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Evaluation of the biological behavior of decellularized pulmonary homografts: an experimental sheep model

Avaliação do comportamento biológico de homoenxertos valvares pulmonares descelularizados: estudo experimental em ovinos

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Abstract

Introduction: **The cryopreserved homograft is a good valve substitute due attributes like excellent hemodynamics, low incidence of thromboembolic events, infection resistance and good mid-term durability. However, progressive homograft degeneration and fibrocalcification may occur, particularly in the childhood and young adults. Their antigenicity triggers an immunological reaction that plays an important role in their degeneration and failure. The decellularization process was proposed to decrease this antigenicity. By the action of detergents and enzymes, this process removes all cellular components from the homograft matrix, diminishing immunogenicity and probably delaying its degeneration.**

Objective: **The objective of this experimental and descriptive study is to evaluate the biological and functional behavior of decellularized pulmonary homografts (Decell-H), treated by a sodium dodecil sulfate solution (0.1%), developed in our University (Pontifícia Universidade Católica do Paraná). For the characterization of Decell-H performance, parameters like recellularization, calcification, and echocardiographic data will be analyzed.**

Methods: **Eight juvenile sheep were submitted to the implantation of the Decell-H sutured into orthotopic position, through a left thoracotomy and with cardiopulmonary bypass support. They were followed-up clinically and by periodical echocardiograms until the explantation, which were performed in different time for**

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every two sheep: seven, 30, 90 and 180 postoperative days. For histological analysis we used Hematoxilin-eosin, Movat and Alizarin-Red staining.

Results: **The sheep reached their follow-up period in a good clinical state. There was no valve regurgitation or stenonis by the echocardiogram. The animals submitted to the explantation in 90 and 180 days had a significant somatic growth and these Decell-H(s) had a diameter increase, without central valve insufficiency. Histologically, all homografts preserved their extra-cellular matrix organization and were progressively recellularized, without calcification.**

Conclusion: **In this experimental model, the Decell-H behaved as an excellent valve substitute.**

Descriptors: **Transplantation, Homologous, Host vs Graft Reaction. Heart Valve Diseases.**

Resumo

Introdução: **Não havendo um substituto valvar ideal, os homoenxertos criopreservados são considerados uma boa opção, pelo excelente perfil hemodinâmico, baixa incidência de tromboembolismo, resistência a infecções e durabilidade a médio prazo. Porém, estão sujeitos à progressiva degeneração, especialmente em crianças e adultos jovens. Sua antigenicidade desencadeia uma resposta imunológica que contribui para sua degeneração, calcificação e falência. Para diminuir esta antigenicidade, desenvolveu-se o processo de descelularização. Pela ação de detergentes e enzimas, este processo remove os componentes celulares do homoenxerto, diminuindo sua imunogenicidade e, provavelmente, retardando sua degeneração.**

Objetivo: **O objetivo deste estudo, experimental e descritivo, é analisar o comportamento histológico e funcional de homoenxertos pulmonares ovinos descelularizados (H-descel) por uma nova solução, composta principalmente de dodecil sulfato de sódio a 0,1% e desenvolvida na PUCPR. Para caracterizar este comportamento, serão avaliados o repovoamento celular, a ocorrência de calcificação e a função valvar ao ecocardiograma.**

Métodos: **A amostra foi constituída de oito ovinos, submetidos ao implante de H-descel em posição ortotópica, através de uma toracotomia esquerda, com auxílio de circulação extracorpórea. Os animais foram acompanhados clinicamente e por ecocardiogramas periódicos até o explante, realizados em prazos predefinidos para cada dois animais: sete, 30, 90 e 180 dias. A análise histológica foi realizada por colorações Hematoxilina-eosina, Pentacrômio de Movat e Alizarina Red.**

Resultados: **Todos os animais sobreviveram ao procedimento e atingiram seus períodos de seguimento. Não houve insuficiência ou estenose destes enxertos ao ecocardiograma. Os animais submetidos aos explantes em 90 e 180 dias tiveram significativos ganhos ponderais e estes H-descel aumentaram de diâmetro, sem desenvolver insuficiência. À histologia, todos mantiveram a organização de sua matriz extracelular, foram progressivamente repovoados e não apresentaram calcificação.**

Conclusão: **Neste modelo experimental, os H-descel mostraram-se excelentes substitutos valvares a médio prazo.**

Descritores: **Transplante Homólogo. Reação Hospedeiro-Enxerto. Doenças das Valvas Cardíacas.**

INTRODUCTION

The use of valve homografts is well established in the surgical treatment of congenital and acquired heart valve diseases. Among the techniques of conservation of homografts, cryopreservation is the most widespread. Described by O'Brien et al. [1], cryopreservation allows longer storage of these grafts and, in some series, is associated with increased durability when compared with other preparation techniques [1]. Still, the grafts are not ideal because they are subject to progressive degeneration, especially in children and young adults [2-5].

The degeneration of homografts is multifactorial, including technical aspects related to the implant, such as distortion, compression of the sternum, under or over sizing, and implant in the anatomic or extra-anatomical position. However, it is well established that homografts trigger an immune response in recipients, and some authors could correlate the intensity of this reaction to the delayed degeneration of the grafts [6,7].

The immune reaction stems from ABO [8,9] and HLA [10-15] systems incompatibility. HLA Class I and II, mainly present in endothelial cells and in interstitial cells of the valves and arterial wall of conduits, trigger donor-specific

immune response, both cellular, with activation of T lymphocytes, such as humoral, with the production of anti-HLA antibodies class I and II [10-12,14,15].

With the advancement of processing technology of biological tissues, the decellularization was proposed as a method to reduce or even eliminate the antigenicity of grafts, and thus improve the late results. This technique was first proposed in 1998 by Bader et al. [16] and subsequently modified by other authors. Among the different methods of decellularization, methods were described using enzymes such as trypsin, and ribonuclease desoxiribonuclease, hypo-or hypertonic solutions, surfactants like sodium dodecyl sulfate (SDS), octilphenotoxietanol (Triton X-100), deoxycholic acid, in addition to ethanol and glycerol [16-20].

In addition to removing all the cells, cellular "debris" and DNA, the method of decellularization should, ideally, preserve the integrity of collagen and elastic fibers of the extracellular matrix, maintaining their biomechanical properties [21-23]. The extracellular matrix intact, not antigenic and without residual cytotoxicity is a prerequisite for biocompatibility and longevity of allografts [24].

Several studies have shown that deccelularized valves can be seeded *in vitro* with different cell types using tissue engineering techniques, as are also repopulated spontaneously *in vivo*, after implantation. Depending on the characteristics of repopulation, the grafts would be remodeled, with regenerative capacity and growth, and could produce all the components of the extracellular matrix, including glycosaminoglycans, collagen and elastic fibers. Such characteristics are found currently only in pulmonary autograft [24-27].

The aim of this study, experimental and descriptive, is to assess the histological and functional behavior of pulmonary homografts from sheep decellularized by a new solution mainly composed of 0.1% sodium dodecyl sulfate developed at PUCPR. To characterize this behavior, the cell repopulation, the occurrence of calcification and valve function on echocardiography will be assessed.

METHODS

Animal model

The sample consisted of eight juvenile Suffolk sheep (*Ovis Aries*), with an average age of four ± 0.5 months (minimum = 3, maximum = 5) and average weight of 22.6 ± 1.9 kg (minimum = 20, maximum = 25). The animals underwent surgery at the Veterinary Hospital of PUCPR and underwent implantation of decellularized homografts in orthotopic position. In addition to clinical exams, the animals underwent serial echocardiograms until the time of explant, these performed with seven, 30, 90 and 180 days postoperatively (two animals in each period).

All procedures followed the protocol for Animal Care "Guide for the Care and Use of Laboratory Animals" (National Academy of Sciences, Washington, DC, 1996) and the Brazilian College of Animal Experimentation (COBEA).

The study was approved by the Ethics Committee on Animal Use (CEUA) of PUCPR (Opinion No. 004.07/CEUA-PUCPR).

Preparation of the graft

The homografts were harvested from juvenile sheep of the same race. The hearts were removed aseptically and transported in isotonic saline solution (at 4°C) to the Center for Cardiovascular Grafts of the Tissue Engineering and Cell Transplantation Laboratory of PUCPR. In continuous laminar flow chamber, the pulmonary valved conduits were dissected and then immersed in a nutrient solution RMPI 1640 ® (Sigma) containing antibiotics (cefoxitin 240 μg/ml, lincomycin 120 μg/ml, polymyxin B and 100μg/ml vancomycin 50 μg/ml) for 24 hours, also at 4°C.

Then, they were decellularized with a solution called PUC I (Brazilian patent required under the number PI0800603-2), consisting predominantly of sodium dodecyl sulfate (SDS) at 0.1%, with the aid of a mechanical shaker (Shaker Table 109M®, Inc. Nova Ética Ltda.). After decellularization, the grafts were preserved in nutrient medium with antibiotics (RPMI 1640®) at 4°C. We performed histological hematoxylin-eosin analyses of a distal segment of each conduit to prove the effectiveness of decellularization process.

Surgical Procedure

Under general anesthesia induced and maintained with propofol and fentanyl, the operations were performed by left thoracotomy in the fourth intercostal space. After systemic anticoagulation using heparin at a dose of 250U/kg, cannulas were introduced into the descending aorta and right atrium for installation of cardiopulmonary bypass performed at normothermia and 2.4 litres/m^2 flow. It was not necessary aortic occlusion, keeping the heart beating.

After proximal and distal occlusion of the pulmonary artery approximately two centimeters of the arterial native conduit and native pulmonary cusps were excised. At this point, the diameters of native pulmonary annulus and grafts rings were measured. The reconstruction was performed with local implantation of the decellularized pulmonary homograft, interposed between the stumps of the pulmonary trunk. The anastomoses were performed using 5.0 polypropylene continuous sutures. With no significant bleeding, cardiopulmonary bypass was discontinued. Reversal of heparin was not necessary. After review of hemostasis and drainage of the left pleural cavity, the chest was closed in anatomic layers. One hour after the operation, the chest drain was removed.

Postoperative care

Analgesia was achieved with flumexil. Aspirin (100mg/day) and dipyridamole (75mg/d) were administered until the time of explant. In the first 24 hours postoperatively, the sheep remained in the Veterinary Hospital and then were sent to the farm from the same institution where they received clinical veterinary follow-up. The animals were also assessed by serial echocardiograms. Each graft was implanted according to the study schedule, or that is, two animals seven days after surgery, two with 30, two with 90 and two 180 days postoperatively.

Removal of homografts

For the removal of homografts studied, it was necessary to sacrifice these animals, performed in the operating room of the veterinary hospital of the institution, with aseptic technique after anesthetic induction with ketamine and halothane maintenance. Through a new left thoracotomy the graft was dissected and isolated. At this point, we assessed both macroscopically and perform photographic documentation. After systemic anticoagulation with heparin, under hypnosis and anesthesia, sheep received intravenous infusion of 40 mEq of potassium chloride to achieve cardiac arrest, allowing the explant the grafts.

Echocardiographic analysis

Echocardiograms were performed by a single observer at a date close to 15, 45, 100 and 170 days postoperatively. With the aid of the continuous and pulsed Doppler, we evaluated the following echocardiographic parameters: flow velocity (m/s), valve gradient (represented by the simplified Bernoulli equation, where: valve gradient= 4.flow velocity ^{2}), valve competence, mobility and thickening of the leaflets, the possible presence of calcifications, vegetations, and thrombi.

Macroscopic analysis

Parameters were assessed, such as adhesion, consistency of the graft, presence and distribution of calcifications, vegetations, thrombi, appearance and mobility of the leaflets and annulus diameter, measured by Hegar candles.

Microscopic analysis

A longitudinal segment of the graft, comprising the proximal and distal anastomoses, the conduit and valve leaflet was fixed with 10% formaldehyde, immersed in paraffin and sliced lengthwise (cuts of 4mm). For histopathological analysis we used stains of hematoxylin-eosin, *Movat's* Pentachrom and Alizarin Red pH 4.2 and 7.0.

RESULTS

Clinical outcome

There was no immediate death and all the sheep reached the predetermined periods of follow-up in good clinical condition, except for one animal (sheep 4), who at the time of euthanasia, 30 days after implantation, presented a case of infective endocarditis with involvement of general condition and weight loss of 16%. The other animals who had undergone explantation in seven and 30 days showed slight changes in body weight. Those already submitted to the explants in longer periods had significant weight gains, with 61 and 71% for the two animals subjected to the explants at 90 days and 73 and 76% for the two animals of 180 days.

Macroscopic analysis

The macroscopic evaluation showed that all grafts maintained their initial characteristics, preserving the elasticity and integrity of the conduits, with no signs of stenosis or dilatation. Regardless of the time of explant, all valves remained thin and with translucent appearance, with normal mobility of leaflets, without macroscopic signs of degeneration or calcification (Figure 1). The exception was the sheep no. 4, who had infective endocarditis of the graft with the presence of vegetation and recent thrombi and slight thickening on the leaflets. In this case, they were also noted discrete calcifications in proximal and distal anastomoses. Despite the subjectivity in analyzing and quantifying the severity of pericardial adhesions on the grafts were similar to those of native structures. Data from the macroscopic analysis are detailed in Table 1.

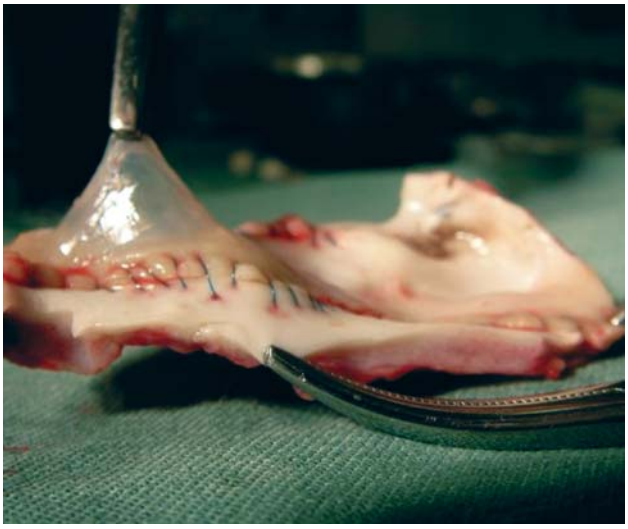

Fig 1 - Photograph showing translucent appearance and integrity of decellularized homograft leaflets explanted after 180 days

Table 1. Macroscopic findings of the explanted decellularized homografts.

Sheep	Time	Adhesions	Thrombi	Calcification	Aspec Leaflets	Mobility	Endocarditis	Observations
1	7	discrete (fibrinous)	small (anast D)	0	Thin/transl	Normal	No	-
2	7	Discrete (fibrinous)	0	0	Thin /transl	Normal	No	Leaflets edema
3	30	mild	0	0	Thin /transl	Normal	No	-
4	30	mild	presente (leaflets)	discrete (anast P/D)	Discrete thickening	Diminuída	Yes	Veget 1mm (leaflets)
5	90	discrete	0	0	Thin /transl	Normal	No	-
6	90	discrete	0	0	Thin /transl	Normal	No	-
7	180	mild	0	0	Thin /transl	Normal	No	-
8	180	discrete	0	0	Thin /transl	Normal	No	-

Time: Time of explant (days); Aspec leaflets: Aspect of the leaflet; Transl: Translucent; Anast D: Anastomosis distal; Anast P/D: Proximal and distal anastomoses; Veget: Vegetation

Table 2. Valve diameters of the native annulus and homograft at the time of implant and explant.

		Implant		Explant	Variation
Sheep	Time (days)	Native annulus (mm)	Graft (mm)	Graft (mm)	(%)
1	7	19	21	21	0
2	7	22	23	23	0
3	30	20	22	22	0
4	30	20	21	21	0
5	90	17	19	23	+15.7
6	90	16	19	23	+15.7
7	180	18	18	24	+33.3
8	180	18	17	24	+38.3

Valve diameter

The mean diameter of the native pulmonary annulus was 18.7 ± 1.9 mm (minimum = 16, maximum= 22), while the mean diameter of the implanted grafts was 20.0 ± 2.0 mm (minimum = 17, maximum = 23), showing the equivalence of graft-recipient ratio. There was no change in the diameter of the grafts explanted after seven and 30 days. However, there was a 15.7% increase in the diameter of the grafts explanted after 90 days. Similarly, there was a mean increase of 35.8% (33.3% and 38.2%) in diameter grafts explanted after 180 days. The valve diameters are detailed in Table 2.

Histological analysis

Hematoxylin-eosin:

Explants of seven days (sheep 1 and 2)

They showed little inflammatory reaction in the periadventitial region consisting predominantly of cells with characteristics of polymorphonuclear cells (neutrophils). The remainder of the arterial conduit's wall was virtually acellular, except for the presence of some cells, also polymorphonuclear, from migration through the anastomosis of the graft lumen. Accompanying this inflammatory activity, we found few monocytes and histiocytes. They could also be observed, rare endothelial cells (one to two cells/field 400X). In this period, there was no neovascularization. The leaflets of these grafts were completely acellular with no signs of inflammation, repopulation, re-endothelialization or neovascularization. There were no signs of thrombosis.

Explants of 30 days (sheep 3 and 4)

At 30 days of evolution, the conduits had already been partially repopulated, especially in the adventitia layer and medium. At this stage, the acute inflammatory reaction was no longer evident, with few polymorphonuclear cells, giving rise to cells with morphology of histiocytes, fibroblasts

and myofibroblasts. The re-endothelialization was also more evident, allowing us to see five to six cells per 400x field.

The homograft removed from the sheep number 3 presented few new vessels, unlike the sheep number 4, with many new vessels in its adventitia, accompanying inflammation secondary to bacterial endocarditis.

The valve leaflets of the sheep number 3 remained virtually acellular. In the sheep four - animal presenting bacterial endocarditis – it was noted an intense inflammatory infiltrate of polymorphonuclear cells and bacteria, probably staphylococci. This inflammatory infiltrate progressed to the conduit, through the insertion of the leaflets.

While there were no signs of graft thrombosis in number 3, there were some thrombi on the leaflets of the sheep number 4.

Explants of 90 days (sheep 5 and 6)

At 90 days, the repopulation of the conduits was abundant and homogeneous, predominantly of cells with characteristics of fibroblasts and myofibroblasts. There were few histiocytes and monocytes. This organized repopulation allowed the differentiation of the adventitia of an intima-media layer. There were many neovessels in adventitia of these conduits. The repopulation of the grafts was complete, even in the leaflets. The repopulation of the leaflets was still limited and is best seen in layer ventricularis and guiding the base to the tip of the leaflets. There were no signs of thrombosis of these grafts.

Explants of 180 days (sheep 7 and 8)

The conduits showed complete repopulation with histological appearance similar to native artery. There were large number of cells with microscopic features suggestive of fibroblasts, myofibroblasts and smooth muscle cells, better organized, allowing the differentiation of the layers of the conduit in adventitia, media and intima. There were many neovessels in adventitia of these conduits.

At 180 days, the valve leaflets have also had significant repopulation in ventricularis, fibrous and spongy layers. The repopulation was also full, with no signs of thrombi.

***Movat's* Pentachrom**

The findings with the Movat staining corroborated with those observed in HE. In all conduits it could be observed the normal organization and integrity of collagen and elastic fibers, regardless of the time of explant. In the leaflets, we identified the preservation of the trilaminar structure of all grafts: fibrous layer - with a predominance of collagen fibers, spongy layer - with the predominance of glycosaminoglycan and ventricularis layers - more cellular.

The only exception was the leaflets of sheep number 4, who had endocarditis due to the total disruption of their structures.

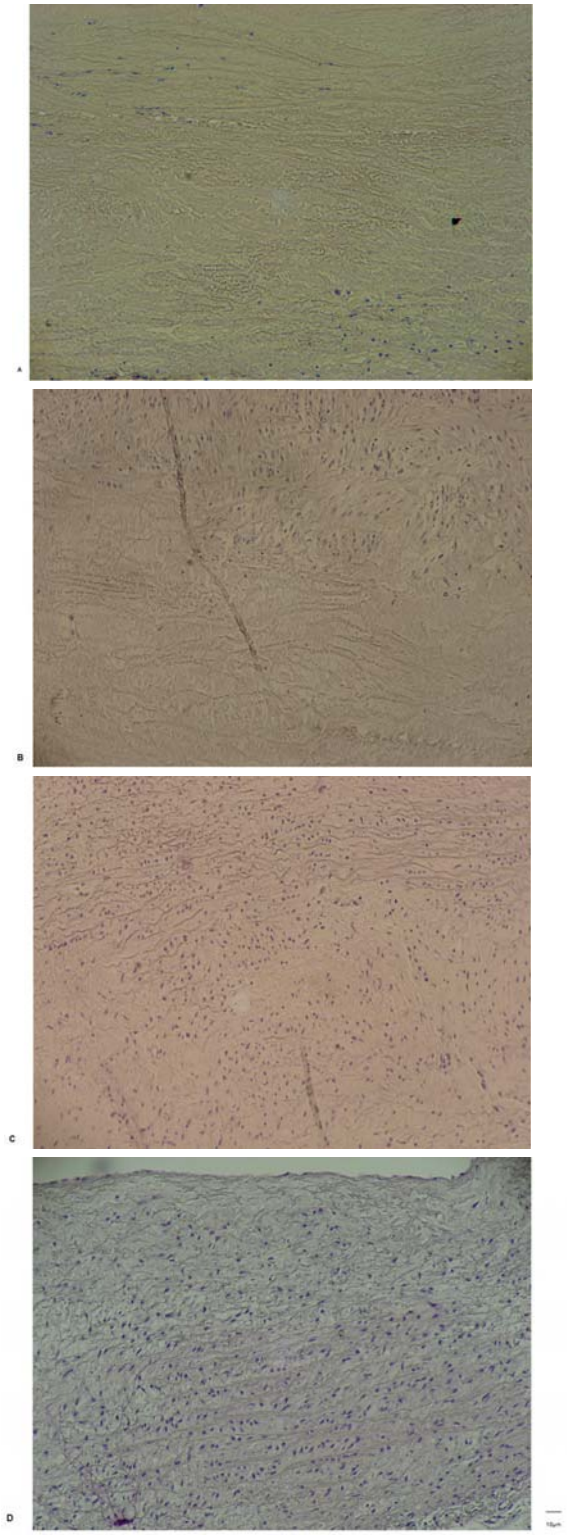

Fig. 2 – Evolution of the repopulation of decellularized homografts from sheep explanted in different periods: A: 7 days - restricted to the adventitia, B: 30 days - reaching half of the conduit, C: 90 days - reaching nearly the entire length of the conduit, D: 180 days - reaching the entire length of the conduit. (all in H.E., 100X

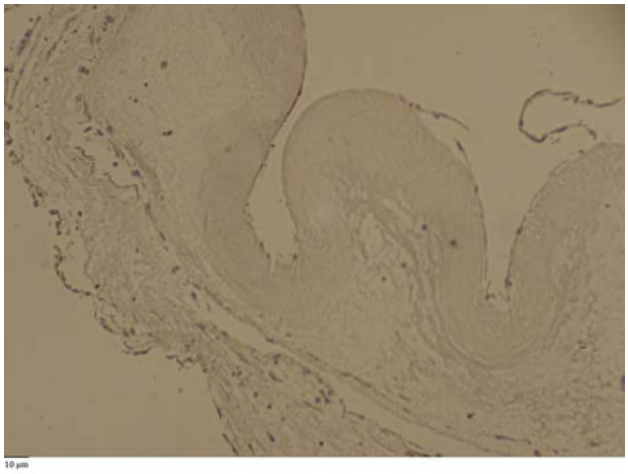

Fig. 3 – Photomicrograph of decellularized homograft explanted after 180 days. Repopulation reaching the distal end of the leaflet. (H.E., 100X)

Alizarin Red 4.2 and 7.0

For these stains it could be noted the absence of calcium both in the conduits and in the valve leaflets, even in animals with 180 days of evolution. Only in sheeps number 3 and 4, we observed the presence of calcium phosphate (HR 4.2) and calcium oxalate (AR 7.0) in small amounts in proximal and distal anastomoses, featuring discrete calcification in these anastomoses (Figures 2-4).

Echocardiographic analysis

The mobility of the valve leaflets remained normal in all examinations throughout the follow-up, with no signs of

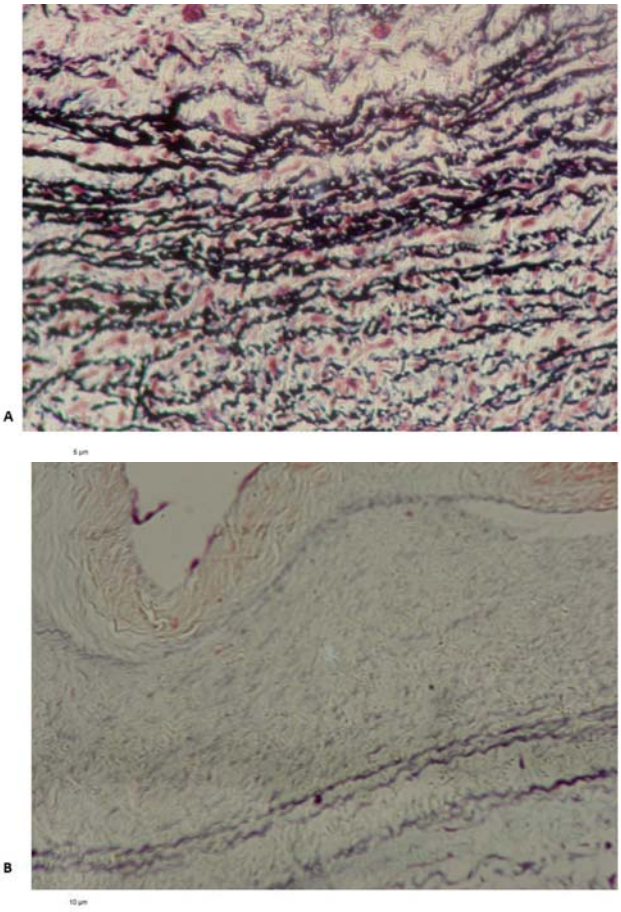

Fig. 4 – Photomicrograph of decellularized homograft explanted after 180 days. A: Intact elastic fibers in the conduit (Russel-Movat, 200X), B: Preserved trilaminar structure of the leaflet (Russel-Movat, 100X).

Table 3. Echocardiographic data of decellularized homografts implanted in the right ventricular outflow tract.

Sheep	Days of P.O.	Max Grad (mmHg)	MeanGrad (mmHg)	Flow Vel (m/s)	Valvar Mobil	Leaflet thick	Pulm Fail	RV Function	Calcific.	Veget.	Thrombi
1	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-
3	14	2.9	2.0	0.86	Normal	0	0	Normal	0	0	0
4	-	-	-	-	-	-	-	-	-	-	-
5	16	3.2	2.2	0.90	Normal	Discrete	0	Normal	0	0	0
	43	5.7	2.9	1.20	Normal	0	0	Normal	0	0	0
6	16	9.6	5.2	1.55	Normal	Discreto	0	Normal	0	0	0
	43	8.1	4.2	1.43	Normal	0	0	Normal	0	0	0
7	7	10.1	6.3	1.59	Normal	0	0	Normal	0	0	0
	99	8.4	5.4	1.45	Normal	0	Minimum	Normal	0	0	0
	167	7.0	4.3	1.33	Normal	0	Minimum	Normal	0	0	0
8	14	3.0	2.0	0.60	Normal	0	0	Normal	0	0	0
	106	6.2	4.3	1.25	Normal	0	Minimum	Normal	0	0	0
	174	4.6	2.8	1.08	Normal	0	Minimum	Normal	0	0	0

PO: postoperative days on which the examination was performed; Grad Max (mmHg): Maximum gradient in millimeters of mercury; Grad Med (mmHg): Mean gradient in millimeters of mercury; Fl Vel (m/s): Flow velocity in meters per second; Mobil.: Mobility, Leaflet thick: thickening of the leaflets; Pulm fail: pulmonary failure, RV: Right ventricle; Calcif: Calcification; Veget.: Vegetations

thickening or calcification. The flow velocities, as well as the mean and instantaneous maximum gradients were consistently low, close to normal. There was also no significant changes in sequential tests, even after 180 days of evolution. Sheeps 7 and 8 showed minimal valve insufficiency in the echocardiograms of 100 and 170 days. In all tests, the right ventricular function was normal. There were no echocardiographic evidence of thrombi, vegetations and calcification. The summary of echocardiographic findings can be found in Table 3.

DISCUSSION

The ideal valve substitute should allow cell viability, maintaining the potential for remodeling and regeneration of the extracellular matrix with growth capacity and preventing progressive tissue degeneration [27]. Currently, only the pulmonary autograft has these characteristics [28].

Although cryopreserved homografts presenting cell viability, the donor cells are precisely those that trigger an immune response by the receiver, mainly related to HLA and ABO incompatibility, which is related to the delayed degeneration [10 to 13.15]. In an attempt to improve results with the use of homografts, Bader et al. [16] proposed the method of decellularization as a way to eliminate or minimize this immune response.

Because this technology is new, there are still many controversies regarding the best method of decellularization, regarding the use of homologous or heterologous matrices, and the need of seeding *in vitro* these grafts with cells seeded in the laboratory.

Our research is directed towards the use of homologous matrices, in view of unfavorable results of heterologous matrices published by Simon et al. [29] and also due to the possibility of transmission of retroviruses to humans [30].

The experimental model in young sheep is well established for the test of valve substitutes, because the pathophysiology of degeneration and calcification is similar to humans, but to a more rapid [31]. It is also possible to compare different bioprostheses, with lower costs than other animal models, and easily reproducible [32]. It is important to note that the assessment of new valve substitutes in sheep in experimental models is a mandatory pre-clinical study, according to the U.S. Food and Drug Administration Replacement Heart Valve Guidance [33].

The histological findings of this study showed that the grafts removed after seven days showed an acute inflammatory infiltrate, consisting mainly of polymorphonuclear cells (neutrophils), mainly located in the adventitia and around the anastomosis. Similar results were described by Elkins et al. [26] using the Synergraft® technology, where the initial and transitory inflammatory infiltrate has not resulted in degeneration of the graft and was important for the chemotaxis of other cells, preparing the graft to the future cell repopulation.

The transience of the acute inflammatory reaction can be detected at 30 days, when the neutrophils were replaced by more mature cells with morphology of histiocytes, fibroblasts and myofibroblasts. During this period, the cell repopulation had already reached the middle layer of the conduit and we observed many neovessels in the adventitia of the graft.

The grafts explanted after 90 days showed a repopulation abundant and homogeneous, even up to the intima of the conduit. The predominant cells had characteristics highly suggestive of fibroblasts and myofibroblasts progressively organized, allowing us to identify an intima-media layer and an adventitial layer, the latter with many vessels. As the valve leaflets of the grafts explanted at seven and 30 days were virtually acellular, after 90 days we observed the onset of repopulation of the ventricularis layer, progressing from the base to end of these leaflets. These findings were very similar to those published by Elkins et al. [23], which showed substantial repopulation of the conduit and an initial layer ventricularis repopulation of the valve leaflets.

After 180 days, the grafts showed a repopulation complete and organized similarly to a native valved conduit. There was a predominance of mature cells, enabling differentiation of three layers of the conduit: adventitia, media and intima. In the adventitia, we found many new vessels and, in the media, muscle cells. While the repopulation of the leaflet from one of the sheep (no. 7) reached less than half of its length, the other sheep (no. 8) reached more than half of the leaflet. These findings are also similar to those described by Elkins et al. [23]. Dohmen et al. [27], in an experimental study in sheep receiving decellularized heterografts with deoxycholic acid, also describe that even six months after the repopulation of the leaflet had not reached its end.

The process of re-endothelialization was gradual, being more evident after 90 days. Although we observed complete re-endothelialization of the conduits, the distal portion of the leaflets still had a few flaws of endothelialization in some animals, even after 180 days. Numata et al. [34] found a reendothelization most uniform in the same period, including the valve leaflets in an experimental model in pigs that received cryopreserved homografts. Moreover, even studies using seeding *in vitro* with endothelial cells of decellularized homografts were unable to prove the reendothelization of the leaflet's end [24].

The presence of a confluent layer of endothelial cells is essential in preserving the subendothelial matrix and to prevent thrombosis due to exposure of basement membrane collagen after decellularization [24]. This may be an argument for seeding of endothelial cells cultured in the

laboratory prior to implantation. Moreover, in our study there was no evidence of thrombosis of the grafts.

Except for sheep no. 4, who had infective endocarditis, all other animals showed clinical improvement. Those who underwent explantation in longer periods, 90 and 180 days, had significant weight gains. Accompanying these weight gains, the decellularized homografts showed an increase in their diameter of 15.7% in 90 days and up to 38.2% in 180 days. However, we can not say whether this increase was proportional to that expected for this animal, due to the lack of a control group. In an experimental study in sheep, Lopes et al. [35] compared the biological behavior of heterografts decellularized by deoxycholic acid (1%) with cryopreserved homografts after 180 days. As in our study, the authors show that the decellularized heterografts showed a significant increase in their diameter valves, unlike conventional cryopreserved homografts, which have maintained their original diameter. Cebotari et al. [36] reconstructed the RVOT of children with homografts decellularized with trypsin-EDTA and seeded *in vitro* with autologous endothelial cells, and also showed an increase in the diameter of these grafts after three years of follow-up with the maintenance of low gradients and valve competence, suggesting the remodeling and growth.

It can be argued that the increase in diameter is due to pathological dilatation of the grafts, however, some evidence supports the real growth:

1. Macroscopic analysis of the grafts showed no signs of thinning of the conduit wall, with normal coaptation of the leaflets.

2. The valve diameters increased, apparently in proportion to the weight gains of animals.

3. The echocardiogram revealed no significant valve insufficiency.

4. Microscopic analysis showed an orderly repopulation. We also observe the integrity of collagen and elastic fibers in addition to their normal organization.

By Silvermann-Movat and Russel-Movat stainings, we could confirm the integrity of collagen and elastic fibers, respectively, in all grafts. This suggests that the method of decellularization used did not compromise the structure of the extracellular matrix, and that these cells were able to remodel the graft appropriately.

As Steinhoff et al. [24], no calcification was observed either in conduit or the valve leaflets. These authors, however, describe calcifications in subvalvar muscle tissue near the proximal anastomosis, in all grafts, with three months postoperatively. It is common to observe calcifications in vascular anastomoses, but these findings may be due to an incomplete decellularization or by some degree of structural impairment due to the process, since the authors used an enzyme (trypsin) as decellularizing agent, as already mentioned by Dohmen et al. [27] as an inferior method for altering the collagen matrix.

Serial echocardiography showed low flow velocity and transvalvular gradients and not increased in serial examinations. The preservation of the mobility of the leaflets and valvular function in the medium term corroborates the hypothesis regarding the viability of cryopreserved homografts.

The biomechanical stability achieved in these homografts after the decellularization process is critical for its function and surgical safety. The loss of tissue stability, which may accompany the decellularization process, was not significant in this study, which used tissues from thin wall, under the pulmonary pressure and in orthotopic position. The use in aortic or heterotopic position may be more critical, requiring further studies in these conditions.

CONCLUSIONS

Decellularized homografts from sheep were gradually repopulated by recipient cells and this repopulation was organized and functional, with no signs of degeneration or calcification. Serial echocardiography showed the maintenance of valve function after 180 days, with low gradients and minimal valve insufficiency. There was an increase in the diameter of these grafts, apparently following the growth of the animal. The cellular organization and preservation of the integrity of elastic and collagen fibers, as evidenced by histology, and the maintenance of good valvar function, evidenced by echocardiography, suggest an excellent biological behavior, with appropriate remodeling.

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