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Decellularization as an anticalcification method in stentless bovine pericardium valve prosthesis: a study in sheep

Descelularização como método anticalcificante em próteses valvares de pericárdio bovino sem suporte: estudo em ovinos

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Abstract

Objective: **The objective was to analyze the decellularization process with SDS in glutaraldehyde-preserved bovine pericardium as an anticalcification method in a circulatory sheep model.**

Methods: **The valved tubs were implanted in pulmonary artery position in sheep by 180 days. The animals were divided in two groups of eight animals: control group - glutaraldehyde-preserved bovine pericardium and the study group - decellularized bovine pericardium with 0.1% SDS and glutaraldehyde-preserved. After explantation the tubs were analized by x-ray macroscopy, hematoxilin-eosin, alizarin-red and Russel-Movatz pentacromic histology. The calcium content was measured by flame atomic absorption spectrometry.**

Results: **There was no early mortality, but two animals in each group died during the study. All cusps in the control group were severely calcified and in some points in the conduits, while the decellularized group did not show macroscopic calcification. Data were proved by x-ray and histologycal exams. The matrix was preserved in histologycal analysis in decellularized group, without gross calcification. The wall conduits calcium content was 35.25±42.13 µg/mg in the control group versus 15.75±10,44 µg/mg in the decellularized one: in the cusps was 264.4±126.16 µg/mg in control group versus 94.29±27.05 µg/mg in decellularized group (*P*=0,009).**

Conclusion: **The decellularization with 0.1% SDS was effective as an anticalcification method in bovine pericardial grafts implanted in a sheep circulatory model for 180 days.**

Descriptors: **Pericardium. Bioprosthesis. Tissue engineering. Transplantation, heterologous.**

Resumo

Objetivo: **Avaliar o processo de descelularização com dodecil sulfato de sódio (SDS) como método anticalcificante em próteses de pericárdio bovino fixadas em glutaraldeído, em modelo circulatório de ovinos.**

Métodos: **Tubos valvulados de pericárdio bovino foram implantados em posição pulmonar de ovinos por 180 dias. Os animais foram divididos em dois grupos com oito animais: grupo controle, com condutos de pericárdio fixado em**

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glutaraldeído e grupo estudo, com pericárdio descelularizado com SDS 0,1% e posteriormente fixado em GDA. Os explantes foram submetidos à análise macroscópica, histológica com hematoxilina-eosina, alizarina-red e pentacrômico de Russel-Movatz, estudo radiológico e quantificação de cálcio com espectrometria de absorção atômica.

Resultados: **Não houve mortalidade imediata, porém dois animais de cada grupo faleceram na evolução tardia. Os enxertos do grupo controle apresentavam intensa calcificação das cúspides e em algumas regiões dos condutos, enquanto que os enxertos descelularizados apresentavam-se preservados, sem calcificações macroscópicas evidentes. Esses resultados foram comprovados por análise histológica e radiográfica. Histologicamente, os enxertos descelularizados tiveram sua matriz melhor preservada e com diminuição acentuada da calcificação. O conteúdo de cálcio nos condutos foi de 35±42 µg/mg de tecido no grupo controle *versus* 15 ±10 µg/mg nos descelularizados. Nas cúspides valvares, esses valores foram de 264±126 µg/mg no grupo controle *versus* 94±27 µg/mg nos descelularizados (*P*=0,009).**

Conclusão: **A descelularização com SDS 0,1% foi efetiva como método anticalcificante em condutos de pericárdio bovino implantados em modelo circulatório de ovinos por 180 dias.**

Descritores: **Pericárdio. Bioprótese. Engenharia tecidual. Transplante heterólogo.**

INTRODUCTION

Biological valve prostheses have very attractive characteristics, such as good hemodynamic function, low incidence of thromboembolic complications, even in the absence of anticoagulant therapy and are associated with good functional recovery and quality of life. Nevertheless, its durability is limited, especially in children and young adults [1-4].

Glutaraldehyde (GDA) is used for the fixation of heterologous biological tissues, because it reduces their antigenicity and increases tissue resistance. Moreover, it is effective as a sterilizing agent [5]. However, progressive tissue degeneration, especially due to the occurrence of dystrophic calcification, still represents a major limitation for the clinical use of biological valve prostheses. The mechanisms responsible for calcification are multifactorial [6-9], however, it is known that one major cause is due to electrostatic attraction among circulating calcium, phospholipids present in membranes and cellular debris from fixed tissues [10,11]. In addition, the release of residual aldehyde is cytotoxic, causing inflammatory reactions and working as an initial site of calcium deposition [11].

Several treatments were tested in order to eliminate or retard the calcification, but no method is quite effective yet [12-16]. The decellularization has been proposed as an alternative during the biological tissue processing of cardiovascular prostheses. In theory, the removal of cellular elements does not only reduces the tissue antigenicity, but it also potentially inhibits or slows calcification for eliminating the initial outbreaks of calcium deposits in membranes and cellular debris [17-20].

The decellularization can be performed by physical, chemical or enzymatic processes. Among the most commonly used decellularizing solutions, those based on sodium dodecyl sulfate (SDS), sodium deoxycholate, Triton X100, trypsin and DNase can be mentioned [18].

Our research group from the Pontifical Catholic University of Parana (PUCPR), developed and patented a method of decellularized homograft valve, based on the SDS solution. The experimental and clinical results showed that the grafts became more biocompatible and presented greater resistance to calcification [22,23]. Using the same technology, we could observe that bovine pericardium decellurized and posteriorly fixed with GDA did not calcify when implanted in the subcutaneous cellular tissue (SCT) of rats for periods of up to 90 days (unpublished data).

This work represents the continuation of this research line and aims to assess whether the decellularization with SDS of glutaraldehyde-fixed bovine pericardium with valvular tubes is an effective anticalcification method in a large animal circulatory model.

METHODS

Sixteen juvenile Soulfok sheeps were used, aged 14 ± 2 weeks (min = 12, max = 16) and weight 24 ± 3 kg (min = 20, max = 30), divided into two groups of eight animals each. In the GDA group (control group), the animals were implanted with 0.5% glutaraldehyde-fixed bovine pericardium with valvular tubes, and in the deccellurized group (DESCEL), valvular tubes of bovine pericardium decellularized with 0.1% SDS solution, and, subsequently, fixed with 0.5% glutaraldehyde. In both groups, the valvular tubes were orthotopically implanted in the outflow of the right ventricle, replacing the pulmonary valve and the initial portion of the pulmonary artery trunk. The animals were followed-up through a period of 180 days, when the grafts explantation was performed. This study was approved by the Animal Research Ethics Committee under the protocol No. 263, opinion No. 173/07 CEUA PUCPR on 03/10/07.

Grafts preparation

The bovine pericardia was collected in slaughterhouses

of the metropolitan region of Curitiba and transported to the Cardiovascular Graft Center of the Tissue Engineering and Cell Transplantation Laboratory at PUCPR, in physiological saline solution at 4 °C, where all the pericardial fat has been dissected in laminar flow. After that, they have been immersed in nutrient medium Roswell Park Memorial Institute (RPMI 1640, Sigma®), containing antibiotics (cefoxitin 240 mg / ml, lincomycin 120 mg / ml, polymyxin B 100 mg / ml and vancomycin 50 mg / ml) for 24 hours.

After decontamination, the pericardia of the GDA group have been fixed in a 0.5% glutaraldehyde solution for 72 hours followed by the routines used by the Cardiac Prosthesis routines in tissue preparation for construction of cardiac prostheses. Twenty-one mm diameter tubes were constructed, containing a tricuspid valve in its proximal portion. All the valved conduits were stored in a buffered solution of methyl paraben to the time of implantation. In the DESCEL group, the pericardia were decellularized with 0.1% SDS, according to the echnology developed and patented at PUCPR (Brazil patent application: PI 800603-2). They were then fixed in 0.5% glutaraldehyde, with the rest of the process similar to that of GDA Group. A tissue segment was processed for histological analysis with hematoxylin-eosin for evaluating the efficiency of decellularization.

Surgical technique

Anesthesia was induced with propofol (Diprivan®, Astra Zeneca) at a dose of 4 mg / kg and a 0.6 mg/kg/min maintenance dose through intravenous procedures.

The operations were performed by left thoracotomy in the fourth intercostal space. After opening the pericardium longitudinally, systemic heparinization was performed at a dose of 200 U / kg and the EC was established by cannulation of the descending thoracic aorta and right atrium. The procedures were performed with extracorporeal circulation (EC) at normothermia using pediatric membrane oxygenator (ECO-1, Braile Biomédica®), blood flow from 2 to 2.4 l / min, with a beating heart and without aortic clamping.

After pulmonary artery cross-section, the native pulmonary cusps were resected and the right ventricle outflow reconstructed with implantation of bovine pericardial valvular tube, with 4.0 polypropylene sutures in the proximal and distal portions. Antibiotic prophylaxis was performed with 1 mg ceftiofur / kg and 4 mg / kg gentamicin 12/12h for 5 days and postoperative analgesia with meglumine 1.5 mg / kg flumexil. The animals were kept on Gralha Azul Farm, and, before sacrifice, underwent transthoracic echocardiography study(SONOS 5500, Philips), to assess the valvular mobility and the presence of calcification, as well as the flow velocity measurements by continuous and pulsed Doppler with their respective calculation of transvalvular gradients.

Explant, macroscopic analysis and histological evaluation

The animals were sacrificed on the 180th day of evolution, and the tubes explanted by another lateral thoracotomy. During the procedure, the adhesion intensity around the grafts was observed.

The explanted conduits were macroscopically evaluated and the photographic documentation done †with a Sony Cyber †-Shot ® 5.1 digital camera. The consistency of the conduit walls, the mobility of valvular cuspids, the intensity and distribution of calcification and the presence of thrombi or vegetations. The conduits were also radiologically evaluated (Mammomat C3, Siemens), with axial and sagittal incidences for the observation of calcification spots, its location and extension of calcified area.

The conduits were sectioned longitudinally to obtain three segments containing a valve cusp and its respective segment of the conduit wall. The most calcified segment has been dehydrated at 60°C for 24 h, crushed with a scalpel blade and hydrolyzed with 6N HCl for 72h and referred to LACTEC (Institute for Technology Development - PR) for calcium content by atomic absorption spectrometry with atomization flame (Perkin Elmer, 4100). The amount of calcium was expressed as mg/mg dry weight of tissue analyzed. The second segment was fixed in 10% formalin, embedded in paraffin, cuttting 4 mm thick. The slides were stained with hematoxylin-eosin to assess the presence of inflammatory infiltrate, alizarin red at pH 4.2 and 7.0 for analysis of calcification with calcium phosphate crystals and calcium oxalate, respectively, and Russel-Movat pentachrome, evaluating the architecture of the extracellular matrix. The third segment was stored in case of further analysis.

Statistical analysis

The obtained results were expressed as mean, median, minimum, maximum values and standard deviation. In order to compare the groups regarding quantitative variable, the amount of calcium was considered the nonparametric Mann-Whitney test. P values <0.05 were considered statistically significant. Data were organized into an Excel spreadsheet and analyzed with the computer program Statistica v.8.0.

RESULTS

Clinical evolution

There was no immediate mortality. Four animals (two in each group) died in late development. Two animals died due to bacterial endocarditis in the valved conduit, both in the first month of evolution. One animal died from bleeding on the second postoperative day, and, the other one, secondary to an abdominal infection caused by abomasal

rupture during the 2nd week. These animals underwent necropsy and were excluded from the study. The others had a satisfactory clinical evolution, with adequate weight and height gain, presenting a final weight of 43 ± 5.6 kg (min = 38, max = 49).

Echocardiographic analysis

The echocardiogram results are shown in Table 1. It was not possible an adequate echocardiographic evaluation in three animals of each group, due to the lack of appropriate transducer for the size of the animals.

Macroscopic analysis

There was a significant difference in postoperative adhesions between the groups. In all the animals of the GDA group, the adhesions were intense, with a remarkable inflammatory reaction around the grafts presenting difficulties with their dissection. On the other hand, the adhesions in DESCEL group were loose, with a clearer plan of dissection.

By palpation, the grafts from the GDA group were hardened, especially in the cusp region, while the DECEL ones had soft consistency, and both were being covered by pannus. It was observed that the cusps were very calcified and immobile in GDA group, while calcification was less evident on the walls of the conduits after longitudinal grafting section. In the DESCEL group, the cusps were movable without gross calcifications, and the duct walls were also well preserved (Figure 1). There were no thrombi or vegetation in any case.

These findings were confirmed by radiographic analysis, with intense calcification of the cusps of the GDA group, while the DESCEL group had only calcification spots distributed near the suture lines (Figure 1).

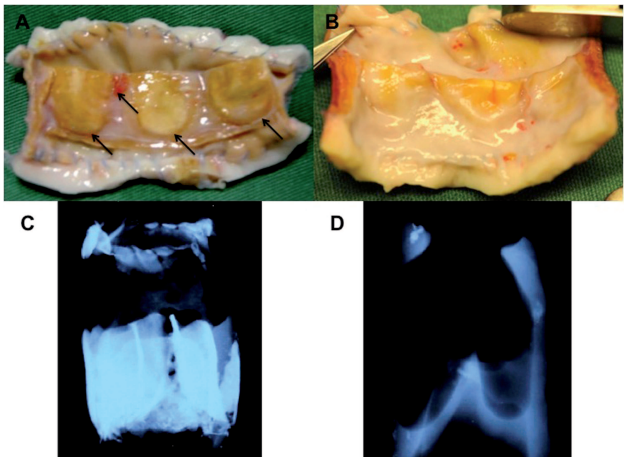

Fig. 1 - Macroscopic analysis. Gross macroscopic and radiographic aspects of the grafts. In A, the GDA valved conduit with calcified cusps (arrows), confirmed by radiography, in C, showing cusp calcification, in B, DESCEL valved conduit with preserved cusps without gross calcification (arrow), and, in D, only calcification spots in suture lines

Histological analysis

HE stain

In the GDA group, either in the cusps or the conduits, the extracellular matrix was disorganized, with areas of necrosis and rupture of collagen fibers. There were chronic inflammatory infiltrate composed of mononuclear leukocytes, phagocytic multinucleated giant cells, lymphocytes, macrophages and histiocytes, suggesting a foreign-body granulomatous reaction. This infiltrate was more evident in its proximal portion and more intense on the adventitial face, where the presence of neovascularization could be noted. In some areas of the

Table 1. Echocardiographic data obtained in late evolution.

Variable	Group	N	Mean	Median	Minimum	Maximum	Standard deviation
Diameter	Control	3	1.7	1.7	1.6	1.9	0.15
	Study	3	1.8	1.8	1.7	1.9	0.11
Δp max	Control	3	58	62.1	41.5	70.9	15.09
	Study	3	30	30.7	23.6	35.8	6.13
Δp mean	Control	3	34	33.8	24.4	45.6	10.62
	Study	3	19	20.5	12.8	23.7	5.60
Max velocity	Control	3	3.7	3.9	3.2	4.2	0.51
	Study	3	2.7	2.7	2.4	2.9	0.28
Mean velocity	Control	3	2.8	2.9	2.3	3.1	0.43
	Study	3	2.0	2.1	1.5	2.3	0.40

Δp max: maximum gradient and Ap min: minimum gradient

conduit, the presence of chondrocytes and fibroblasts with formation of osteoid metaplasia areas in the early stages can easily be observed. In the cusps, the inflammatory infiltrate was more evident in their basal and middle portions, enabling us to visualize large areas of calcification (Figure 2A). There was formation of neointimal layer in the cusps and conduits, consisting of loose connective tissue of probable hematological origin, containing inflammatory cells and focal points of endothelium.

The DESCEL group had the same type of inflammatory infiltrate, however, the extracellular matrix was preserved with less fragmentation of collagen fibers. Unlike the control group, the cusps and conduits showed only focal points of calcification located near the suture lines. The formation of neointimal tissue in this group was more intense than in the control group (Figure 2B).

Russel-Movat pentachrome stain

In the GDA group, there was loss of tinctorial affinity, suggesting that the tissue was necrotic and devitalized. The collagen fibers that were stained yellow proved themselves to be wrinkled and disorganized. The amount of glycosaminoglycans, stained blue or bluish green, was greatly reduced. The neointimal layer was well-characterized, with small amounts of glycosaminoglycans and few inflammatory cells (Figure 2C). On the other hand, the DESCEL group had a higher tinctorial affinity (metachromasia) compared to the control group. The extracellular matrix also showed some degree of disorganization, however, the collagen fibers were aligned in parallel and the elastic fibers were relatively intact with little fragmentation, with higher amount of glycosaminoglycans. The neointimal formation was also intense, with the same characteristics as the control group and more glycosaminoglycans (Figure 2D).

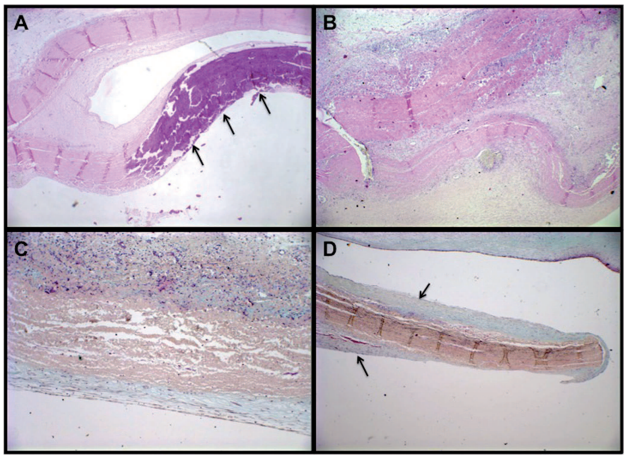

Fig. 2 - Histological analysis. Histologic evaluation of the grafts. In A, photomicrographs of hematoxylin-eosin 40x of GDA group showed degeneration of the cusp (arrow). In B, DESCEL group with the same staining showing inflammatory infiltrate with the matrix preserved. In C, Russel-Movat pentachrome with degenerate matrix and few glycosaminoglycans. In D, DESCEL graft with the same staining, with the matrix preserved and large amount of glycosaminoglycans in intimal hyperplasia (arrows)

Alizarin red-staining pH 4.2 and 7.0

In the GDA group, there was an accentuated deposition of phosphate crystals and calcium oxalate in the valvular cusps and some points of the conduit. On the contrary, these deposits were absent in the DESCEL group, and only a few focal points of calcification along the suture lines could be noted (Figure 3).

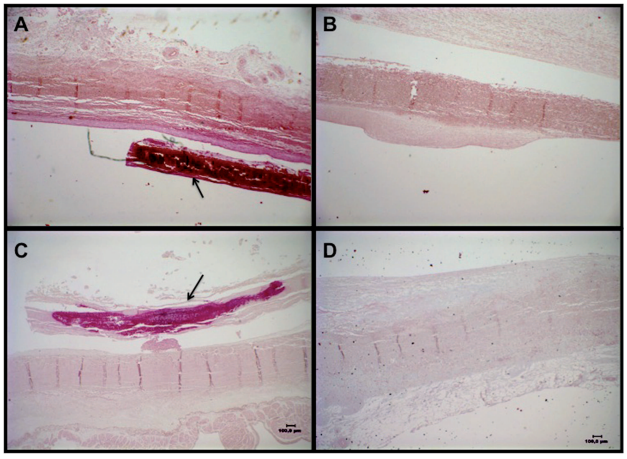

Fig. 3 - Histological analysis. Photomicrographs in alizarin red-40x. In A and B, pH 4.2, and C and D, pH 7.0. In A, GDA group presents cusp calcification (arrow). In B, DESCEL group with no calcification. In C, the GDA Group with calcified cusp (arrow); in D, DESCEL group with no gross calcification

Table 2. Measurement of calcium in valve cusps and and conduits.

Variable	Group	N	Mean	Median	Minimum	Maximum	Standard deviation	*P* Value*
Calcium conduct	GDA	6	35.25	19.93	10.00	119.22	42.13	0.297
	DESCEL	6	15.75	13.75	5.76	34.24	10.44	
Calcium cusps.	GDA	6	264.4	227.94	180.56	517.58	126.16	0.009
	DESCEL	6	94.29	102.23	48.49	120.22	27.05	

** Non-parametric Mann-Whitney test, P<0.05. Values expressed in µg of ca/mg tissue*

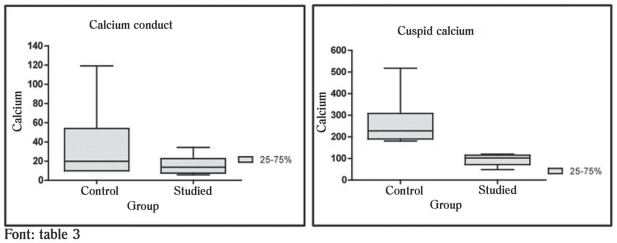

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Fig. 4 - Graphics with amount of calcium measured in the valved conduits and cusps

Quantification of calcium

The values †for the amount of calcium in both groups are detailed in Table 2 and Figure 4. The amount of calcium was higher in the valve cusps than in the conduits, in both groups. Comparatively, there was a reduction of calcium content in the cusps and conduits of the DESCEL group, however, this difference was not statistically significant for the valvular cusps ($P = 0.009$).

DISCUSSION

Biological valve prostheses have several advantages over the mechanical ones, and the indications for its use have increased worldwide in recent years [2]. Despite technological advances in the prostheses design and chemical treatment methods of biological tissues, tissue degeneration due to dystrophic calcification is still problematic. It is well established that the presence of cells and cellular debris facilitate the beginning of the calcification process. Taking into consideration these aspects, the decellularization emerges as a promising alternative to eliminate or retard dystrophic calcification [17,18].

In this study, we could demonstrate the effectiveness of decellularized bovine pericardium with 0.1 % SDS, with complete removal of cells and adequate preservation of the extracellular matrix, confirming the remarks of Oswal et al. [19].

The use of decellularized pericardium resulted in significant differences in macro and microscopic assessments between the two groups analyzed in this study. Due to the explants, we observed that adhesions around the decellularized tubes were less intense than in the GDA group, suggesting that the decellularized tissue was more biocompatible. These findings confirm the observations of Costa et al. [23] with decellularized porcine heterografts implanted in pulmonary position in sheep.

Histologically, the largest cellular tissue with best preservation of the extracellular matrix and absence of calcification also appear to indicate improved biocompatibility of the DESCEL group toward the GDA group, however, this interpretation is limited by the lack of a more detailed immunohistochemical analysis. Moreover, the greatest amount of glycosaminoglycans in the DESCEL group may have been an additional factor in reducing calcification, since they exert protective effects to the matrix for decreasing the impact of stress, as well as modulating the inflammatory reaction [18].

We observed, in both groups, that the calcification was more intense in the valvular cusps than on the conduit walls, confirming the observations of Gabbay et al. [7] and Pires et al. [6] that calcification depends not only on the implantation site, but also on the mechanical stress over the tissue. This was also evident in the radiographic analysis, where we could observe, in the GDA group, more intense concentrations of calcium in the commissural regions and the in leaflet base along to its insertion into the conduit, with patterns similar to those described by Flameng et al. [22] in bovine pericardial bioprosthesis implanted in mitral position of sheep. The decellularization was effective as a anticalcification method in the DESCEL conduit cusps that were radiographically calcium-free, with only a few focal points of calcification in the regions traumatized by the proximal suture lines. According to Veseley et al. [8], the calcification in these regions that suffer more mechanical stress is a consequence of the progressive breakdown of collagen fibers.

The efficiency of anticalcification methods has been varied in the literature, depending on the tissue and experimental model used. Most studies used the implant model in TCSC of rats, which limits its interpretation because it does not assess the influence of bloodstream and tissue stress effects on calcification [12,13,15,16,21]. Our research group evaluated the effect of SDS decellularization in GDA-fixed bovine pericardium and implanted in the TCSC of rats for 90 days, with a reduction of 98% in the final amount of calcium (65.91 μg/mg tissue versus 1.24 μg/mg tissue) (unpublished data). Costa et al. [21] found an absence of calcification in decellularized bovine pericardium implanted in rats for 90 days.

In the literature, there is only one experimental work with a sheep model that used treatment with detergents in bovine pericardium as an anticalcification method, aimed at phospholipid removal[22]. Flameng et al. [22] found values of 1.05 mg calcium/mg tissue in the cusps of biological prostheses with bovine pericardium treated with the nonionic detergent Tween-80 implanted in mitral position of sheep for 5 months. Our study showed that, in the valvular cusps, the amount of calcium was significantly reduced by 65% after 6 months of evolution (GDA Group 264 mg calcium/mg tissue versus DESCEL group 94 mg calcium/mg tissue). It is important to emphasize that, for a methodological limitation, the cusp segments analyzed encompassed the proximal suture line, which certainly contributed to an increase in the final concentration of

calcium. We believe that, if the cusps had been cut along its implantation base, the final levels of calcium in the DESCEL group would have been even lower due to the absence of histological calcification in the valvular cusps.

Despite the fact that the echocardiographic evaluation was limited by the small number of observations, it was shown that GDA group conduits had calcified cusps with a significant reduction of their mobility and high transvalvular gradients. On the other hand, in the DESCEL group, the cusps were movable with a slight thickening, and presenting lower transvalvular gradients in relation to the control group. The presence of moderate obstruction with some elevation gradients in the prostheses morphologically preserved from the DESCEL group was secondary to the "prosthesis/animal disproportion", as a result of significant weight and height gain of the animals that have doubled their weights in 6 months of postoperative evolution.

In conclusion, this study demonstrated, in a circulatiory sheep model, that valvular tubes of decellularized bovine pericardium with 0.1% SDS had better biocompatibility, and their extracellular matrix was better preserved when compared with a conventional glutaraldehyde-fixed valvular tube. The decellularization with 0.1% SDS was effective as an anticalcification method, especially in the valve cusps, which showed more susceptible to degeneration by dystrophic calcification when compared to the conduit wall. Once confirmed that the durability of valvular prostheses constructed from decellularized bovine pericardium is equivalent to those of conventional GDA-fixed bovine pericardium, its clinical application should be considered, and could represent a significant advance, especially for younger patients, where early calcification is still problematic with the current valvular prostheses.

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