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BRIEF COMMUNICATION

Evaluation of humoral immune response to donor HLA after implantation of cellularized versus decellularized human heart valve allografts

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Abstract

We have evaluated the development of antibodies in response to donor allograft valve implant in patients who received cellularized and decellularized allografts and determined possible immunogenic epitopes considered responsible for antibodies reactivity. Serum samples from all recipients who received cellularized allografts or decellularized allografts were collected before valve replacement and at 5, 10, 30 and 90 days post-operatively and frozen until required. Tests were performed using the Luminex-based single human leukocyte antigen (HLA)-A, -B, -C and HLA-DR, -DQ antigen microsphere assay. To determine possible immunogenic epitopes, we used the HLAMatchmaker (HLAMM) software if applicable. Decellularized grafts elicited lower levels of anti-HLA class I and II antibody formation after implantation than cellularized allografts. All patients from cellularized group presented donor-specific antibodies class I and II within 3 months of observation period. In HLAMM analysis, the cellularized group had significantly higher numbers of immunogenic epitopes than decellularized group for both class I and II (p: 0.002 – cl I / p: 0.009 – cl II / p: 0.004 – cl I and II). Our findings demonstrate that the anti-HLA antibodies detected in the cellularized group were against donor HLA possible immunogenic epitopes and that in the decellularized group the anti-HLA antibodies were not against donor HLA possible immunogenic epitopes. These findings lead us to suggest that choosing sodium dodecyl sulfate decellularization process is the best alternative to decrease the immunogenicity of allograft valve transplant.

Valve transplant with biological tissue have been used since 1962 (1, 2). Allograft valves are the most useful biological prostheses for valve replacement (3–5). Although these prostheses are efficient and reduce substantially the morbidity and mortality, there are still design issues and a non-effective response of the body to the implanted materials (6). In aortic position, the allograft presents a significant structural deterioration in the first 10 years post-implantation (7, 8), and in pulmonary position, graft stenosis can prevent long-term durability (9). Most of the patients who received cryopreserved allograft developed humoral antibodies against human leukocyte antigens (HLA), which are specific against transplanted tissues (10–12).

In solid organ transplantation, the presence of donor-derived dendritic cells in allogenic tissue has been appointed to play an important role in the immune activation of recipient by direct antigen presentation (13). Studies have shown that the absence of dendritic cells (antigen presenting cells) in cryopreserved valve allografts is compensated by the preservation of other cells expressing HLA class II molecules predominantly in the endothelium which may be responsible for the initiation of a specific immune response against heart valve allograft (14).

Tissue engineering has been used to overcome these limitations and promising approaches using decellularized heart valve allografts (15) intend to avoid the immune response (5). Experimental and clinical experience with decellularization process have been gained with porcine tissue (16), ovine tissue (17) and subsequently, human tissue (5). Several methods have been developed to produce completely acellular heart valve tissue matrices using multistep detergent-enzymatic

extraction (18), Triton detergent (16), trypsin/ethylenediaminetetraacetic acid (19), deoxicolic acid (20), RNAse and DNAse (15). Meyer et al. (21) studied the reduction of immune response to aortic valve allografts by the decellularization process in rats. They concluded that decellularization significantly reduces the cellular and humoral immune response to allograft tissue.

On the other hand, Zehr (22) and Bechtel et al. (23) provides convincing evidence that the SynerGraft™ decellularization technology successfully removed antigens from an aortic and pulmonary allograft. Another protocol, using sodium dodecyl sulfate (SDS) in the presence of protease inhibitors, was successful for heart valve decellularization (24). Costa et al. (25, 26) demonstrated that decellularized allografts are less immunogenic than cryopreserved allografts and had normal and stable hemodynamic performance up to 18 months post-operatively. They compared the immunological and echocardiographic data of decellularized (AutoTissue Ltd™, Berlin, Germany) *vs* cryopreserved allografts used for right ventricular outflow tract (RVOT) reconstruction during Ross operation.

To evaluate the efficacy of the decellularization process in decreasing or preventing the development of humoral antibodies in response to donor allograft valve implant, the presence of anti-HLA antibodies was analyzed using the Luminex-based single HLA-A, -B, -C and HLA-DR, -DQ antigen microsphere assay in patients implanted with a cryopreserved allograft valve (CAV) or AutoTissue Ltd™-treated allograft valve or SDS-treated allograft valve. Additionally, HLA class I and II mismatches between recipient and donor were analyzed with HLAMatchmaker (HLAMM) algorithm (27), to determine the possible immunogenic epitopes that were responsible for antibodies reactivity.

Twelve patients, who underwent an aortic or pulmonary valve replacement between October 2005 and March 2009, were studied prospectively. Group 1 consisted of six patients who received cellularized allograft valve (median age 59 years, age range 30–75 years; four males, two females, five aortic valves and one pulmonary valve) and Group 2 with six patients who received SDS 0.1% decellularized allograft valve (median age 38.6 years, age range 24–49 years; one male, five females, one aortic valve and five pulmonary valves). The preoperative and surgical characteristics of the patients are listed in Table 1. Despite the group heterogeneity, no major differences that would influence the results were found between the two groups. The choice of allograft size was done in accordance to patient surface area, but as a general rule the biggest allograft available was implanted with a deliberate over sizing policy. The study was conducted in accordance with institutional guidelines and has been approved by the Ethical Committee of Pontifical Catholic University of Parana (PUCPR) registered as number 1305. Before being enrolled, patients signed the informed consent to participate in the study.

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 10–30 min. The pulmonary autografts were 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. No extension of the allograft with pericardial patches in the proximal suture line was used.

Allografts preparation

The allografts were obtained from donation after cardiac death and were prepared by the Human Cardiac Valve Bank of Santa Casa of Misericordia of Curitiba (BVCHSC). The cryopreservation was done according to previously published methods (26). The valves were prepared following standard

Table 1 Group 1 – cellularized patient information

Patient	Gender	Age (years)	Valve origin	Indication	Valve size	Hemoderivative	ABO
PAC1C	M	56	Aortic	Aortic stenosis	26	Erythrocytes	O−
PAC2C	F	51	Aortic	Aortic insufficiency	23	Platelets, plasma, erythrocytes	A+
PAC3C	M	69	Aortic	Aortic aneurysm	22	Plasma, erythrocytes	A+
PAC4C	F	75	Aortic	Aortic stenosis	22	Platelets, plasma, erythrocytes	NI
PAC5C	M	73	Aortic	Double aortic lesion	22	Platelets, plasma, erythrocytes	NI
PAC6C	M	30	Pulmonary	Aortic insufficiency	24	Erythrocytes	O−
PAC1D	F	48	Pulmonary	Aortic stenosis	23	Plasma, erythrocytes	O+
PAC2D	F	23	Pulmonary	Aortic stenosis	24	Erythrocytes	O+
PAC3D	F	44	Pulmonary	Aortic insufficiency	23	Plasma, erythrocytes	O+
PAC4D	M	25	Pulmonary	Aortic stenosis	24	Platelets, plasma, erythrocytes	NI
PAC5D	F	49	Pulmonary	Aortic insufficiency	24	Platelets, plasma, erythrocytes	O+
PAC6D	F	43	Aortic	Aortic insufficiency	23	Erythrocytes	B+

NI, not informed.

protocols and decellularized by a proprietary process (28). All allografts were first cryopreserved and when required were decellularized. The choice of the valve prostheses occurred according to patient needs, allograft availability and implantation technique.

HLA typing of valve donor and recipient

Blood samples with ethylenediaminetetraacetic acid (EDTA) anticoagulant were collected from all valve donors and obtained from allograft valve bank. Recipients' blood samples with EDTA anticoagulant were obtained before or after valve transplant. All recipients and valve donors were typed for HLA-A, -B and -DR (PCR-SSO LABType® Class I and II; One Lambda Inc., Canoga Park, CA) following the recommendation of provider.

Anti-HLA antibodies detection

Serum samples from all recipients were collected before valve replacement and at 5, 10, 30 and 90 days post-operatively and stored at -20°C until required. Anti-HLA antibodies detection tests were done using Luminex Single antigen bead assay (LABScreen™ Single Antigen Class I and II; One Lambda Inc.). These methodology uses microbeads coated with purified class I or class II HLA antigens and pre-optimized reagents for the detection of class I or class II HLA antibodies in human sera. In Luminex Single antigen bead assay, singles beads are used to focus on reactions against one or a few antigens, e.g. to compare reactivity of different serum samples from the same individual (29). Data acquisition was done by LABScan® 100 flow analyzer (One Lambda Inc.) that detects fluorescent emissions of each bead. Results were analyzed using HLA Fusion® software. The cut-off used was any MFI (medium of fluorescent intensity) value over 500.

Antibody reactivity patterns with HLAMatchmaker

HLAMM is a computer software that determines the HLA compatibility at the structural level (27, 30). In HLAMM, each HLA antigen is viewed as a string of epitopes represented by short sequences involving polymorphic amino acid residues in antibody-accessible positions and by longer sequences and amino acid residues in discontinuous sequence positions which are called eplets. These eplets are considered as key elements of epitopes that can elicit specific alloantibodies (30).

Positive antibody detection results of this study were submitted to HLAMM (2008 version) analysis to determine HLA compatibility at the structural level (30). The first step of a HLAMM serum analysis is to identify alleles that are responsible to reveal negative antibodies reactions. Such alleles can be expected to have eplets that are not recognized by patient's antibodies and from these eplets together with the patient's own eplets we can identify the ones that may be responsible for the positive antibodies reactions and, furthermore, if the possible immunogenic eplets are from donor-specific molecules.

The methodology used for donor and patient HLA typing does not give high resolution four digit alleles. But for HLAMM analysis, four digit alleles information is necessary. Because of that, the HLA-DRB1 four digit information was determined by the most frequent allele in a comparable population. The allele frequency used was that one described in the HLAMM program. Moreover, patient and donors did not have their HLA typing for -DRB3/4/5, -DQA1 and DQB1 alleles. In order to realize a complete HLAMM class II analysis, the HLA-DRB3/4/5 and −DQA/-DQB alleles were determined using the most frequent allele association between alleles HLA-DRB1 and alleles HLA-DRB3/4/5 and -DQA/-DQB, described at HLAMM software.

Statistical analysis

Results were expressed by median, minimum values and maximum values or by frequencies and percentage. To compare groups regarding the number of antigenic epitopes we used Mann–Whitney test. To prove the probability of antigenic epitopes presence, groups were compared using Fisher test. $P < 0.05$ was statistically significant. The data were organized in a Microsoft Excel spreadsheet and analyzed by Statistica 8.0.

Results

This pilot study compares the two methods of valve preservation, with and without donor cells. For that, initially, the focus was the HLA compatibility between recipients and donors in both groups (Table 2). We observed higher than five HLA antigens mismatches between recipients and donors for class I and II in cellularized and decellularized group. The next step was the Luminex Single antigen bead assay analysis. In group 1, we observed that all recipients were reactive for both class I and II. In this group, two recipients presented class I DSA (donor-specific antibodies) in pre-transplant and developed class II DSA during post-transplant monitoring. The other four recipients of the same group developed class I and II DSA after the transplant. In the group 2, one recipient did not present anti-HLA antibodies in any analyzed sample. One presented class I DSA already in pre-transplant sample and class II DSA within 3 months of the observation period. The other four patients developed class I DSA during post-transplant monitoring. Two of them presented class II DSA during the observation period. After statistical analysis, we observed that the development of DSA in post-transplant of both groups (1 and 2) was statistically significant only for

Table 2 HLA compatibility between recipients–donors in both groups and antibody detection information[a]

			A*	A*	B*	B*	DRB1*	DRB1*	DRB	DRB	DQA1*	DQA1*	DQB1*	DQB1*	MM	DSA before TX	DSA after TX
Group 1	PAC1C	Recipient	01:01	68:01	08:01	44:02	03:01	11:01	3*02:02	3*01:01	05:01	01:02	02:01	03:01	5	No	Yes
		Donor	01:01		08:01	57:01	03:01	07:01	3*02:02	4*01:01	05:01	02:01	02:01	03:03			
	PAC2C	Recipient	01:01	23:01	44:02	81:01	07:01	11:01	4*01:01	3*02:02	02:01	05:01	03:03	03:01	11	No	Yes
		Donor	31:01	33:01	39:01	44:03	08:01	15:01		5*01:01	04:01	01:02	04:02	06:02			
	PAC3C	Recipient	24:02	25:01	15:01	44:02	13:02	14:01	3*03:01	3*02:02	01:02	01:04	05:01	05:03	12	Yes	Yes
		Donor	02:01	68:01	40:01	51:01	04:01	13:01	4*01:01	3*01:01	03:02	01:03	03:01	06:03			
	PAC4C	Recipient	25:01	32:01	18:01	27:05	01:01	15:01	5*01:01		01:02	01:01	05:01	06:02	10	Yes	Yes
		Donor	02:01	31:01	07:02	50:01	11:01	13:01	3*01:01	3*02:02	01:02	05:01	03:01	06:02			
	PAC5C	Recipient	29:01	30:01	41:01	44:03	04:03	07:01	4*01:01		02:01	03:01	02:02	03:02	10	No	Yes
		Donor	02:01	11:01			11:01	14:01	3*02:01	3*02:02	01:02	03:01	05:01	06:02			
	PAC6C	Recipient	02:01	01:01	40:01	570:1	04:08	11:04	4*01:01	3*02:02	03:02	05:01	03:01	03:01	7	No	Yes
		Donor	02:01	03:01	07:02	40:04	04:11	09:01	4*01:01		03:02		03:02	04:02			
Group 2	PAC1D	Recipient	02:05	24:02	08:01	57:01	03:01	16:02	3*02:02	5*01:01	05:01	01:02	05:02	02:01	11	Yes	Yes
		Donor	02:01	03:01	15:17	51:01	04:07	13:01	3*02:02	4*01:01	03:01	01:03	06:03	03:02			
	PAC2D	Recipient	01:01	24:02	08:01	35:04	03:01	04:04	3*02:02	4*01:01	01:02	03:01	02:01	03:02	8	No	No
		Donor	03:01	29:02	15:10	44:03	03:01	11:01	3*02:02	3*01:01	01:02	05:01	02:01	03:01			
	PAC3D	Recipient	02:01	30:02	44:02	57:03	11:01	13:01	3*02:02	3*02:02	05:01	01:03	03:01	06:03	8	No	Yes
		Donor	02:01		35:01	52:01	03:01	15:02	3*02:02	5*01:01	01:02	01:03	02:01	06:01			
	PAC4D	Recipient	01:01	26:01	35:01	51:01	07:01	11:04	3*02:02	4*01:01	02:01	05:01	02:02	03:01	9	No	Yes
		Donor	02:05	30:02	35:03	50:01	07:01	14:01	3*01:01	4*01:01	01:04	02:01	02:02	05:03			
	PAC5D	Recipient	03:01	02:01	07:02	51:01	04:01	11:01	4*01:01	3*02:02	01:02	03:02	03:01	06:02	8	No	Yes
		Donor	03:01	24:02	07:02	52:01	04:04	15:01	4*01:01	5*01:01	01:03	03:01	03:02	06:02			
	PAC6D	Recipient	26:01	29:01	44:02	51:02	07:01	08:01	4*01:01		02:01	04:01	04:02	02:01	11	No	Yes
		Donor	03:01	24:02	07:02	52:01	04:04	15:01	4*01:01	5*01:01	01:02	03:01	03:02	06:02			

MVI, mismatches.

[a]Gray shade indicates the matching between recipient and donor HLA molecules.

class II ($P = 0.015$). The detection of *de novo* DSA occurred in different monitoring periods (5, 10, 30 and 90 days after transplant) for each patient. All patients received hemoderivatives which included platelets concentrate, fresh plasma and/or erythrocytes concentrate (Table 1). The kind of hemoderivatives received did not matter in the analysis.

DSA present in pre-transplant samples could not be considered developed against the allograft, since already existent before transplant. However, there was an increase of the MFI values of DSA in post-transplant monitoring for both groups.

Analyzing Luminex Single antigen bead assay results with HLAMM, we observed that HLA molecules, which we found antibodies against in the post-transplant serum of group 2, did not share or share only few eplets with donor-specific molecules in comparison to group 1 (Tables 3–5). In group 1, we could observe that almost all antibodies reactions shared possible immunogenic eplets with donors HLA molecules (Tables 3–5).

Additionally, we observed that the number of donor-specific immunogenic eplets was significantly higher in group 1 than in group 2 (Figure 1A–C). In group 1, we found a median of 12.5 for class I, 25.5 for class II and 37 for class I/II. In group 2, we found a median of 1 for class I, 0 for class II and 3 for class I/II. The P value was significant for class I ($P = 0.002$), class II $P = 0.009$) and also for class I/II ($P = 0.004$).

Discussion

The specificity of DSA is determined by the result of the Luminex Single antigen bead test and the typing of the valve donor (29). All the patients of the study were analyzed by this way. In the post-transplant of cryopreserved allografts, transplant are directly related to donor-specific immune response, which we have not seen in decellularized allografts transplant, which can be explained by a reduced immune response following decellularized valve transplant and that the use of cryopreserved allografts could induce a higher donor-specific immune response.

The allografts induce anti-HLA antibodies even in this rather short follow-up period. Group 1 showed in many cases an increase in the mean value of fluorescence intensity (MFI) of these alloantibodies during the sera monitoring. The MFI of antibodies in group 2 did not present this pattern.

According to Fischlein et al. (31), the cryopreservation of allograft valve represents a cell- and tissue-protective preservation. In their study, they showed that all allograft valves caused immunologic reactions post-operatively, probably because of graft endothelium cell membranes are human

Table 3 Possible immunogenic eplets present on donor class I HLA molecules that were the targets of recipient anti-HLA antibodies (gray colored)

		Class I																		
		Recipient HLA	Donor HLA	Eplets																
Cellularized	PAC1C	*A*0101*	*A*0101*																	
		*A*6801*	x																	
		*B*0801*	*B*0801*																	
		*B*4402*	*B*5701*	45RMA	62GE	71SA	76ENI	79RI	113HD	116S	163LW									
	PAC2C	*A*0101*	*A*3101*	9T	56R	73ID	76VDT	80VGT	113YQ	166TEW	193AV	245AS	253Q							
		*A*2301*	*A*3301*	9T	73ID	76VDT	80VGT	113YQ	166TEW	193AV	245AS	253Q								
		*B*4402*	*B*3901*	116F	158T	166TEW														
		*B*8101*	*B*4403*																	
	PAC3C	*A*2402*	*A*0201*	9F	62GE	66RKH	73TD	76VDT	79GT	80VGT	107W	142MT	145KHA	166TEW						
		*A*2501*	*A*6801*	66RNQ	71QS	73TD	76VDT	79GT	80VGT	113YR	142MT	145KHA	166TEW	245VA						
		*B*1501*	*B*4001*	9H	113HN	143ISQ	147L	163EW	177DT	180E										
		*B*4402*	*B*5101*	44RT	113HN	193PV														
	PAC4C	*A*2501*	*A*0201*	62GE	66RKH	70KAH	76VDT	79GT	80VGT	127K	105S	107W	116Y	142MT	145KHA	150AAH	151AHV			
		*A*3201*	*A*3101*	9T	56R	73ID	76VDT	79GT	80VGT	105S										
		*B*1801*	*B*0702*	70IAQ	116Y	152RE	177DK	180E												
		*B*2705*	*B*5001*	12AMR	41T	44RK	103L	113YN	116L	152RE	163LW									
	PAC5C	*A*2901*	*A*0201*	9F	62GE	66RKH	69RAHT	70KAH	71HS	127K	107W	113YH	142MT	145KHA	150AAH	151AHV				
		*A*3001*	*A*1101*	9Y	90D	144KR	150AAH	151AHA	152HA	163RW										
		*B*4101*	x																	
		*B*4403*	x																	
	PAC6C	*A*0201*	*A*0201*	66RNQ	71QS	151AHE	161D													
		*A*0101*	*A*0301*	9Y	44RE	62RN	65QIA	70IAQ	152RE	177DK										
		*B*4001*	*B*0702*	103L																
		*B*5701*	*B*4004*																	
Decellularized	PAC1D	*A*0205*	*A*0201*	9F																
		*A*2402*	*A*0301*	9F	62QE	66RNQ	71QS	113YR	116D	151AHE	161D	275EL								
		*B*0801*	*B*1517*	116D	152RE															
		*B*5701*	*B*5101*	44RT	152RE	193PV														
	PAC2D	*A*0101*	*A*0301*	66RNQ	71QS	73TD	76VDT	80VGT	151AHE	161D										
		*A*2402*	*A*2902*	9T	62LQ	66RNQ	71QS													
		*B*0801*	*B*1510*	9Y	113HD	152RE														
		*B*3504*	*B*4403*	9Y	32L	41T	44RK	76ENT	79RT	113YD	167ES	199V								
	PAC3D	*A*0201*	*A*0201*																	
		*A*3002*	x	44RT	62RN	73TS	80ERN	103L	113HD	116S	193PV									
		*B*4402*	*B*3501*	44RT	152RE	193PV														
		*B*5703*	*B*5201*																	
	PAC4D	*A*0101*	*A*0205*	44RM	62GE	66RKH	70KAH	73TD	76VDT	80VGT	127K	105S	107W	113YH	142MT	145KHA	150AAH	151AHV	166TEW	
		*A*2601*	*A*3002*	9S	56R	17RS	76ENT	80EGT	105S	152RR	166TEW									
		*B*3501*	*B*3503*	116F																
		*B*5101*	*B*5001*	9H	32L	41T	44RK 113YN	116L												
	PAC5D	*A*0301*	*A*0301*	9S	62EE	66GKH	163TG	167DG												
		*A*0201*	*A*2402*																	
		*B*0702*	*B*0702*																	
		*B*5101*	*B*5201*																	
	PAC6D	*A*2601*	*A*0301*	9F	62QE	73TD	76VDT	80VGT	144KR	150AAH	161D	275EL								
		*A*2901*	*A*2402*	9S	62EE	66GKH	70KAH	127K	113YH	144KR	150AAH	151AHV	163TG	167DG						
		*B*4402*	*B*0702*	44RE	65QIA	70IAQ	73TS	80ERN	113HD	163EW	177DK	180E								
		*B*5102*	*B*5201*																	

HLA, human leukocyte antigen.

Table 4 Possible immunogenic eplets present on donor class II HLA molecules that were the targets of recipient anti-HLA antibodies (gray colored) in the cellularized group

		Class II																			
		Recipient HLA	Donor HLA	Eplets																	
Cellularized	PAC1C	*DRB1* 0301*	*DRB1* 0301*																		
		DRB1 1101*	*DRB1* 0701*	4Q	26QF3	67IR	76GDT	180VM	98ES												
		DRB3 0202*	*DRB3* 0202*																		
		DRB3 0101*	*DRB4* 0101*	4Q	32IYN	135S	48YQ6	67LR	70LRRA	71RRA	73AEDT	74RRAE	98KN	120N							
		DQB1 0201*	*DQB1* 0201*																		
		DQB1 0301*	*DQB1* 0303*	45GV	185I																
		DQA1 0501*	*DQA1* 0501*																		
		DQA1 0102*	*DQA1* 0201*	25FT	34HE	47EK2	48LF	75ILR	80IRS2												
	PAC2C	*DRB1* 0701*	*DRB1* 0801*	14GEY	25YRF	47DYR	57SA	73ALDT													
		DRB1 1101*	*DRB1* 1501*	142M3	26KFD	71QAA	96QV														
		DRB4 0101*	x																		
		DRB3 0202*	*DRB5* 0101*	31QDIY	40HFD	96EV	108T3														
		DQB1 0303*	*DQB1* 0402*	26G	57LD	66DI	70ED	74SV													
		DQB1 0301*	*DQB1* 0602*	52PQ	56RPD	70GT	71VGT	84EV	85EVAG	87AF	90AGI	125GQ	140A2								
		DQA1 0201*	*DQA1* 0102*	44TA2	47QRW	50EF8	75IMR														
		DQA1 0501*	*DQA1* 0401*	69T																	
	PAC3C	*DRB1* 1302*	*DRB1* 0401*	26KFD	71Qk/rA	71QKA	96YL4	120N													
		DRB1 1401*	*DRB1* 1301*																		
		DRB3 0301*	*DRB3* 0101*	12RKS	25HRY	26RY	26KYD	73GRDN													
		DRB3 0202*	*DRB4* 0101*	4Q	32IYN	33LYNQ	135S	48YQ6	98KN	120N											
		DQB1 0501*	*DQB1* 0301*	14AM	26Y	30YYA	45EV	52PL	55PPP	a56PPD	57PD	67WT	70RT	71VRT	74EL	77DT	84QL2	116V	140T2	167HG	
		DQB1 0503*	*DQB1* 0603*	14GM	26L	30HYA	56RPD	57PD	67WT	70GT	71VGT	74EL	77DT	87AF	116V	125GQ					
		DQA1 0102*	*DQA1* 0103*	25FT	129HA2																
		DQA1 0104*	*DQA1* 0302*	48LF	56RR5	60QF	64TI4	69L	80IRS2	160DD											
	PAC4C	*DRB1* 0101*	*DRB1* 1101*	12STS	14SEH	26TFD	31YYFY	57DE	96HV	140T2	149H										
		DRB1 1501*	*DRB1* 1301*	12STS	14SEH	26TFD	31YYFH	32FHN	33LHNQ	71DEA	74DEAA	96HV	140T2	149H							
		DRB5 0101*	*DRB3* 0101*	12RKS	14SEH	25HRY	26RY	26KYD	31LYFH	32FHN	33LHNQ	47DYR	57VA	67LK	71QKG	73GRDN	76GDN	96HV	98QS	104AK	
			DRB3 0202*	12LKS	14SEH	26KFE	32FHN	33LHNQ	67LK	71QKG	74QKGQ	76GDN	51R	96HV	98QS	104AK	189S				
		DQB1 0501*	*DQB1* 0301*	14AM	26Y	5EV	52PL	55PPP	56PPD	70RT	71VRT	84QL2	140T2	167HG							
		DQB1 0602*	*DQB1* 0602*																		
		DQA1 0102*	*DQA1* 0102*																		
		DQA1 0101*	*DQA1* 0501*	41GR3	56RB	60QF	64TI4	69L	75SL4	160AE											
	PAC5C	*DRB1* 0403*	*DRB1* 1101*	12STS	14SEH	26TFD	31YYFY	47DFR	48FR	57DE	67FR	70FDRA	71DRA	73AADT	74DRAA	98KS	149H				
		DRB1 0701*	*DRB1* 1401*	12STS	14SEH	26TFD	31YYFH	32FHN	33LHNQ	57AA	112Y	98KS	149H								
		DRB4 0101*	*DRB3* 0201*	12LKS	14SEH	32FHN	33LHNQ	67LK	71QKG	74QKGQ	76GDN	51R	98QS								
			DRB3 0202*	12LKS	14SEH	32FHN	33LHNQ	67LK	71QKG	74QKGQ	76GDN	51R	98QS								
		DQB1 0202*	*DQB1* 0501*	14GL	26G	30HYV	52PQ	57PV	70GA	74SV	84EV	85EVAG	87AY	90AGI	116I	125SQ					
		DQB1 0302*	*DQB1* 0602*	52PQ	56RPD	57PD	70GT	71VGT	84EV	85EVAG	87AF	90AGI	125GQ								
		DQA1 0301*	*DQA1* 0301*																		
		DQA1 0201*	*DQA1* 0102*	25YT	34HQ	44TA2	47QRW	50EF8	75IMR												
	PAC6C	*DRB1* 0408*	*DRB1* 0411*	57SA	74QRAE																
		DRB1 1104*	*DRB1* 0901*	14FEH	25HRY	26RY	26KYH	31QGIY	40HFD	57VA	180VM	98ES									
		DRB4 0101*	*DRB4* 0101*																		
		DRB3 0202*	x																		
		DQB1 0301*	*DQB1* 0302*	14GM	26L	45GV	56PPA	57PA	167RG	185I											
		x	*DQB1* 0402*	14GM	26G	45GV	57LD	66DI	70ED	74SV	167RG	185I									
		DQA1 0302*	*DQA1* 0302*																		
		DQA1 0501*	x																		

HLA, human leukocyte antigen.

Table 5 Possible immunogenic eplets present on donor class II HLA molecules that were the targets of recipient anti-HLA antibodies (gray colored) in the decellularized group

		Class II															
		Recipient HLA	Donor HLA	Eplets													
Decellularized	PAC1D	*DRB1*0301*	*DRB1*0407*	71Qk/rA	71QRA	73AEDT	74QRAE	96yl4									
		*DRB1*1602*	*DRB1*1301*	26TFD	71DEA	74DEAA											
		*DRB5*0101*	*DRB4*0101*	4Q	48YQ6	70LRRA	71RRA	73AEDT	74RRAE								
		*DRB3*0202*	*DRB3*0202*														
		*DQB1*0201*	*DQB1*0302*	30YYA	52PL	55PPP	56PPA	67VVT	70RT	71VRT	74EL	77DT	140T2	185I			
		*DQB1*0502*	*DQB1*0603*	30HYA	56RPD	57PD	67VVT	70GT	71VGT	74EL	77DT	87AF	125GQ				
		*DQA1*0501*	*DQA1*0301*	34HE	48LF	56RR5	80IRS2										
		*DQA1*0102*	*DQA1*0103*	25FT	129HA2												
	PAC2D	*DRB1*0301*	*DRB1*0301*														
		*DRB1*0404*	*DRB1*1101*	26TFD	31YYFY	32FYN	57DE	67FR	70FDRA	71DRA	74DRAA						
		*DRB3*0202*	*DRB3*0202*														
		*DRB4*0101*	*DRB3*0101*	12RKS	26KYD	31LYFH	57VA										
		*DQB1*0201*	*DQB1*0201*														
		*DQB1*0302*	*DQB1*0301*	14AM	26Y	45EV	56PPD	57PD	167HG								
		*DQA1*0102*	*DQA1*0102*														
		*DQA1*0301*	*DQA1*0501*	41GR3	56RB	75SL4	160AE										
	PAC3D	*DRB1*1101*	*DRB1*0301*	25HRY	26RY	26TYD	73GRDN										
		*DRB1*1301*	*DRB1*1502*	142M3	26KFD	71QAA	96QV										
		*DRB3*0202*	*DRB3*0202*														
		x	*DRB5*0101*	31QDIY	32IYN	40HFD	135S	96EV	98KN	108T3	120N						
		*DQB1*0301*	*DQB1*0201*	45GE5	56LPA	57PA	66DI	77DR									
		*DQB1*0603*	*DQB1*0601*	3P3	66DI												
		*DQA1*0501*	*DQA1*0102*	41ER													
		*DQA1*0103*	*DQA1*0103*														
	PAC4D	*DRB1*0701*	*DRB1*0701*														
		*DRB1*1104*	*DRB1*1401*	31YYFH	47DYR	57AA	112Y										
		*DRB3*0202*	*DRB3*0101*	12RKS	25HRY	26RY	26KYD	31LYFH	47DYR	73GRDN							
		*DRB4*0101*	*DRB4*0101*														
		*DQB1*0202*	*DQB1*0202*														
		*DQB1*0301*	*DQB1*0503*	14GL	26G	30HYV	45GV	52PQ	56RPD	70GA	74SV	84EV	85EVAG	87AY	90AGI	116I	125SQ
		*DQA1*0501*	*DQA1*0104*	2G	44TA2	47ERW	50EF8	75IMR									
		*DQA1*0201*	*DQA1*0201*														
	PAC5D	*DRB1*0401*	*DRB1*0404*	71QRA	74QRAA												
		*DRB1*1101*	*DRB1*1501*	142M3	71QAA	96QV											
		*DRB4*0101*	*DRB4*0101*														
		*DRB3*0202*	*DRB5*0101*	31QDIY	40HFD	96EV	108T3										
		*DQB1*0301*	*DQB1*0302*	56PPA	57PA	185I											
		*DQB1*0602*	*DQB1*0602*														
		*DQA1*0102*	*DQA1*0103*	25FT	129HA2												
		*DQA1*0302*	*DQA1*0301*														
	PAC6D	*DRB1*0701*	*DRB1*0404*	16HFR	25HRF	26KFD	71Qk/RA	71QRA	73AADT	74QRAA	96YL4						
		*DRB1*0801*	*DRB1*1501*	142M3	16HFR	25HRF	26KFD	47DFR	48FR	71QAA	73AADT	96QV					
		*DRB4*0101*	*DRB4*0101*														
		x	*DRB5*0101*	16HFR	25HRF	31QDIY	40HFD	73AADT	74DRAA	96EV	108T3						
		*DQB1*0402*	*DQB1*0302*	55PPP	56PPA	66EV	67VVT	70RT	71VRT	74EL							
		*DQB1*0201*	*DQB1*0602*	52PQ	56RPD	57PD	66EV	67VVT	70GT	71VGT	74EL	84EV	85EVAG	87AF	90AGI	125GQ	
		*DQA1*0201*	*DQA1*0102*	44TA2	47QRW	50EF8	75IMR										
		*DQA1*0401*	*DQA1*0301*	56RR5													

HLA, human leukocyte antigen.

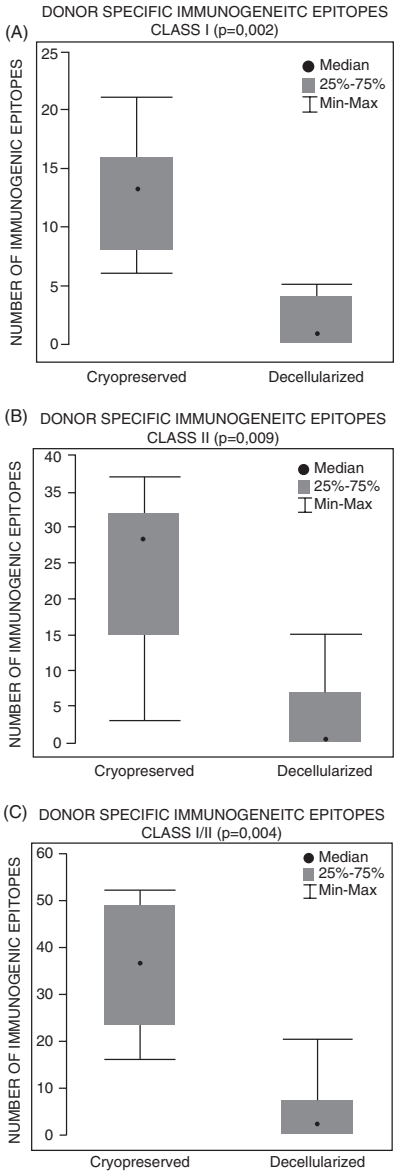

Figure 1 Comparison of number of donor-specific immunogenic epitopes: (A) for class I, (B) for class II and (C) for class I/II.

lymphocyte antigen class I and II positive and endothelial antigens present the primary immunologic stimulus.

Moreover, Welters et al. (32) demonstrated that cryopreservation allows preservation of endothelial and valve architecture and viability of presumed improved durability and convenience of long-term storage. Immunologically, however, better preservation of allograft and endothelium viability may actually sustain immunogenicity and elicit a more vigorous immunologic reaction from the recipient; this response can theoretically contribute to accelerated degeneration of allograft valves or patch material. In the other hand, Yap et al. (33) studied the influence of anti-HLA antibodies in CAV implantation and concluded that the clinical significance of their findings was unclear, as no correlation was found between the prevalence of anti-HLA antibody and echocardiographic parameters of valve dysfunction at a mean of 3.5 years follow-up.

In order to evaluate the best choice of valve allograft treatment, we analyzed the alloantibodies development during the post-transplant period. We observed that in a first moment (between 3 months), decellularization protocol presented more benefits for valve allograft transplant than only cryopreservation protocol because the development of alloantibodies in patients who received decellularized valve allograft was lower than in the patients who received cryopreserved valve allograft. It could be determined because of the number of DSA specificities after transplant was higher for cellularized group. This finding corroborates Elkins et al. (5, 17) who demonstrated that decellularization process is a method of choice in attempting to reduce the antigenic response in cryopreserved tissue.

Tissue-engineered heart valves have several potential advantages over currently used prostheses, such as a potential growth capacity, greater durability and the opportunity to use viable, autologous cells that can utilize body's mechanisms to repair and remodel (16). Several distinct methods of decellularization have been employed which can explain disparities in the experimental and clinical outcomes (17). Meyer et al. (21) demonstrated that decellularization of aortic valve allografts is associated with a significant reduction in cellular and humoral immune responses to levels shown with non-immunogenic syngenic tissue. They thought that this could prolong the durability of valve allografts and might prevent immunologic sensitization of allografts recipients. In the same way, Costa et al. (25) evaluated ELISA PRA results and echocardiographic exams. They observed that decellularized allografts (AutoTissue Ltd™) were less antigenic than cryopreserved allografts, exhibited normal hemodynamic performance in the right side of the circulation and have yielded stable results up to months post-operatively.

Dignan et al. (12) showed that HLA class II antigens mismatch was significantly associated with structural degeneration in patients receiving an aortic allograft valve who were followed for ≥years. They also demonstrated a trend toward increased structural deterioration in patients with two or more mismatches of class I, B antigens. Likewise, in our findings, the HLA antigens mismatches for class I and II, we found that they were higher than five mismatches, and they appeared more for HLA class II in both groups.

In addition to the analysis of the presence of DSA, we also analyzed the presence of possible immunogenic epitopes specific to donor HLA molecules. This analysis was done by the HLAMM program, which can be used not only for HLA compatibility studies but also to analyze serum screening for sensitized patients (38).

One of the concepts of HLAMM is that HLA typing differences between antibody producer and immunizer will

define the mismatched eplet repertoire which the patient has been exposed to and this information facilitates the interpretation of serum-screening results (39). Analysis of antibody reactivity patterns with HLA panels may distinguish reactive and non-reactive eplets so that specific donor HLA response can be confirmed and the responsible eplet for the response determined.

Our data showed that the immune response, especially in the group 1, was managed by donor-specific epitopes. These findings were possible because we had patient and valve allograft donor HLA typing information. When we compared the donor eplets with the possible immunogenic eplets recognized by the recipient's antibodies, we observed that the majority of these eplets were shared with donor HLA molecules, and this observation was significant in group 1. Cai and Terasaki (34) reported that the identification of the HLA epitopes should be helpful distinguishing DSA from natural antibodies, which appear to be produced in response to non-HLA environmental stimuli.

This kind of analysis is pioneer in valve allograft transplant, but we can find data about other kinds of transplant as heart transplant and most commonly renal transplant. Kosmoliaptsis et al. (35) demonstrated that the number of epitopes mismatched between an alloantigen and the host HLA type determined using the HLAMM algorithm, correlates closely with both development and strength of an alloantibody response. Dankers et al. (36) performed further analysis of sera from patients who had rejected kidney allografts and indicated that the chance for a patient to develop donor-specific alloantibodies directed against mismatched HLA class I antigen is directly related to the number of mismatched epitopes present on that allogenic HLA molecules. Similarly, Peräsaari et al. (37) studied eplet mismatches defined by HLAMM program in pediatric heart transplant and suggested that the eplet mismatch number was associated with the development of HLA antibody-mediated complications such as coronary artery disease.

Despite the small number of patients studied in our report, we could demonstrate with Luminex Single antigen bead results and HLAMM analysis that choosing the SDS decellularization (PUC solution) process can be an effective alternative to decrease the immunogenicity of allograft valve transplant. Furthermore, the finding of high presence of anti-HLA antibodies in patients underwent cellularized allograft valve transplant shows the importance of donor and recipient HLA compatibility analysis before valve transplantation. The clinical significance of these findings requires further investigation.

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Conflict of interest

Dr FDAC has a conflict of interest as owner of the decellularization protocol patent used in this study.

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