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In vivo endothelialization of cardiac bioprostheses: conventional versus non-aldehyde preservation

Endotelização in vivo das biopróteses cardíacas: preservação convencional versus não-aldeídica

Vinicius José da Silva NINA, Pablo Maria Alberto POMERANTZEFF, Ivan Sérgio Joviano CASAGRANDE*, David CHUNG*, Carlos Manuel de Almeida BRANDÃO, Sérgio Antonio Barbosa do NASCIMENTO, Luiz Alberto BENVENUTI, Sérgio Almeida de OLIVEIRA

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Abstract

Objective: **A protective layer of endothelial cells (EC) of host origin on the entire surface of bioprosthetic heart valves has never been reported. Current commercial bioprosthetic heart valves are commonly preserved in glutaraldehyde (GA) and are cytotoxic to host cells preventing spontaneous endothelialization. The aim of this study is to demonstrate the potential for *in vivo* endothelialization of heart valves treated by the L.-Hydro™ preservation process.**

Method: **L-Hydro™ preservation process consists of mild extraction of antigenic substances by the action of polyethyleneglycol and incorporation of an anti-inflammatory and a anti-thrombotic agent. Seven stented porcine valves treated by the L-Hydro™ process and three GA-fixed porcine valves were implanted in the mitral position of juvenile sheep. The valves were evaluated by echocardiography and angiography prior to sacrifice at five months. Recovered valves were also histologically and histochemically evaluated.**

Results: **There were no hemodynamic differences between the groups. However, scanning and transmission electron microscopy showed a nearly complete coverage of EC on the surfaces of all leaflets in the L-Hydro™ treated valves. The EC were in direct contact with the underlying collagen layer and expressed von Willebrand-related antigens (vW).**

The surfaces of the GA-treated valves were covered by fibrin deposition, macrophages, calcium and thrombotic material. Only sparse EC were observed and contact of the EC where the underlying tissue was incomplete.

Conclusion: **These data indicate that L-Hydro™ treated porcine valve tissues are capable of inducing spontaneous endothelialization with evidence of strong cell attachment of the new endothelium to the collagen matrix.**

***Descriptors*: Bioprosthesis. Heart valve prosthesis. glutaraldehyde.**

Work performed in the Heart Institute, Hospital das Clínicas, Medical School of the University of São Paulo, SP, Brazil and *The International Heart Institute of Montana, Missoula, MT, USA.

Correspondence address:
Vinicius José da Silva Nina
Rua Sebastião Archer, 101,Olho D´Água
CEP: 65065-480 São Luís-MA Tel: (98) 248-0320
Fax: (98) 232-8700 E-mail: rvnina@aol.com

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Resumo

Objetivo: **O revestimento endotelial in vitro das biopróteses com células do hospedeiro parece ter ação protetora contra a calcificação, trombose, inflamação e o desgaste mecânico. O objetivo deste estudo é analisar o potencial para endotelização in vivo com um processo alternativo de preservação tecidual (L-Hydro™).**

Método: **A preservação L-Hydro consiste na extração controlada de substâncias antigênicas pela ação do polietilenoglicol e na incorporação de um agente antiinflamatório e antitrombótico. Para testar a re-endotelização in vivo, foram implantadas em posição mitral de ovelhas jovens sete próteses porcinas L-Hydro (grupo teste) e três convencionais preservadas com glutaraldeído (GA - grupo controle). Estas próteses foram explantadas com 150 dias após avaliação ecocardiográfica e angiográfica. A avaliação histológica consistiu em microscopia de varredura e transmissão, e imuno-histoquímica (von Willebrand) para detecção da presença e viabilidade das células endoteliais, respectivamente. Utilizou-se o teste-t não pareado para análise estatística.**

Resultados: **Não houve diferença hemodinâmica significativa nos dois grupos (p>0.05). Entretanto, a microscopia mostrou no grupo teste um revestimento endotelial quase completo formado por células confluentes, viáveis com expressão do fator vW, as quais encontravam-se em contato direto com a matriz colagênica subjacente. No grupo controle (GA), as superfícies valvulares estavam recobertas por fibrina, macrófagos, cálcio, material trombótico e células endoteliais esparsas com expressão fraca do fator vW, e com pouco contato direto com o colágeno.**

Conclusões: **Estes dados indicam que o processo L-Hydro™ permite endotelização espontânea com boa adesividade celular à matriz colagênica, o que favoreceria maior durabilidade às biopróteses porcinas.**

Descritores: **Bioprótese. Prótese das valvas cardíacas. Glutaral.**

INTRODUCTION

Over the years it has been demonstrated that the incapacity of valvular prostheses to reconstitute a viable cardiac tissue seems to be the main determinant factor of thrombogenicity, inflammation and a limited long-term durability [1].

Faced with this situation, the preservation of the structure and natural function of the tissues has gained importance and is one of the greatest aims in research and in the development of future valvar substitutes [2].

Previous studies demonstrated that cell extraction and the removal of cellular debris from the inside the valvar cuspids substantially reduce the antigenicity and dispense with the necessity of fixation using glutaraldehyde (GA) [3,4]. And, that the maintenance of the integrity of the matrix can be assured by the possibility of re-endothelialization by host cells. [5-8]

There are no published reports of complete spontaneous endothelialization of the valvar leaflets after their implantation with the utilization of the conventional methods of tissue preservation.

This study reports on the potential of *in vivo* re-endothelialization of stented porcine valvular prostheses treated by a new non-aldehydic process of tissue preservation whose main agent of tissue stabilization is polyethyleneglycol.

METHOD

Preparation of the prostheses

The porcine valves were obtained from an abattoir approved by the Ministry of Agriculture and the Public Health Inspectors. After being mounted on a stent, the prostheses were preserved using the L-Hydro ™ process (Philogenesis Inc., Monrovia, USA), which consists of three distinct stages as described in general terms as follows (patent applied for):

1st Stage: Extraction of the porcine antigens and masking of the remaining antigens under controlled chemical oxidation using polyethyleneglycol acid;

2nd Stage: Incorporation of a non-steroidal anti-inflammatory agent (similar to aspirin) and an antithrombotic agent (similar to heparin) to the valvar tissue.

3rd Stage: Sterilization of the tissue in hydrogen peroxide solution (H_2O_2).

Concluding the preservation process, the prostheses were stored in a solution of 50% ethanol until use.

Implantation in animals

The experiments in animals were performed after approval of the Ethics Committee on Scientific Research of the Medical School of the University of São Paulo and following the norms of the American Association for Accreditation of Laboratory Animal Care. [9]

The sample consisted of 10 Santa Inês sheep with ages ranging from 4 to 6 months old and body weights of 25 to 35 kg.

The animals were divided in two groups:

1) Study Group: 7 animals implanted with non-aldehydic porcine bioprostheses (L-Hydro).

2) Control Group: 3 animals implanted with conventional porcine bioprostheses preserved in GA.

The prostheses were 25 mm in diameter and were implanted in the position of the mitral valve by left lateral thoracotomy in the 4th intercostal space utilizing cardiopulmonary bypass (CPB) with hypothermic anoxic arrest induced by ventricular fibrillation. The operation was concluded with pleural drainage and closure of the cavity by planes. The animals were maintained alive for 150 days.

Hemodynamic performance

The hemodynamic performance was compared between the two groups using 2-dimensional Doppler echocardiography with an ATL Ultramark 6 echocardiograph (Philips, Drachten, Netherlands). Using echocardiography the mean transvalvar gradient, the valvar area, the mobility and competence of the leaflets and the degree of valvar regurgitation were determined.

The hemodynamic evaluation was completed by angiography at death. Right and left catheterizations were performed through the jugular and right carotid veins employing Swan-Ganz and Pig-Tail catheters respectively (Baxter Healthcare Corporation, Irvine, USA). A ventriculogram was made, central venous, systemic arterial and pulmonary blood pressures were taken and the heart outflow was measured by thermodilution for each animal.

Radiologic evaluation

After explantation of the valves, a macroscopic study of the foci of calcification of the valvar leaflets by the mammographic technique utilizing a Senographe DMR mammograph (GE, Buc, France) was performed.

Histologic and immunohistochemical evaluation

The explanted prostheses were fixed in histo-choice (Amresco Inc. Solon, USA) and cut in fragments that included the base, the middle portion and the free margin of the leaflets, which were embedded in paraffin, sectioned in 4 micrometer-slices and stained using hematoxylin-eosin (HE) and von Koosa stain. The slides were examined using an Olympus CBA optical microscope (Olympus, Tokyo, Japan).

The valvar ultra-structure was analyzed by transmission electronic microscopy using an EM 301 electronic microscope (Philips, Drachten, Netherlands).

The investigation of endothelial repopulation was performed by scanning electronic microscopy, in which the specimens were dehydrated with acetone to the carbon dioxide critical point, coated with a gold compound and examined in a 240 Stereoscam (Cambridge Instruments, Cambridge, UK).

The study of the endothelial cellular activity was achieved by immunohistochemical staining for factor VIII (Factor von Willebrand Policlonal Dako, Grostrup, Denmark).

Statistics

The quantitative data were expressed as means and standard error of the means. A comparison between the groups was made using the non-matched Student t-test. The qualitative data were described by absolute and relative frequencies and compared between the groups by the Fisher exact test. Statistical significance was established for p-values < 0.05.

Data was processed and statistical analysis was made utilizing GraphPad InStat 3.0 for Windows 95 (GraphPad Software, San Diego, USA).

The supposition of normal distribution and equal variances were tested.

RESULTS

Echocardiographic and hemodynamic evaluation

The 2-dimensional echocardiography demonstrated that the prostheses functioned normally without detectable regurgitation, except in 1 control case in which there was a reduction in the mobility of the leaflets secondary to extensive calcification.

There was no statistical difference (p-value > 0.05) between the two groups in respect to the transvalvar gradients, valvar area, pulmonary and systemic pressures and the cardiac outflow (Table 1).

Table 1. Means and standard errors of the echocardiographic and hemodynamic measurements in the two groups

Variable	Study	Control	p-value
Peak gradient (mmHg)	6 ± 1.30	4.3 ± 0.88	0.40
Mean gradient (mmHg)	3 ± 1.04	2.33 ± 0.33	0.65
Valvar area (cm^2)	2.5 ± 0.34	1.66 ± 0.33	0.17
MAP (mmHg)	88.9 ± 9.75	85 ± 11,38	0.82
PAP (mmHg)	23.8 ± 2.37	41.3 ± 8.20	0.12
PCP (mmHg)	12.8 ± 1.74	34.8 ± 7.90	0.06
CO (L/min)	5 ± 0.70	5.2 ± 0.85	0.82

cm^2 = SQUARE centimeters
CO = cardiac outflow
mmHg = millimeters of mercury
p-value = level described for the non-matched student t test for different variables
PAP = pulmonary arterial pressure
MAP = mean arterial pressure
PCP = pulmonary capillary pressure

Macroscopy

The valves of the study group did not present with endocarditis, thrombosis, hematomas, abrasions or structural lesions. In just one case, a small commissural tear was observed. In the three controls there were thrombi organized on the atrial surface with extensive areas of calcification and hardening of the leaflets. This was not evidence of this on the study valves.

Radiological evaluation

There was no calcification detected by the mammographic technique on the valves preserved by the

L-Hydro™ process. With the three control valves (GA) there were foci of calcification.

Optic macroscopy

Staining by HE demonstrated a layer of confluent endothelial cells lining 70% of the surface of the leaflets of the non-aldehydic (L-Hydro) valves, which were in direct contact with the subjacent collagen matrix. For the Control Group leaflets (GA) the covering of endothelial cells was restricted to only the base of the leaflets and in some areas there was no direct contact with the collagen due to the interposition of fibrin, mononuclear cells, calcium and thrombotic material (Figure 2). Staining by von Kossa confirmed calcification in this group. This staining was negative in the valves of the study group.

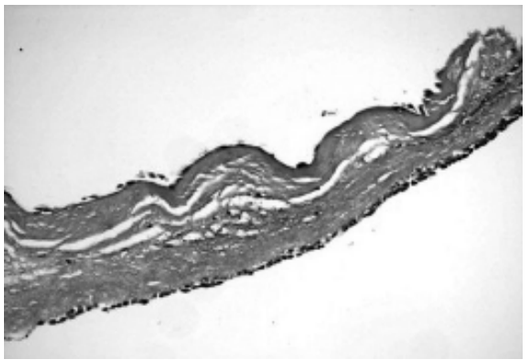

Fig. 1 – Optical microscopy of a non-aldehydic valve (L-Hydro). Staining by HE. The valvar surface is recovered by endothelial cells and fibroblasts are incorporated in the valvar matrix (magnification 10x)

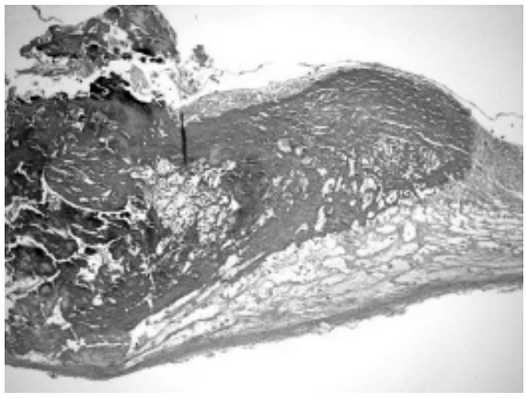

Fig. 2 – Optical microscopy of a conventional valve (GA). Staining by HE. Note the absence of cell repopulation of the matrix surface (magnification 10x)

Scanning Microscopy

The valves treated using the L-Hydro process demonstrated a cell lining confluent with the morphology typical of endothelium (diamond-shaped cells) (Figure 3).

The conventionally treated valves (GA) showed large areas formed by fibrils of nude collagen and sparse endothelial cells present only at the base of the leaflets (Figure 4).

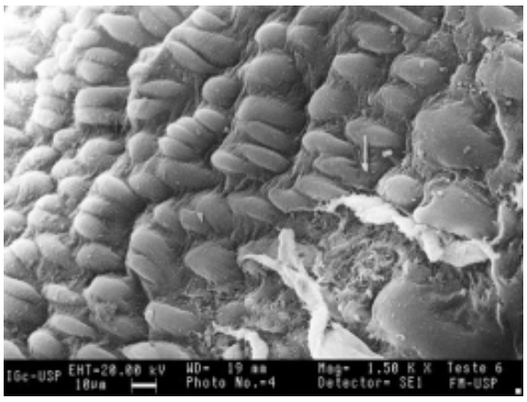

Fig. 3 – Scanning electronic microscopy of a non-aldehydic valve (L-Hydro). Layer of confluent endothelial cells (magnification 1500x)

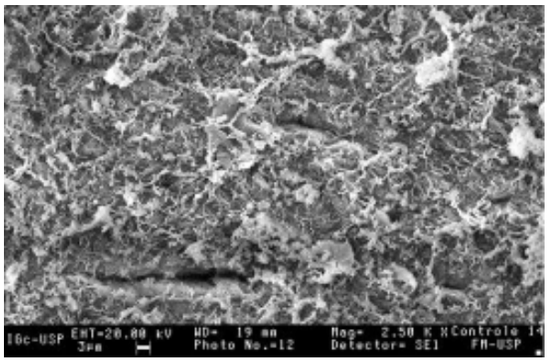

Fig. 4 – Scanning electronic microscopy of a conventional valve (GA). Absence of endothelial cells – nude collagen (magnification 2500x)

Transmission electronic microscopy

In the study group (L-Hydro) repopulation of cells of collagen by viable fibroblasts was observed, which was not seen in the control group (Figure 5).

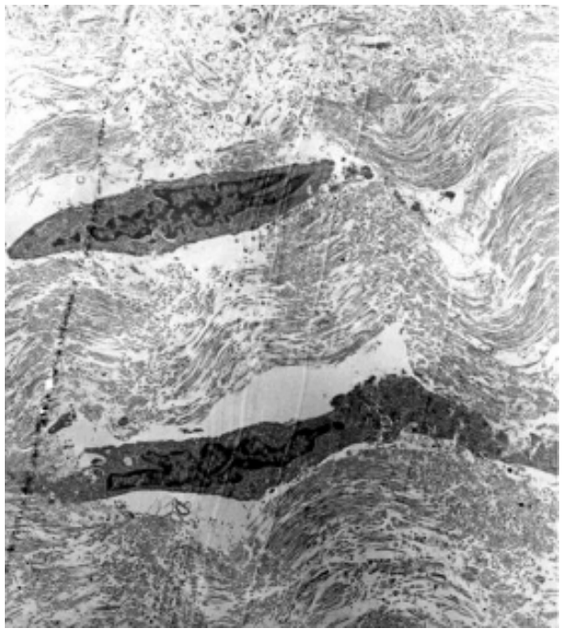

Fig. 5 – Transmission electronic microscopy of a non-aldehydic valve (L-Hydro). Viable fibroblasts incorporated into the collagen matrix (magnification 28,000x)

Immunohistochemistry

Expression of the factor VIII (von Willebrand) confirmed the findings of the staining by HE in the study group (L-Hydro), demonstrating that the cellular lining was composed of viable endothelial cells (Figure 6).

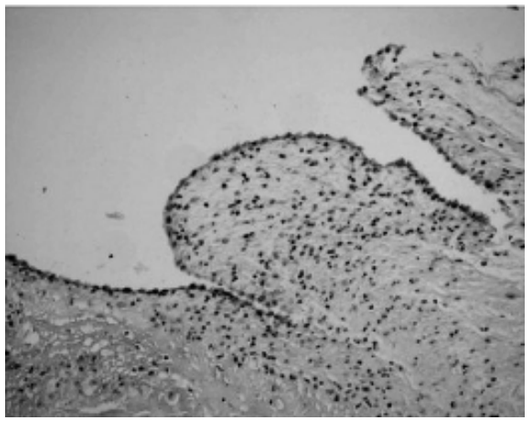

Fig. 6 – Immunohistochemical staining of a non-aldehydic (L-Hydro) valve. Expression of the endothelial viability of polyclonal factor VIII (magnification 20x)

There was a weak expression of factor VIII in the control group.

The histologic findings 150 days after implantation are summarized in Table 2.

Table 2. Absolute and relative frequencies of the histologic findings 150 days after valve implantation

Variable	Category	Study	Control	p-value
Re-endothelialization	Yes	7(100.0%)	0(0.0%)	0.008
	No	0(0.0%)	3(100.0%)	
Thrombosis	Yes	0(0.0%)	3(100.0%)	0.008
	No	7(100.0%)	0(0.0%)	
Calcification	Yes	1(14.3%)	3(100.0%)	0.033
	No	6(25.7%)	0(0.0%)	

p-value = level described for the Fisher exact test

COMMENTS

The main disadvantage of heterologous bioprostheses its limited durability due to tissue degeneration, which occurs, at least in part, as the result of an immunogenic reaction to the xenogenic tissue mediated by the cytotoxin of the glutaraldehyde [10].

Glutaraldehyde reduces the relaxation and increases the hardness of the valvar leaflets leading to an abnormal mechanical functioning of the valvular prostheses, which results in local tissue damage that is predisposed to thrombogenicity [11-13]. Currently, it is believed that the development of a layer of endothelial cells has a protecting action against these tissue injuries assuring a greater resistance and durability [14,15].

However, the endothelial growth is only possible if the valvar leaflets are processed utilizing an atoxic method of tissue preservation, as the aldehydes continue to be toxic against endothelial cells in concentrations of as low as three parts per million thereby impeding the conventional biological valves acquiring an endothelial lining [16].

ISHIHARA et al. [17] demonstrated that only 23% of the biological valves explanted in a period of 12 to 60 months presented with any endothelial coating and even then restricted to the base of the leaflets.

This incapacity of endothelial repopulation of the valvar leaflets predisposes them to insudation by proteins and salts that, eventually leads to calcification [18].

Published studies have demonstrated that the repopulation of the surface and the collagen matrix with

autologous endothelial cells can confer a potential for regeneration and growth of the valvar tissue transforming it into a live structure [5,6,8,12,14]. In a recent preliminary clinical study using a porcine pulmonary prosthesis obtained by tissue engineering for the reconstruction of the left ventricle outflow tract, DOHMEN et al. [19] also suggested the potential benefits of *in vitro* valvar endothelialization when a non-aldehydic matrix is utilized.

In this study the non-aldehydic preserved valves are more resistant to thrombosis, calcification, inflammation and to mechanical wear. The calcification was more evident in the group treated with GA, which was responsible for the reduction in the mobility of the leaflets whose hemodynamic repercussion was translated by the elevation, although not significant, of the mean pulmonary and pulmonary capillary blood pressures. This finding is consistent with the study by SCHOEN et al. [20], who utilized the same experimental model (sheep) over a similar period (150 days).

The re-endothelialization was not observed in the control group (GA), except for 'pannus' at the base of the leaflets, with little contact with the subjacent collagen matrix and without a viable expression of factor VIII. On the contrary, in the study group (L-Hydro) the valves demonstrated a potential of spontaneous endothelialization with excellent adhesiveness and cellular viability similar to those described for valves treated by enzymatic decellularization or by photo-oxidation [21].

The endothelialization observed in the current study was favored by the preservation using L-Hydro™ whose principal agent of tissue stabilization is polyethyleneglycol (PEG). PEG is produced by the heterogeneous catalytic polymerization of ethylene oxide monomers and is solubility in water, cetones, glycerol and ethanol; however, it is less hygroscopic and resists decomposition better in these last two compounds. Its toxicity is reduced greatly as it is an inert chemical; and its excretion, when in contact with the blood circulation, is total through the kidney without being metabolized, which guarantees its clinical application as a vehicle to dissolve drugs that are little soluble in water such as reserpin and nitrofurantoin or those easily hydrolysable such as alkaline barbiturates [22].

The lower toxicity of PEG was demonstrated by WICOMB et al. [23], who reported that with the acute administration of high doses in animals of as much as 16 g/kg over 12 to 16 hours, adverse effects were not detected and that the long-term administration was equally innocuous.

PEG was efficacious in several *in vitro* experimental studies when added to a myocardial protection solution, guaranteeing the functional viability of the organ for longer periods (up to 24 hours) than in conventional cardioplegia solutions such as Saint Thomas and the one from the University of Wisconsin (from four to six hours). This greater capacity of preservation is due to the osmotic action of PEG which stabilizes the membrane making it more permeable to extracellular solutes and consequently preventing cellular edema [24].

The immunosuppressant property which is fundamental to L-Hydro™ preservation utilized in this study is also attributed to PEG. It has been demonstrated that antigens that link to PEG, manifest a reduction of their antigenicity; as was reported in a study by COLLINS et al. [25], in which a reduction of 30% in the incidence of rejection in a group of heart transplantation recipients was observed when the donor hearts were preserved in a solution containing 5% PEG. In a subsequent study by TOKUNAGA et al. [26], a statistically significant increase in the survival (11.9 vs. 9.6 days) was demonstrated in liver transplanted rats whose organs were previously irrigated with solutions of high molecular weight PEG (20,000 Daltons). For those authors this immunosuppressant action occurs due to the binding of PEG with the lipids of the antigen's cellular membrane forming reversible complexes, which alter or mask the cellular surface of these antigens in a manner analogous to what was described for specific chemical combinations with allergens. That is, it interferes with the activation of the macrophages and consequently with the activation of the $T\text{-}_{Helper}$ cells, inducing the state of tolerance of the donor's antigens, whose immunogenicity is reduced. Similarly, in L-Hydro™ preservation some porcine antigens are extracted and the remaining antigens are masked by the controlled chemical oxidation of PEG.

The current study, as well as others which utilized the same animal model, in the evaluation of new technology for tissue fixing/stabilizing, have the limitation of not being able to reproduce the effect of heart disease and the profile of comparable coagulation of humans [7,12,19]. However, endothelialization as a mechanism to minimize of delay the structural degeneration is a real possibility, as long as the valvar tissue can be preserved by an agent with the characteristics of PEG making it favorable to repopulation by cells of the host owing to its low toxicity,

to its capacity of reducing the porcine xenoantigens and to preserve the valvar histologic architecture. These properties may guarantee a better performance and durability of biological prostheses. However, only clinical trials with long-term follow up can validate the premises of this study.

CONCLUSIONS

The results indicate:

1. The L-Hydro (non-aldehydic) process allows spontaneous endothelialization with evidence of a good adhesiveness of the new endothelium to the collagen matrix;

2. The possibility of an improvement in the performance and the durability of the heart valvar prostheses owing to re-endothelialization with host cells is very promising as it prevents the thrombogenicity and structural degeneration.

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