Immunological and echocardiographic evaluation of decellularized versus cryopreserved allografts during the Ross operation

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Abstract

Objective: Compare the immunological and echocardiographic data of decellularized versus cryopreserved allografts used for RVOT reconstruction during Ross operation.

Methods: From 16/01/03 thru 07/10/03, 20 Ross operations were performed using decellularized (n=11) or cryopreserved (n=9) allografts. Echocardiography was done at discharge, 1, 3, 6 and 12 months and annually thereafter. Samples for determination of antibodies against HLA class I and II were obtained preoperatively and at days 5, 10, 30, 90 and 180 postoperatively. These samples were tested by the ELISA method in LAT-M dishes (unspecific) for identification of circulating antibodies and the results expressed as mean sample values (IS=DO/cutoff). If positive, LAT-E (specific) was performed and PRA levels determined.

Results: There was no mortality. Cryopreserved allografts showed marked IS values elevations for class I and II antibodies which started at the first month and remained elevated up to 6 months. In contrast, of the patients receiving decellularized allografts, seven remained negative, two patients had only marginal elevation of class I antibodies and two patients showed abnormal elevations of PRA levels. This response happened earlier than in the cryopreserved group, starting on the 5th postoperative day and has returned to baseline levels in one case. Echocardiography showed mild, but significant, elevation of gradients in cryopreserved valves but none in the decellularized.

Conclusions: Decellularized allografts had normal function up to 18 months and showed important reduction of the immunogenic response when compared to cryopreserved valves.

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Keywords: Allograft heart valve; Humoral immune response; Short-term valve function

1. Introduction

The Ross operation is considered by many as the best option for aortic valve replacement in children and young adults. Being a living structure with physiological hemodynamic performance, the pulmonary valve autograft is durable, has almost no incidence of thromboembolic complications, permit a more complete regression of left ventricular hypertrophy and has been shown to be associated with better long-term survival and quality of life when compared to other types of valve substitutes [1,2].

The operation includes reconstruction of the right ventricular outflow tract, which is most often accomplished with a cryopreserved pulmonary or aortic valved conduit. Although the immediate and short-term function of these allografts is very satisfactory, they are prone to degenerative changes with subsequent stenosis and/or insufficiency, which may ultimately necessitate surgical reintervention. Freedom from reoperation for allograft valve failure was 90% at 12 years follow-up in Elkins experience [1], and 80% at 20 years in the pioneer series of Ross as reported by Chambers et al. [2].

Although allograft valve failure is multifactorial, including patient age, surgical techniques, allograft size and type, procurement and processing methods, there is increasing experimental and clinical evidence suggesting an immunological basis for the destruction of normal allograft architecture [3].

Expression of HLA class I and II antigens on viable endothelial, interstitial and dentritic cells present in the donor valve are capable of triggering both humoral and cellular immune response by the host [4-6]. It has been postulated that tissue antigenicity can be abolished or significantly reduced by decellularization methods, and this could enhance long-term durability [7-9]. Additionally,
acellular allografts could be repopulated ‘in vitro’ by tissue engineering techniques or ‘in vivo’ by the host after implantation [10,11].

Results of decellularized allografts in humans are still limited and somewhat contradictory [11–13]. We herein present our short-term results with decellularized pulmonary allografts for RVOT reconstruction during the Ross procedure, comparing the clinical and echocardiographic data and immunological profile against a similar group of patients receiving conventional cryopreserved allografts.

2. Materials and methods

2.1. Patients

Between 16/01/2003 and 07/10/2003, 24 patients underwent a Ross operation at our institution using either decellularized or conventional cryopreserved allograft for the reconstruction of the RVOT. This was not a randomized study, but rather, decellularized allografts were used according to patient acceptance and the availability of the decellularized allograft at the occasion of the operation. To be enrolled in the study, patients had to accept to have blood samples withdrawn at specific time intervals for the immunological analysis.

In this way, the study population compromised 20 patients, of whom 11 received decellularized allografts (Group A) and nine had a cryopreserved allograft (Group B). The preoperative and surgical characteristics of the patients are listed in Table 1, there being no major differences between the two groups. Group A consisted of five males and six females with a mean age of 23.0 ± 9.04 years (min = 9; max = 37) and group B was constituted by seven males and two females with a mean age of 24.3 ± 8.06 years (min = 16; max = 36). The study was conducted in accordance with institutional guidelines and has been approved by the Ethical Committee of Pontificia Universidade Católica do Paraná (PUC-Pr). Before being enrolled, patients signed the informed consent to participate in the study.

2.2. Operative technique

All operations were done through a median sternotomy with cardiopulmonary bypass and mild to moderate systemic hypothermia (30–32 °C). Myocardial protection was achieved with administration of doses of intermittent antegrade cold blood cardioplegia through the coronary ostia every 20–30 min.

The pulmonary autograft was implanted as a root replacement in all cases and the RVOT was reconstructed with interposition of an allograft with running sutures of polypropylene 4-0 for both the proximal and distal sutures lines. There was no extension of the allograft with pericardial patches in the proximal suture line in any case. Allograft size selection was done according to patient surface area, but as a general rule we tried to implant the biggest allograft available with a deliberate over sizing policy.

2.3. Allografts

All allografts were obtained from heart beating donors and were processed and cryopreserved according to previously published methods. The decellularized allografts were prepared from regular cryopreserved grafts available in the heart valve bank freezer. They were thawed according to standard protocols and decellularized by a proprietary process (AutoTissue Ltd®), which involves the use of a solution of 1% deoxycoll acid and ethanol 70% (Fig. 1). The processed decellularized conduits were then kept in RPMI nutrient medium up to 30 days before implantation.

2.4. Study methods

Blood for the determination of circulating antibodies was obtained at the following times: preoperatively, 5, 10, 30, 90 and 180 days after the operation. Samples were initially tested by the ELISA method with LAT-M (Lambda Antigen Tray—unspecific). The results were expressed as mean sample values Is(Sample index)=DO(optical density)/cutoff value. When the Is was positive (values greater than 1), LAT-E (specific) was performed also by the ELISA method using the 1288 kit and the PRA values determined. This technique uses soluble class I and class II antigens from 30 different cell lines that are coupled individually to latex beads and then pooled together to create a panel that represents the majority of serologically recognized HLA class I and class II alloantigens. The beads were incubed with 0.01 ml of patient serum and then washed and stained with fluorescein anti-human immunoglobulin G (secondary antibody). The percentage of fluorescent positive beads was the indicative of the percentage of PRA. Both the LAT-M and LAT-E were determined with the aid of an ELX 800 NB—Universal Microplate Reader—BIO-TEK Instruments Inc.).

Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A decellularized (n = 11)</th>
<th>Group B cryopreserved (n = 9)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>23.0 ± 9.04 (9–37)</td>
<td>24.3 ± 8.06 (16–36)</td>
<td>ns</td>
</tr>
<tr>
<td>Male:female ratio</td>
<td>5:6</td>
<td>6:3</td>
<td>ns</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>Aortic stenosis (7), aortic insufficiency (4)</td>
<td>Aortic stenosis (5), aortic insufficiency (4)</td>
<td>ns</td>
</tr>
<tr>
<td>Ethiology</td>
<td>Rheumatic (7), congenital (4)</td>
<td>Rheumatic (6), congenital (3)</td>
<td>ns</td>
</tr>
<tr>
<td>Previous operation</td>
<td>1</td>
<td>2</td>
<td>ns</td>
</tr>
<tr>
<td>ECC time</td>
<td>102.8 ± 16.4</td>
<td>99.1 ± 11.4</td>
<td>ns</td>
</tr>
<tr>
<td>Clamp time</td>
<td>84.4 ± 12.3</td>
<td>86.4 ± 10.9</td>
<td>ns</td>
</tr>
<tr>
<td>Allograft size</td>
<td>24.3 (23–25)</td>
<td>24.4 (23–26)</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns, not significant; ECC, extracorporal circulation.
All patients had an echocardiographic study before hospital discharge and were asked to return at 3, 6 and 12 months and annually thereafter for further echocardiographic evaluation, which consisted of standard bidimensional and Doppler evaluation with a Hewllet-Packard ImagePoint machine. The peak velocity across the allografts was obtained with pulsed or continuous-wave Doppler and the gradient determined with the modified Bernoulli equation. Pulmonary valve regurgitation was graded according to conventional described methods.

3. Statistical analysis

For parametrical data we performed the Fisher test to verify if the variances between the groups were different. As the variances were different between the groups in all cases, a Students t-test for comparing two groups with different variances were applied.

Non-parametric comparison was performed with the $\chi^2$-test with the Yates correction to compare differences between small samples.

4. Results

There was no operative mortality. Apart for reoperation for bleeding in one patient in the decellularized group, there was no other major complications. During the follow-up period all patients are clinically well in functional NYHA class I.

All patients receiving a cryopreserved allograft developed a strong humoral response against HLA class I and II antigens, which started around the 15th postoperative day, reached a peak around the third month and remained elevated during the study period.

In contrast, in the decellularized group, nine patients had none ($n=7$) or very mild ($n=2$) humoral response and only two patients had significant increase in antibodies levels. When such a response existed, it was faster than in the cryopreserved group, with antibodies levels being detected as soon as the 5th-10th postoperative day.

The two cases with abnormal humoral responses in the decellularized group had different profiles. One patient exhibited elevations for both class I and II antibodies that remained elevated up to 180 days. This patient had an abnormal elevation of class II antibodies preoperatively. The second patient had only a transient elevation for class I antibodies that returned to normal baseline levels 6 months after the operation. Two additional patients in the decellularized group showed a discrete and marginal elevation of class I antibodies at one specific time, but both had normal levels afterwards.

Detailed results on the humoral response in both groups are presented in Table 2 and Figs. 2 and 3.

All the patients in the cryopreserved group as well as the four patients who were LAT-M positive in the decellularized group had abnormally elevated PRA levels. These results are shown in Table 3. There was a tendency for greater PRA class II levels in the cryopreserved group as compared to the four patients in the decellularized group, however, the number of patients is too small for conclusive statistical analysis.

Detailed evaluation of LAT-E results revealed that in the two patients with marked elevated PRA levels in the decellularized group, there were antibodies reacting specifically against donor HLA antigens.

Echocardiographic data demonstrated that cryopreserved allografts presented a significant increase in peak gradients during the follow-up period. At hospital discharge the mean value for the peak gradient across the allograft was 9.3 mmHg and rose to 15.5 mmHg at late follow-up. In contrast, the decellularized allografts were stable during the study period, with a mean value for the peak gradient of 8.6 mmHg at hospital discharge and 9.7 mmHg at the late period. Fig. 4 illustrates the differences in functional performance of the allografts. There were no cases of pulmonary insufficiency.

Table 2
LAT-M class I and class II (cryopreserved $\times$ decellularized)

<table>
<thead>
<tr>
<th>Time since allograft implantation</th>
<th>Group A decellularized allografts ($n=11$)</th>
<th>Group B cryopreserved allografts ($n=9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class I</td>
<td>Class II</td>
</tr>
<tr>
<td>-------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Before operation</td>
<td>0.57 ± 0.13</td>
<td>0.56 ± 0.47</td>
</tr>
<tr>
<td>5 d</td>
<td>0.92 ± 1.25</td>
<td>0.82 ± 1.38</td>
</tr>
<tr>
<td>10 d</td>
<td>1.10 ± 1.22</td>
<td>0.49 ± 0.21</td>
</tr>
<tr>
<td>30 d</td>
<td>2.10 ± 2.31</td>
<td>1.43 ± 2.21</td>
</tr>
<tr>
<td>90 d</td>
<td>1.52 ± 1.57</td>
<td>1.19 ± 1.64</td>
</tr>
<tr>
<td>180 d</td>
<td>1.22 ± 1.69</td>
<td>1.04 ± 1.59</td>
</tr>
</tbody>
</table>

ns, not significant.
5. Discussion

Standard cryopreserved allograft valved conduits possess a high rate of viable fibroblasts and endothelial cells, especially when ischemic time between procurement and cryopreservation is short [14]. Although initially thought that these cells could, at least partially, be functional and contribute to extracellular matrix remodeling, and thus preventing valve failure, long-term explants have shown that both the cusps and the arterial wall are largely acellular presenting matrix hyalinization, fragmentation and degeneration of the collagen and the elastic fibers and gradual loss of proteoglycans and glicosaminoglycans [14].

In fact, a higher index of viability can be even detrimental for the long-term function because it is capable of triggering a stronger immune response [5,6]. Our data confirm previous studies showing that cryopreserved allografts are associated with production of both class I (HLA A-B) and class II

(HLA DR/DQ) anti-HLA antibodies. In our series, antibodies serum levels started rising 15-30 days after the operation, reaching a peak around the third month which was maintained for at least the first 6 months postoperatively in all cases, which is in accordance to the data presented by Hawkins et al. [5].

Although several studies have documented an immune response to heart valve allografts, a direct relationship of this response to valve failure is not clear and as yet not completely understood. Implicit in any definition of rejection is not only a humoral or cellular immunologic response

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group A decellularized n=4</th>
<th>Group B cryopreserved n=9</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRA class I</td>
<td>63% ± 31.38</td>
<td>68% ± 31.62</td>
<td>ns</td>
</tr>
<tr>
<td>PRA class II</td>
<td>31% ± 37.49</td>
<td>49.2% ± 38.28</td>
<td>ns</td>
</tr>
</tbody>
</table>

ns, not significant.
Fig. 4. Functional behaviour of the allografts, with mean values for peak gradients at the early and late follow-up in both groups. At late follow-up there is a statistically significant greater gradients for patients receiving a cryopreserved allograft.

(antigenicity) but also some functional consequence of this immunologic reaction to the graft (immunogenicity). Mitchell et al. [15] could not demonstrate evidence for antibody mediated injury in explanted allografts showing no more than mild to sparse mononuclear inflammatory cell infiltrate composed of T cells and macrophages in the valvular tissue. One possible explanation could be a state of immunological tolerance, as valve interstitial cells may induce T-cell anergy on the host as suggested by Batten et al. [16]. On the other hand Rajani et al. [17] found multiple foci of inflammation consisting of clusters of either T or B-lymphocytes in valves from infants who had premature degeneration in less than 8 months, suggesting a rejection phenomena.

Although HLA or ABO blood type cross-matching is not routinely used, and even disregarded, when choosing an allograft for an individual patient, more recent evidence may indicate the opposite. Some series with long observation times have suggested that that HLA induced allograft rejection may contribute to the observed morphologic changes in valvular tissue and eventual long term deterioration of allograft function [3,18]. The same uncertainty exists for ABO blood type compatibility. Although it has been suggested that valvular endothelial cells do not express ABO surface antigens, Christenson et al. [19] demonstrated accelerated degeneration and allograft fibrocalsifications in children with blood group incompatibility.

In the present series, only two patients in each group were ABO compatible. Eight out of the nine patients in group A who were ABO incompatible had no HLA antibody elevation, suggesting that blood type compatibility had no major impact in the analysed parameters.

Blood transfusion was required in four patients in group A and in three of group B. Although not included in the present study, we have a control group of patients undergoing open-heart operation without the use of any prosthetic material. Using the same methodology as here, we could not detect HLA antibody elevation, independent of the requirement of blood transfusion. This suggests that blood transfusion and extracorporeal circulation may have little influence in our results.

Theorically, a limited period of immunosuppression could be beneficial after allograft valve implantation. In the experimental set, the use of cyclosporine A has successfully arrested the immune response to allograft implantation in rats [20]. In humans, valves from explanted heart transplants have shown normal architecture and preserved cellularity, even when the organ has been rejected, suggesting that immunosuppressive therapy can be important for long-term valve function [21]. However, a trial of azathioprine in 13 children receiving cardiac valve allografts failed to reduce the immune response and was associated with undesirable side effects [22].

Decellularization has been proposed as a method to diminish or even abolish allograft antigenicity and thus having a neutral behavior. Experimentally these grafts not only appeared immunologically inert but also became progressively repopulated by host cells that resembled fibroblasts actively synthesizing new collagen and participating in matrix remodeling. These functional, living grafts should impact long-term results favorably [9,10].

The ideal decellularization technique should be able to completely eliminate all the cellular components of the graft while causing minimal damage to the extracellular matrix, maintaining normal tissue biomechanics and also being non-citotoxic and biocompatible [10]. Several distinct methods of decellularization have been employed, including different combinations and concentrations of trypsin, sodium dodecylsulphate, Triton X-100, deoxicolic acid, RNAse and DNase, ethanol, glycerol and hypo/hypertonic solutions. These methodological differences are an obvious explanation for the disparities in either the experimental as well as the clinical outcomes [10,23].

The decellularization method employed in our allografts is a proprietary process (AutoTissue Ltd†), which is based in a combination of deoxicolic acid and ethanol treatment. This technology has been extensively tested in vitro and in vivo and the results published elsewhere [11,24].

To start our clinical experience with decellularized allografts we have chosen to restrict its use to the right side of the circulation and with exclusive use of homologous matrices. The first limitation was due to the fact that there is still some concern that treated matrices may not properly withstand systemic pressures and are more susceptible to conduit dilatation [17,23]. The second restriction was based in some experimental data demonstrating that decellularized heterologous matrices are still able to elicit a strong immunogenic response due to an interspecies incompatibility [25]. According to Allaire et al. [25], decellularized heterologous aortic grafts implanted in rats demonstrated disintegration of the elastin network of the media and developed aneurysmal dilatation in most cases. This may explain the premature failures with decellularized heterografts reported by Simon et al. [13].

Our data demonstrates that decellularized allografts is less antigenic than its cryopreserved counterpart, exhibited normal hemodynamic performance in the right side of the circulation and has yielded stable results up to 18 months.
postoperatively. In contrast to the cryopreserved allografts, we could not detect any elevation in antibody and PRA levels in seven cases and only marginal elevation in two others. In two patients, however, abnormal PRA levels with specificity for donor antigens could be demonstrated, and this may indicate failure of the process to completely eliminate the cells. Experience has shown that some variables as, conduit wall thickness, completeness of adventitial layer dissection and exposure time to decellularizing agents may be critical and could result in some cell remnants, especially in the conduit wall.

Others have made similar observations. Hawkins et al. [7] have studied 14 cases of decellularized homografts, detecting no PRA elevations in nine cases, two patients had abnormal levels of class I and II antibodies and in three cases they observed elevations of only class II antibodies. In Elkins et al. experience [9], 8% of the cases developed newly elevated PRA levels after a pulmonary Synergraft implantation. Betchel et al. [12] found two cases of elevated PRA levels, however, this response turned out to be unspecific associated with IgM production.

Functionally our decellularized allografts had normal hemodynamic performance up to 18 months, and, more important, we could not observe any increase in gradient and no new case of pulmonary insufficiency during this period. Although the number of cases is small and follow-up is short, these observations are encouraging. In contrast, the experience of Betchel et al. [12] evidenced an increase in gradients even in the absence of an immunological response. This difference appears somewhat intriguing but may be related to the decellularization technology.

In summary, decellularized allografts were significantly less immunogenic than cryopreserved allografts and had normal and stable hemodynamic performance up to 18 months postoperatively. Although larger number of patients and longer follow-up times are needed, decellularized allografts may be a superior alternative for right ventricular reconstruction during the Ross operation.

References

Appendix A. Conference discussion
Dr M. Antunes (Coimbra, Portugal): This is a nicely presented paper, follows on that presented yesterday. Naturally, the main aim of your paper is to demonstrate that there is a lack of immune response to the decellularized grafts. You are comparing it to cryopreserved, which presumably would not be live tissues, or do you plan to do that? Because I assume that
decellularizing of the homografts is quite a complex procedure. If plain antibiotic-preserved grafts could do the same, then the value of this process would be far reduced.

**Dr Costa:** I talked to Dr Tirone David some time ago about this constricting response on the right sided homografts, and we were commenting about not using cryopreserved and very viable valves during the Ross operation. We thought about thawing the grafts and leaving them alone before implantation for some time to make them less viable. But I think this is not enough. Even if you leave the graft, let’s say, for two weeks, three weeks or four weeks, you are going to still have some cell remnants and you are still going to have an immune response. So I think you should really decellularize to have a complete inert graft from this standpoint.

**Dr S. Aharinejad** (Vienna, Austria): Looking at your data, one would gain the idea that in cryopreserved grafts the cells would somehow gain the ability to be immunologic. If yes, how did you evaluate this?

**Dr Costa:** I guess I didn’t understand your question. If the cells are viable or immunogenic, is that what you asked?

**Dr Aharinejad:** Well, what I am asking is, if these patients with cryopreserved grafts develop antibodies, as you showed in your ELISA test, then the cells on the surface of the grafts should be somehow immunologic. So how did you prove this? Looking at the ELISA data would give you a clue about what is going on that surface of the graft.

**Dr Costa:** I think the cryopreserved grafts may have endothelial cells on the surface and also fibroblasts and dendritic cells in the matrix, and this will simulate an immune response. If you make a complete decellularization, I think there are no cells, or at least we cannot see any cells on microscopy, so this would diminish the immune response markedly.

**Dr S. Cebotari** (Hannover, Germany): You observed an immunological response in two patients in the decellularized group. How can you explain this? Was it an immunological response to the extracellular allograft matrix or probably this graft was not completely decellularized?

**Dr Costa:** This is a very intriguing question. We elected in these patients to use this technology just with homografts. We are still not confident to use heterografts with this, because I think the matrix, for example, the heterologous matrix, can be immunogenic, and also the matrix, the homologous matrix can also have some immunogenicity as well. But in these two reported cases I am very confident that this represents incomplete decellularization of the grafts. Depending on the thickness of the wall of the graft to be decellularized, you have to leave the graft a little more or a little less in the decellularization solution for you to be able to complete decellularize it, and probably in these two cases we had some cell remnants there which was responsible for this immune response.

**Dr M. Bechtel** (Luebeck, Germany): I think I missed something: Were these decellularized allografts cryopreserved too?

**Dr Costa:** Yes, they were regular cryopreserved grafts from the freezer. We thawed the grafts some days before the operation, decellularized them, and then we just kept them in a nutrient medium until the operation, like some days after.

**Dr Bechtel:** And they were cryopreserved according to the same protocol as were the conventional allografts?

**Dr Costa:** The same protocol. All the grafts in our bank, they are from heart-beating donors and cryopreserved very shortly after. So they are probably very viable grafts.

**Dr Bechtel:** So the effect you see is really due to the decellularization, not to the absence of any cryopreservation or any difference in the cryopreserved protocol?

**Dr Costa:** They are absolutely from the same origin. The only difference between one group and the other is the decellularization.

**Dr G. Gerosa** (Padova, Italy): One technical question. After the decellularization process, is the basal membrane maintained, and second, did you have any chance to observe an increase in thickness of the cusps after the implantation over time that can be explained by some extracellular matrix remodeling in vivo because of a host infiltration inside the cusps?

**Dr Costa:** By histology we could see that the basal membrane was still there, so it was protected. Your question is very important because we have tested several solutions at the university, and we saw that depending on the concentration of the solution and so on, you can make a lot of damage to the collagen and to the elastic fibers. So I think we could obtain a solution which maintains the extracellular matrix in a nice way, and this will enable cells to repopulate the graft and that is what we saw in the experimental setting. And that is what Dr Dohmen also saw in one patient that he explanted in humans and saw that the graft was completely recellularized and endothelialized in humans.

**Dr D. Ross** (Edmonton, Alberta, Canada): I didn’t see any data on pulmonary insufficiency. Do you have any evidence that the decellularized valves are actually any less structurally intact, any evidence of pulmonary insufficiency or dilatation of the pulmonary homografts in the decellularized group?

**Dr Costa:** We didn’t see any dilatation. That was a concern as well because there is some concern that these grafts are a little more extensible and could have some dilatation. We didn’t see any dilatation. We didn’t do MRI on those patients, but by echo, the diameter of the graft is similar to the hospital discharge. So there is no constricting response but also there is no dilatation as well. So I think the graft is working very nicely.