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Decellularized heterografts versus cryopreserved homografts: experimental study in sheep model

Análise do comportamento biológico de heteroenxertos descelularizados e homoenxertos criopreservados: estudo em ovinos

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Abstract

Objectives: **The aim of this study is to assess the biological behaviour of porcine decellularized heterografts (Desc group) compared with cryopreserved homografts (Crio group) implanted in juvenile sheep.**

Methods: **Decellularized porcine pulmonary heterografts were implanted in five animals and cryopreserved pulmonary homografts in another five. The animals were followed-up for a mean of 280 ± 14 days. The valve diameter was measured by echocardiography, which was performed at the 30th postoperative day, and before the explantation. The valves were also assessed macroscopically. Histological evaluation was performed using H.E., Gomori and Weigert staining. Immunohistochemistry specified different cell types (Factor VIII, CD3, Vimentin and CD68). Calcium quantity was analyzed using atomic absortion spectometry.**

Results: **There was one death in the Desc group due to endocarditis. The valves of Crio group showed decrease in the cellularity whereas the valves of Desc group showed matrix repopulation with endothelial and interstitial cells. Loss of collagen density and disarrangement of the normal fiber architecture was observed in Crio group. Calcium content demonstrated higher levels on the cusps and conduits in Crio group comparatively with Desc group. (*P*=0.016). The mean valvular diameter at the explantation was significantly increased (*P*=0.025) in the Desc group.**

Conclusions: **Decellularized heterografts had a different biological behaviour when compared to cryopreserved homografts and become repopulated by cells with fibroblasts and endothelial cells characteristics. The matrix was preserved and some regenerative potential was present**

Descriptors: **Transplantation, homologous. Transplantation, heterologous. Cryopreservation. Tissue engeneering.**

Resumo

Objetivo: **Este estudo avalia o comportamento biológico dos heteroenxertos porcinos descelularizados (Grupo Desc) comparados com os homoenxertos criopreservados (Grupo Crio) implantados em carneiros jovens.**

Métodos: **Foram implantados em cinco animais heteroenxertos pulmonares porcinos descelularizados e em outros cinco, homoenxertos pulmonares criopreservados. Os animais apresentaram seguimento médio de 280 ± 14 dias. O diâmetro valvar foi medido por ecocardiografia, a qual foi realizada no 30º pós-operatório e antes do explante. As valvas foram também avaliadas macroscopicamente. A avaliação histológica foi realizada utilizando-se coloração de H.E., Gomori e Weigert e imunohistoquímica (Fator VIII, CD3, Vimentina e CD68). A quantificação de cálcio foi realizada utilizando-se espectrometria de absorção atômica.**

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Resultados: **Houve um óbito no Grupo Desc por endocardite. As valvas do Grupo Crio apresentaram decréscimo na celularidade, enquanto que as valvas do Grupo Desc demonstraram repovoamento da matriz com células endoteliais e intersticiais. No grupo Crio, observou-se perda na densidade e desarranjo da arquitetura das fibras colágenas. A espectrometria de absorção atômica demonstrou maior calcificação no conduto e nas cúspides dos enxertos criopreservados quando comparados aos descelularizados (*P*=0,016). O diâmetro médio valvar no explante foi significantemente maior no Grupo Desc (*P*=0,025).**

Conclusão: **Heteroenxertos descelularizados apresentam um comportamento biológico diferente quando comparados aos homoenxertos criopreservados, tornando-se repovoados por células com características de fibroblastos e células endoteliais. A matriz permaneceu bem preservada, o que possibilitou um processo de regeneração celular.**

Descritores: **Transplante homólogo. Transplante heterólogo. Criopreservação. Engenharia de tecidos.**

INTRODUCTION

The cryopreserved grafts are frequently used to treat different types of heart valve diseases and various types of complex congenital heart diseases. In such cases the homografts present a better hemodynamic profile, low incidence of thromboembolism and greater resistance to infection, however, its durability is limited, especially in children and young patients [1]. There are evidences that homografts failure is caused, - at least in part - by immunological reactions caused by cellular elements remaining in the homograft's matrix, that trigger immune reaction by the receiver. The endothelial cells and fibroblasts in the homograft are able to express Class I and II histocompatibility complex, that are recognized by the receiver's immune system and can cause tissue degeneration and graft failure [2].

The decellularization of homologous or heterologous matrices are promising techniques to prevent the graft failure induced by immune response. Recent studies suggest that decellularized heterografts lead to a reduced inflammatory response by the receiver and are able to be progressively repopulated by autologous cells. These factors allow, at least in theory, improvement of late survival of the grafts implanted [3-5].

Different methods of decellularization were proposed, among them the use of detergents such as Triton X-100, sodium dodecyl sulfate and sodium deoxycholate, among others. The main purpose of decellularization is to remove all cellular components, by maintaining intact the main structural elements of the extracellular matrix. The maintenance of collagen matrix integrity is important to provide a suitable environment for cell migration and enable the tissue reorganization [6,7]. Previous studies indicate that the process of remodeling the extracellular matrix is directly related to the regenerative capacity and the valves's growth potential [8].

The aim of this study is to analyze the biological behavior and growth potential of porcine decellularized pulmonary heterografts by using sodium deoxycholate and ethanol compared to conventional cryopreserved homografts implanted in the right ventricle outflow tract of young sheep.

METHODS

This study consists of a comparative study of cryopreserved homografts and decellularized heterografts implanted in orthotopic position in the right ventricle outflow tract of of sheep by using cardiopulmonary bypass. We selected ten young sheeps aged between 14 to 18 weeks (16.6 ± 1.1) and weighing between 33 and 41kg (37.1 ± 2.5), which were divided into two groups of 5 animals, according with the type of valve implanted. The first group (Desc Group) consisted of five animals, which received decellularized homografts, and in the second group (Crio Group) cryopreserved homografts were implanted. The animals were clinically followed-up under echocardiographic exam and the valves explanted after a mean period of follow-up of 280 days. The experimental study was approved by the Ethics Committee on Animal Research - PUC-PR, under the research protocol No. 913/03.

Porcine decellularized heterografts

The heterografts were harvested from young pigs weighing between 25 and 30 kg. The hearts were aseptically obtained and pulmonary conduits were dissected in laminar flow, by maintaining a small segment of subvalvar myocardium. The internal diameter of pulmonary conduits was measured with Hegar's dilator and the valves with 17 mm diameter were selected. The valves were then maintained in Hanks solution (Sigma Chemical, St. Louis, MO, USA) with antibiotics (penicillin 100U/ml^{-1}, 100µg/mL-1 streptomycin and 250ng/mL^{-1} amphotericin B) at 4°C, between 5 to 7 days to ensure the grafts sterility. The

decellularization of porcine pulmonary valves was performed using a 1% sodium deoxycholate, for 24 hours under continuous agitation, and 70% ethanol for 30 minutes (Auto Tissue^{GmbH}). The valves were then maintained at Roswell Park Memorial Institute medium (RPMI 1640, Sigma) until the date of implantation.

Cryopreserved homografts

The cryopreserved homografts were harvested from young sheep weighing between 25 and 30 kg. The hearts were collected and dissected in the same conditions described above and five valves with internal diameter of 17mm were selected. The valves were maintained in Hanks medium (Sigma Chemical, St. Louis, MO, USA) with the same antibiotics solution described previously, for 48 hours. Then the valves were washed in a 0.9% saline solution and maintained in RPMI medium with 10% dimethylsulfoxide (Gibco ® cod.:102270-106) and cryopreserved in a programmable freezer (Planer III, KRYO 10 series) with freezing gradient of -1ºC/min up to -80°. The valves were then maintained at a -150ºC temperature in mechanical storage freezer (Sanyo, Ultra-low temperature).

The valves thawing before implantation was performed using 0.9% saline solution at 42-50°C, using gradual dilution of RPMI and fetal calf serum (10%).

Surgical technique

All animals received a dose of 0.5 mg/kg of valium (Diazepam®, Nova química-Sigma Pharma) intravenously as anesthetic premedication. Then, cardiac and invasive pressure monitoring were performed, in addition to anesthetic induction with 4mg/kg of propophol (Diprivan®, Aztra Zeneca) and maintenance anesthesia with propophol (0.6 mg/kg/min). Mechanical ventilation was performed in a volume respirator - Oxigel 1722 (Oxigel Mats. Hosps. Ind. e Com Ltda).

The animals were positioned in right lateral decubitus position and, under sterile conditions, left lateral thoracotomy, exposure of the pericardium and full heparinization with intravenous dose of 250U/kg were performed. After, the right atrium and descending thoracic aorta were cannulated and normothermic cardiopulmonary bypass - with the aorta opened – was established. The pulmonary artery trunk was transsectioned and native leaflets were removed. The grafts were then orthotopically implanted, by performing the distal and proximal suture line using 4-0 prolene. Cardiopulmonary bypass was discontinued and after appropriate hemostasis the chest was closed through layer suturing technique. The thoracic drain was removed one hour after the procedure.

After surgery, the animals were referred to the Veterinary Hospital PUC-PR. 1.5 mg/kg of flunixin meglumine (Banamine®, Shering Plow) was used as analgesic agent. As antibiotic prophylaxis 1mg/kg of Ceftiofur (Ceftiofur Exenel®, Pfizer) and gentamicin (Gentamicin® Neo-chemical) 4mg/kg q 12/12h were used for a period of 5 days. The control transthoracic echocardiography (Philips SONOS 5500 system) was performed on the 30rd postoperative day and before the explantation in all cases. The transvalvular gradient and pulmonary systolic diameter in longitudinal sectioning of the pulmonary artery were obtained. Echocardiography was also used to estimate the valve function, mobility and thickening of the leaflets.

Valve explantation and analysis

The animals were sacrificed nine months after surgery. The procedures were performed using general anesthesia and under aseptic conditions. The valves were resected including the distal muscle portion of the right ventricle outflow tract and a distal segment of the native pulmonary artery.

Macroscopic analysis was performed at the time of explantation for detection of thrombus, valve rupture or calcification. Then, each graft was longitudinally divided into three segments along its commissures, each one containing a leaflet and a corresponding segment of the valve wall. The first segment was sent for analysis of calcium and the other two for histological and immunohistochemistry analysis.

Atomic absorption spectrometry was performed to quantify the calcium content in the valve. The specimens removed from the conduit and leaflet were lyophilized, pulverized and separated into samples of equal weight. Thereafter, hydrolysis was performed in 6N HCl for 8h. The calcium content was measured by atomic absorption (Perkin Elmer, 4100).

For microscopic examination the specimens were divided longitudinally in the middle of the pulmonary leaflet after embedded in paraffin. Segments of 4µ in thickness were stained with hematoxylin and eosin (HE) for characterization of the cellular distribution and they were also submitted to Weigert staining - for analysis of elastic fibers - and Gomori for analysis of collagen fibers.

The immunohistochemical studies included staining for factor VIII (Dako-cod. 0082) for identification of endothelial cells, CD3 for characterization of T lymphocytes (Dako-cod. A0452) and CD68 (Dako-cod. M0725) for identification of interstitial mesenchymal cells.

Statistical analysis

The comparison of the calcium contents, the valve diameter and mean valve gradient between the two groups was performed by Mann-Whitney test, used for analysis of non-parametric values. The *P*d"0.05 value was considered as statistically significant.

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RESULTS

All animals survived the surgery and presented good evolution in the immediate postoperative period. There was one late death in Desc Group on the 40th postoperative day due to endocarditis. All remaining animals showed satisfactory clinical evolution until the time of explantation. The valves in both groups were explanted after a mean time of 280 ± 14 days. In Crio Group, the postoperative mean weight increased from 36±2.7kg to 62.7±3.3kg at the time of explantation. In Desc Group, the mean preoperative weight was 38±2.23 kg and in the explantation was of 60.2±2.58kg.

Valve macroscopic morphology

The morphological inspection of the valves in both groups showed no evidence of thrombus or rupture. Calcification at the suture line was present in all valves in both groups. In Crio Group, two conduits presented focal points of calcification and another conduit presented rough calcification. In Desc Group only one conduit showed focal point of calcification.

Although calcification was not observed in the leaflets in both groups, the leaflets in Crio Group were more thick (Figure 1) when compared with the Desc Group (Figure 2), however, with no signs of deformation, shrinkage or collapse.

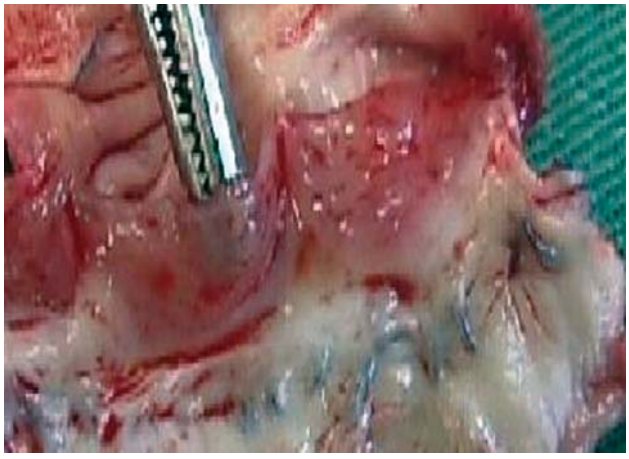

Fig. 1 - Macroscopic analysis of the cryopreserved homograft leaflet. Photo showing thickening of the cryopreserved homograft leaflet explanted with 9 months

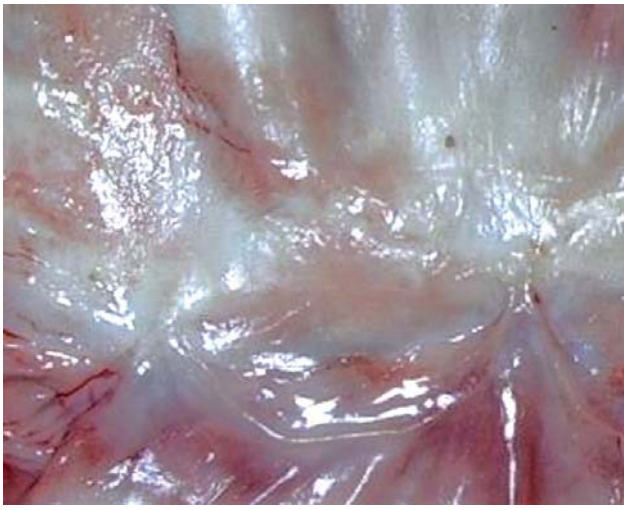

Fig. 2 – Macroscopic analysis of decellularized heterograft leaflet. Photo showing absence of thickening of the decellularized heterograft leaflet explanted with 9 months

Histology and immunohistochemistry

Cryopreserved homografts (Crio Group)

Conduits

Electronic microscopy showed loss of cellularity and trilaminate structure of the pulmonary conduit and basement membrane was not identified. The collagen and elastic fibers presented fragmented and with focal calcification (Figure 3). Multifocal areas of necrosis and calcification were observed in the middle layer of the valve wall.

There was predominance of inflammatory cells in the adventitia layer of conduit next to the suture line on the donor-recipient interface. This inflammatory infiltrate was predominantly lymphocytic (CD3+) with cell rate by micrometer square of 13.81 ± 4.80, located mainly in the adventitia, extending up to the middle layer. It was also identified macrophages and monocytes through specific marker CD68 in the proportion of 3.00 ± 1.53 cells/μm^2 and interstitial cells were not identified with characteristics of fibroblasts (Figure 3). The valves presented endothelial layer on its surface, not homogeneous, characterized by immunostaining for Factor VIII (Figure 3). The distribution of endothelial cells in the valve surface was 1.39 ± 1.06 cells/μm^2.

Leaflet

Electronic microscopy showed changes of the normal architecture of the collagen fibers, with structural damage and rupture of the fibers. The elastic fibers presented fragmented and without the usual laminar architecture. There was also loss of the trilaminate leaflet structure.

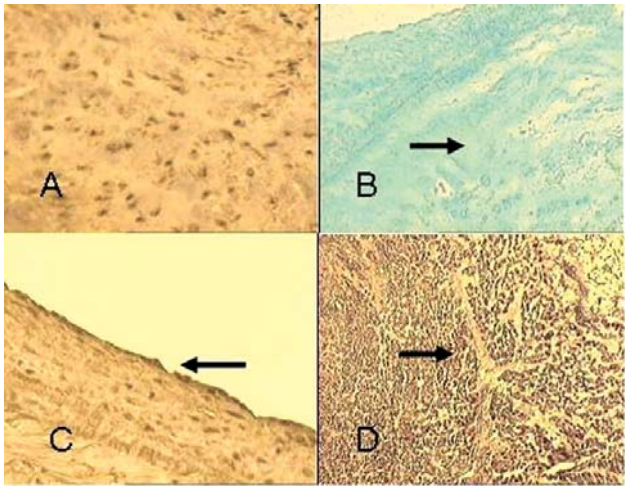

Fig. 3 - Histological analysis by optical microscopy of the cryopreserved grafts. Photomicrographs of histological suturing of the pulmonary conduit, decellularized heterograft, at 40x magnification. A: Immunohistochemistry for vimentin; it is noted presence of cells with fibroblasts characteristics. B: Gomori staining shows intact collagen fibers. C: Immunohistochemistry for factor VIII, showing endothelial cells. D: Weigert staining; it is noted presence of intact elastic fibers

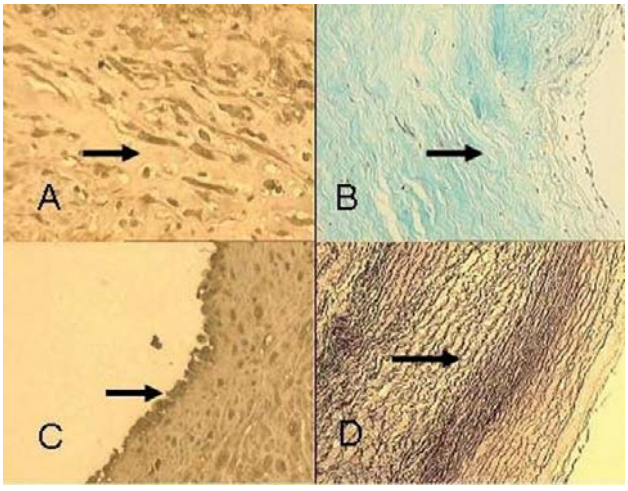

Fig. 4 - Histological analysis by optical microscopy of the decellularized grafts. Photomicrographs of histological suturing of the pulmonary conduit, cryopreserved homograft, at 40x magnification. A: Immunohistochemistry for vimentin, absence of interstitial cells with fibroblasts characteristics. B: Gomori staining shows degeneration of collagen fibers. C: Immunohistochemistry for factor VIII; endothelial cells are shown. D: Weigert staining with presence of rupted elastic fiber

The leaflets showed significant reduction of cellularity by staining with H&E. The interstitium showed few mesenchymal cells with morphology of fibroblasts, at a ratio of 0.20 $\pm$ 0.07 cells/μm^2 and presence of inflammatory cells positive for CD3, at a ratio of 4.34 $\pm$ 1.51 cells/μm^2. By means of factor VIII, endothelial cells with focal distribution at a ratio of 0.72 $\pm$ 0.42 cells/μm^2 were noted.

Decellularized heterograft (Desc Group)

Conduit

The middle layer of the valve was predominantly repopulated by interstitial cells with characteristics of fibroblasts, with distribution of 3.19 $\pm$ 1.26 cells/μm^2 (Figure 4A).

The Gomori staining showed appropriate preservation of the extracellular matrix structure. The collagen fibers presented well preserved without evidence of increased interfibrilar spaces (Figure 4B). The Weigert staining showed no distortion or fragmentation of elastic fibers (Figure 4D).

Near the adventitia of the conduit was noted lymphocytes with scattered distribution 4.40 $\pm$ 0.16 cells/μm^2 and monocytes surrounding blood vessels at the ratio of 7.83 $\pm$ 4.69 cells/μm^2.

In the conduit surface were found endothelial cells of focal distribution of 6.97 $\pm$ 3.96 cells/μm^2 under staining for factor VIII (Figure 4C).

Leaflet

The optical microscopy showed maintenance of normal trilaminate structure of the valve. Pattern of cell migration from the base of the leaflet towards the free margin was noted. In the leaflet was noted presence of interstitial cells - predominantly mesenchymal cells - immunomarked with Vimentin, with morphology for fibroblasts and distribution of 0.91 $\pm$ 0.36 cells/μm^2. The cell population in the leaflet was also characterized by the presence of inflammatory cells at the rate of macrophages (CD68): 2.24 $\pm$ 1.34 cells/μm^2 and lymphocytes (CD3): 1.26 $\pm$ 0.27 cells/μm^2.

The base of the leaflet showed greater amount of interstitial cells as compared to middle and distal portion. In the middle portion of the leaflet, there was a predominance of inflammatory cells, characterized by monocytes and lymphocytes.

The distal portion of the leaflet was almost acellular and the few cells found in this area were tipically inflammatory cells with characteristics for granulocytes, representing an initial non-specific inflammatory reaction.

Atomic absorption spectrometry

The atomic absorption spectrometry showed high content of calcium in Crio Group when compared to Desc Group. The arterial wall of the cryopreserved grafts showed a mean calcium content of 1.59 $\pm$ 1.02 µg/mg, while the decellularized group presented calcium content of 0.26 $\pm$

0.15 µg/mg (*P*= 0.016) (Figure 5). Leaflets in Crio Group presented calcium content of 1.66 ± 0.49 µg/mg, whereas in the Desc Group it was of 0.49±0.30µg/mg (*P*=0.016) (Figure 6).

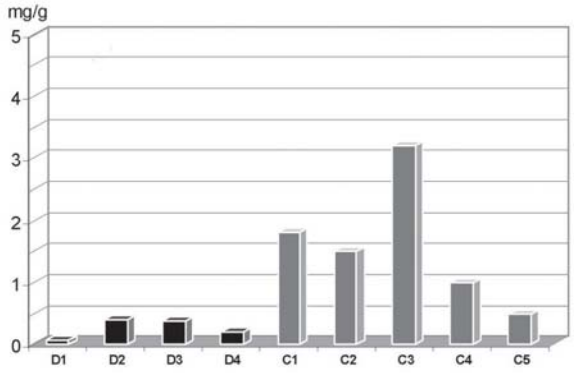

Figure 5 – Calcium quantification in the decellularized heterografts conduits (D) and cryopreserved homografts (C). Graphic demonstration of the calcium content of the valve conduits of Crio Group (C) and Desc Group (D), P = 0.014

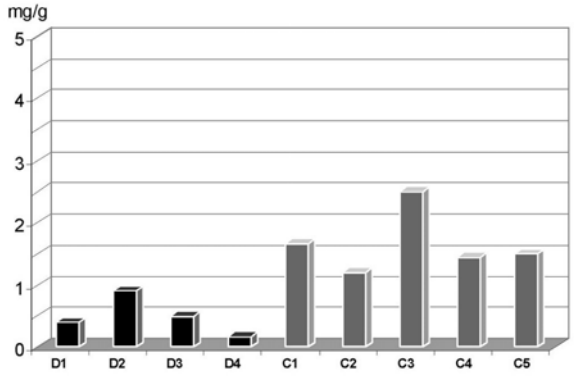

Fig. 6 – Calcium quantification in the decellularized heterografts leaflets (D) and cryopreserved homografts (C). Graphic demonstration of the calcium content in the valve leaflets of Crio Group (C) and Desc Group (D), P = 0.016

Echocardiography

There was no significant pulmonary insufficiency in any of the groups. In Desc Group, morphology and mobility of the leaflets were unchanged. In two animals of Crio group was noted small thickening of the leaflets. Regarding the valve diameter after 8 months, was found in Crio Group a mean diameter of 22.5 ± 1.4 mm, while in Desc Group a mean diameter of 27.7 ± 2.1 mm (*P* = 0.025). The mean transvalvular gradient after 8 months in Crio Group and Desc Group was, respectively, 2.24 ± 0.97 and 2.56 ± 0.85 (*P*> 0.05).

DISCUSSION

The ideal heart valve must have appropriate hemodynamic profile, low incidence of thromboembolism and appropriate durability. The homografts present better hemodynamic profile when compared to conventional valve replacement, however, they still present limited durability, especially in young patients. Moreover, it is known that homografts present no capacity for growth, which is important especially in the pediatric population. Despite cryopreserved homografts are viable structures, they quickly lose their cellularity after implantation and do not contribute to tissue renewal. Indeed, the cellular elements present in homografts can be considered detrimental because they are antigenic and awake the immune response by the receiver [9]. The pulmonary self-graft is currently the only valve replacement with regenerative and growth capacity, however, Ross surgery is technically complex and performed in few capable centers.

Several groups described the effectiveness of the decellularization technique aiming at reducing the antigenicity of the valve homografts [10]. It also was shown that these acellular matrices were able to be repopulated *in vivo* by receptor cells after implantation. Although controversial, the use of decellularized heterografts is a very promising technique due to low availability of human tissues.

Several methods to remove the cells from heterograft and homografts have been proposed, with various levels of effectiveness with regard to decellularization and maintenance of the matrix structure. Grauss et al. [7] showed that changes in formation of extracellular matrix after chemical decellularization of the graft could lead to problems in the cell repopulation by cells of host origin, mainly depending on the method used for decellularization. The use of sodium deoxycholate was previously reported as a suitable method for decellularization of porcine pulmonary and aortic valves [11]. In this study, the cell extraction by using sodium deoxycholate resulted in no significant structural changes in configuration of the collagen fibers of the extracellular matrix, which is important to offer a suitable environment for the *in vivo* process of remodeling of the valve. Scebacher et al. [12] showed that, in addition to an effective graft decellularization, maintaining biomechanical properties of the matrix has fundamental importance for the valve durability.

The structural changes occurring in cryopreserved homografts after *in vivo* implantation have largely been approached in literature. Koolbergen et al. [13] showed that during the first year after implantation, an important reducing in valve cellularity occurs and, after a year, the tissue valve is almost acellular. In this study, we confirmed these notices, however, we found that the valve

endothelium was well preserved on the surface of most of the cryopreserved valves. Other studies showed that the capacity of reendothelization in sheeps is greater than that observed in humans. Jonas et al. [14] compared microscopically fresh and decellularized homografts implanted in pulmonary position of young sheeps and found in both groups the persistence of the endothelium in covering the valve leaflet.

In this study, we also found large numbers of lymphocytes infiltrating the leaflets and the conduits of the cryopreserved homografts when compared with decellularized heterografts. Neves et al. [15] showed in an experimental study, that the lymphocytic infiltrate in homografts persisted more than 2 years after implantation. The authors proposed that the inflammatory infiltrate would be part of the immune reaction caused by the graft, which would lead to a decline in the interstitial cells and subsequent histological changes.

In contrast, in the decellularized group, we found a process of gradual repopulation by interstitial mesenchymal cells, with a pattern of cell migration of the conduit toward the leaflet. The cell population at the base of the leaflet was characterized as interstitial cells, in greater quantity when compared to the middle and distal portion of the leaflet. In contrast, in the distal portion of the leaflet was found a larger population of inflammatory cells. This suggests that the base of conduit represents an older stage of the cellular remodeling process and indicates a pattern of cell migration toward the free margin of the leaflet. Elkins et al. [16] also described the process of cellular remodeling in an experimental study with decellularized porcine valves. The authors suggested that the middle and distal portion of the leaflet represent a more recent response to cellular remodeling, where a population of mononuclear inflammatory cells are activated.

Another important aspect is the presence of endothelial cells on the surface of the decellularized grafts, scattered in islands - and sometimes confluent. The monolayer of endothelial cells have the function of preserving the protein components of the subendothelial matrix, in addition to prevent the fibrous proliferation on the graft surface. The absence of an antithrombogenic endothelial surface exposes the matrix to the blood and increases the risk of thrombosis [17].

Steinhoff et al. [5] suggest that an active reorganization of the tissue in a well preserved matrix is an important prerequisite for the growth potential of the grafts. Our study showed a significant difference between groups, regarding the diameter of the pulmonary grafts obtained by echocardiography in the eighth month after implantation. One possible hypothesis to explain this difference in diameter between the groups could be the dilation of the graft wall. Grauss et al. [7] showed loss of glycosaminoglycans after the chemical decellularization process with Triton X-100 - specifically loss of chondroitin sulphate. This component is located between the layers of collagen fibers of the matrix and has the function of reducing the stress between layers and prevents the deformation and dilation of the valve. In this study, we could not confirm the hypothesis of the valve dilation, since the graft wall showed no decrease in thickness at the time of explant when compared to the native valve. Also, in echocardiographic findings central failure of the graft was not found, which would be expected in the case of valve dilation.

In conclusion, the decellularized heterografts present different biological behavior when compared to cryopreserved homografts. They became partially repopulated by endothelial and interstitial cells of host origin and the extracellular matrix remained well preserved, allowing appropriate cellular remodeling process. Although decellularized grafts have shown greater diameter when compared to cryopreserved grafts, other studies - including electronic microscopy for evaluation of the matrix and greater sampling - are still required to assess the potential growth of decellularized grafts.

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