment lead to that positive effect. PNGase F treatment, however, leads to a significant reduction in glycans present on decellularized porcine valves (will be published elsewhere). Thus, the PNGase F-mediated glycan removal from dpPHV might be responsible for the drop in CD3-positive T cells present in the explants (Figure 6) and therefore indicative for increased tolerability.

In relation to previous studies investigating non-fixed porcine valves transplanted into sheep, the inflammatory reaction observed in our study seems to be much weaker. Ice-free cryopreserved porcine pulmonary valves tested by Biermann and colleagues exhibited intensive calcifications and a strong T cell-mediated immune reaction after 3 months in vivo. On the contrary, other in vivo studies of decellularized porcine heart valves in sheep were reporting very good preclinical results. Goldstein and colleagues reported successful implantation and function of SynerGraft valves made from decellularized porcine aortic valve tissue for up to 336 days in the sheep model. Erdbruegger and colleagues also reported very good results of decellularized porcine heart valves implanted into sheep for up to 11 months. Unfortunately, both, Synergraft and Matrix P prostheses, manifested a very poor outcome in clinical trials and failed due to massive immunologic reaction in man.

Therefore, the important questions are whether these encouraging results can be transferred to the human system and whether these results could justify further preclinical testing in non-human primates or even first clinical trials of decellularized xenogeneic heart valves?

Through thorough in vitro analysis, it became evident that these decellularized porcine valves failed due to incomplete decellularization. As antigens-like αGal epitopes are present on components of the insoluble extracellular matrix, a complete elimination of xenoantigens by decellularization is impossible. Generating genetically based absence of αGal epitopes, however, results in abolishing of binding of preformed human antibodies to porcine decellularized heart valves. This result and the availability of genetically modified pigs lacking not only αGal epitopes, but also neu5Gc and β2GalNT2
makes us confident that decellularized heart valves generated from such pigs will not undergo acute humoral rejection, when transplanted into humans.

A major limitation of this study is the low number of animals per experimental group, but in total, only three out of the 15 implanted xenogeneic decellularized heart valves failed. The failing was based on dilatations, most likely caused by suboptimal trimming and positioning of the graft during surgery resulting in unphysiological blood flow through the grafts. Remarkably, only three other valves showed the development of mild stenosis and insufficiency.

As shown in this study and already before, decellularized matrices may retain SDS, when SDS was used as a decellularizing agent. Since SDS is cytotoxic, residual SDS, strongly bound to the matrix and potentially released during the remodeling process of the matrix in vivo, may interfere with the repopulation of decellularized matrices in vivo, and therefore, the lack of SDS in trypsin + TX decellularized heart valves compared with TX + SDS decellularized valves may account for a better outcome. As trypsin + TX-treated implants showed the best tolerability, it can be speculated that a higher repopulation could lead to less inflammation, mediated by an enhanced remodeling of the xenogeneic extracellular matrix.

The fact that an additional treatment of TX + SDS valves with PNGase F and DNase I considerably reduced the degree of inflammation, while not improving the repopulation, however, gives evidence that the two processes inflammation and repopulation can be separated. Both enzymatic treatments potentially help to remove xenoantigens. DNase I by destroying highly charged DNA molecules and PNGase F by removing N-linked glycans from glycosylated proteins being part of the matrix.

Since the known xenoantigens for humans, αGal and Neu5Gc are carbohydrates, we can speculate that others, which have not identified so far, may act as xenoantigens as well and the PNGase F treatment may have removed unknown xenoantigens resulting in less inflammation in the treated TX + SDS valves.

Conclusively, we could demonstrate that by efficient decellularization combined with enzymatic removal of DNA and N-linked glycans, the immunological barrier causing rapid destruction of xenogeneic tissues could be overcome.

Based on this study, trypsin + TX + DNase I + PNGase F treatment is the most promising approach that should be further investigated. Thus, extended in vivo observation periods, maybe up to several years, should be considered in the sheep model, while pre-clinical tests of decellularized xenogeneic heart valves in non-human primates such as baboons should be started.

The fact that trypsin damages the extracellular matrix must be kept in mind and the mechanical stability of the valves must be ensured prior to implantation. In this study, the trypsin treatment was limited to 90 minutes resulting in valves that showed excellent mechanical stability for 6 months in vivo.

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CONFLICT OF INTEREST

Axel Haverich is shareholder of Corlife oHG, company producing decellularized human heart valves using the SDS/SD decellularization protocol described in the manuscript. All other authors declare to have no conflict of interest.

AUTHOR CONTRIBUTIONS

RR, TG, KT, SS, KH, KF, AC, SC, and IT performed the experiments; RR and TG analyzed the DATA; AH and AH designed the experiments and secured funding; RR, TG, and Andres Hilfiker wrote the manuscript.

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