ORIGINAL ARTICLE

Braz J Cardiovasc Surg 2005; 20(1):14-22

Comparison between the decellularized bovine pericardium and the conventional bovine pericardium used in the manufacture of cardiac bioprostheses

Comparação entre o pericárdio bovino decelularizado e o pericárdio bovino convencional utilizado na confecção de biopróteses valvares cardíacas

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RBCCV 44205-724

Abstract

Introduction: **In this paper, our objective was to compare the decellularized and conventional pericardium mechanical resistance and also its capability of inducing inflammatory response in an animal experimental model.**

Methods: **In order to study these properties, we divided the pericardia into two groups: Group I - pericardium conventionally treated with GTA and Group II – pericardium previously decellularized and then treated with GTA in the conventional way. After the chemical treatment, Group II samples were histologically evaluated to confirm the efficacy of the decellularization process. Then, only for the analysis of mechanical resistance, pericardia were divided in: Groups 1 (conventional pericardia with criteria of approval), 2 (conventional pericardial with criteria of rejection) and 3 (decellularized pericardia). The capacity of inducing inflammatory response was tested in a rat experimental model using 50 Wistar rats, in which rats of each group received patches of the pericardia in the abdomen. Our third step of analysis was to manufacture three decellularized pericardium bioprostheses which were submitted to hydrodynamic evaluation together with a conventional bioprosthesis test.**

Results: **The histological analysis showed complete decellularization. Mechanical resistance gave statistical differences in the "tension of rupture" and "tenacity index" tests. We found no difference in the inflammatory activity in the animal model. Hydrodynamic performance was similar and all prostheses reached 150 million cycles. The final histological analysis assessed the standard microscopic pattern and no rupture or abnormal fragmentation was caused by mechanical stress.**

Conclusion: **The decellularization technique maintains the physical resistance of the pericardium when compared with the conventionally prepared pericardium. And also, there was no difference in both groups regarding to inflammatory response studied in the animal model.**

Descriptors: **Heart valve prosthesis. Bioprosthesis. Pericarduim. Comparative study.**

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Article received in December, 2004
Article accepted in March, 2005

Resumo

Objetivo: **Neste estudo, tivemos como objetivo comparar a resistência mecânica do pericárdio decelularizado com o pericárdio convencional, assim como avaliar sua capacidade de induzir resposta inflamatória em modelo experimental com ratos.**

Método: **Dividimos os pericárdios em: Grupo I – pericárdio submetido a tratamento convencional com glutaraldeído e Grupo II – pericárdio submetido a tratamento de decelularização, previamente ao tratamento convencional. Após o processamento químico, as amostras do Grupo II foram histologicamente avaliadas para confirmar a eficácia da decelularização. A seguir, apenas para análise da resistência mecânica por testes de tração e de desnaturação térmica, os pericárdios foram divididos em: grupo 1 (pericárdio convencional com critérios de aprovação), grupo 2 (pericárdio convencional com critérios de reprovação) e grupo 3 (pericárdio decelularizado). A capacidade de induzir resposta inflamatória foi avaliada em estudo experimental em 50 ratos Wistar, os quais foram submetidos a implante subcutâneo de fragmentos dos pericárdios. Nossa terceira etapa de avaliação consistiu em confeccionar três biopróteses com o pericárdio decelularizado e que foram submetidas à avaliação hidrodinâmica, juntamente com uma bioprótese convencional de teste.**

Resultados: **A análise histológica inicial demonstrou decelularização completa. A resistência mecânica mostrou diferença significativa com relação às variáveis "tensão de ruptura" e "índice de tenacidade". Não encontramos diferença quanto à atividade inflamatória em modelo experimental com ratos. O desempenho hidrodinâmico foi semelhante e todas biopróteses atingiram a marca de 150 milhões de ciclos. A avaliação histológica ao fim da ciclagem mostrou padrão microscópico habitual, não havendo ruptura ou fragmentação anormal induzida por estresse mecânico.**

Conclusão: **A decelularização mantém a resistência física do pericárdio, além de não induzir resposta inflamatória diferente daquela habitualmente encontrada no pericárdio convencional.**

Descritores:

Prótese das valvas cardíacas. Bioprótese. Pericárdio. Estudo comparativo.

INTRODUCTION

The treatment of valvar heart disease has been one of the big challenges of Cardiac Surgery over the last few decades. Native valve replacement by prostheses has been the most important advance in the treatment of patients with this disease, even though this is not without complications [1].

Mechanical prostheses offer satisfactory hemodynamic function and excellent durability over the long term, but as they are thrombogenic, they require permanent anticoagulation, which increases the risk of bleeding [2-5]. Bioprostheses present a hemodynamic profile even better, however, both replacement valves made from porcine aortic valves and bovine pericardium fixed in glutaraldehyde induce activation of the immune system [6,7], triggering an inflammatory reaction [7,8] that leads to infiltration of the collagen matrix, rupture of the collagen-elastin network and calcification [9-11]. These events invariably result in valvar dysfunction and the necessity of reoperation [2-5].

Faced with this, there has been much study aiming at finding a strategy that creates a biocompatible prosthesis which is resistant over the long term. Existing evidence suggests that decellularization of biomaterials makes the tissue less antigenic, reducing the inflammatory response and causing less tissue degeneration [12-14]. However, until now, the decellularization process of bovine pericardium has not been studied with the objective of producing heart bioprostheses.

In this work, the physical proprieties of decellularized bovine pericardium were compared to conventional bovine pericardium. Also, its capacity of inducing inflammatory response in an experimental *in vivo* model and the accelerated durability of prosthesis produced from this material were investigated.

METHOD

Procurement of the biological material

Samples of bovine pericardium were obtained in abattoirs immediately after the slaughter of under 37-month-old animals.

The material was then prepared in plastic containers containing a hypertonic solution of NaCl with 8 mOs $MgSO_4$ buffered at pH 7.4 using a 0.13-M phosphate buffer and transported to the laboratory at 4ºC within a maximum 4 hours [15].

In the laboratory, the pericardia were washed an isotonic 0.9% NaCl solution and all the fat and adherences were removed.

Decellularization process

The bovine pericardium was placed on a support to be submitted to the decellularization treatment. It was

treated at 20ºC for 24 hours in an alkaline solution (3 mL/g) containing (v/v) 6% sulphoxide dimethyl, salts (chloride and sulfate), alkaline bases (1.19M K^+ and 1.74M Na^+) and alkaline earth (0.86M Ca^{2+}). The resulting material was placed in a solution of Na_2SO_4, NaCl, KCl and $CaSO_4$ (96 mL of solution per gram of tissue) for a period of 6 hours and the excess of residual salts was removed by successive washings with 3% boric acid, distillated water and 0.3% EDTA at pH 11, followed of stabilization of the material in a 0.14 mo1L^{-1} phosphate buffer at pH 7.4. After this procedure the pieces of pericardium were washed (3 x for 15 minutes in distilled water) [16].

Reticulation with glutaraldehyde

The materials were then treated in 0.05% glutaraldehyde in a 0.14 mo1L^{-1} phosphate buffer at pH 7.4 for 15 minutes, followed by a glycine borate buffer solution. After this the pericardium was washed six times in a 0.14 mo1L^{-1} phosphate buffer at pH 7.4 and sent to Braile Biomédica for processing. All this procedure was made in the Chemistry School of USP, São Carlos at the temperature of 5ºC [16].

At Braile Biomédica, the pericardium was washed in an isotonic 0.9% NaCl solution. Then, it was submitted to fixing in purified 0.5% glutaraldehyde in a phosphate buffer 0.13 M at pH 7.4. Subsequently, the samples were washed for six times of 15 minutes each in a phosphate buffer and treated with 0.005 M borate buffer solution / 0.025M glutamic acid adjusting the pH alkaline using 0.1N NaOH and left for 24 hours at room temperature.

Completing the tanning period, the pericardium was preserved in a solution of 4% formaldehyde with a 0.2M acetate buffer at pH 5.4 for three days when it was submitted to quality control [15].

Quality control

Macroscopic anatomy

All the pericardial samples was analyzed in respect to the macroscopic aspect using a polarized light. Those with abnormal infiltrations or thicknesses or any type of defect were discarded [15].

Histologic examination

Microscopic evaluation by random sampling of different pericardial fragments was performed with the objective of establishing the histologic standard in the studied samples, even considering that this procedure is not routine in the manufacture and preparation of heart bioprostheses. This study also served to demonstrate the effectiveness of decellularization of the samples.

The histologic study by evaluation of microscopic anatomy utilizes the normal methods of embedding in paraffin wax, using 3- to 5-micron sections and staining using hematoxylin-Eosin (HE), Gomori's trichrome (TG) and Verhoeff stain (VH).

Mechanical resistance of the pericardium

This evaluation consisted in static traction tests (rupture tension, stretching and tenacity index) and a shrinking test. Standardized samples of 6 x 20 mm, fixed using tweezers on special apparatuses, were utilized [17,18].

Just for this evaluation, the pericardium was divided into three groups with 10 fragments in each. Group 1 – conventional bovine pericardium suitable for the production of bioprostheses: Group 2 – conventional pericardium rejected for the manufacture of bioprostheses and Group 3 – decellularized bovine pericardium.

Hydrodynamic assessment

The fatigue or accelerated durability were tested in a Shelhigh FTS 300 fatigue tester – USA, with four evaluation chambers (Figure 1) and analysis of the hydrodynamic performance was tested in an automatic pulse duplicator system of Shelhigh model V. 4.0 – USA (Figure 2). Three decellularized pericardial bioprostheses (mitral nº 29) and one conventional bovine pericardial bioprosthesis (mitral, nº 29 – Braile Biomédica) were placed in the fatigue tester for evaluation of mechanical wear. The test was interrupted at every 50 x 10^6 cycles to assess the hydrodynamic performance. The hydrodynamic profiles were assessed at zero, 50 x 10^6, 100 x 10^6 and 150 x 10^6 of cycles.

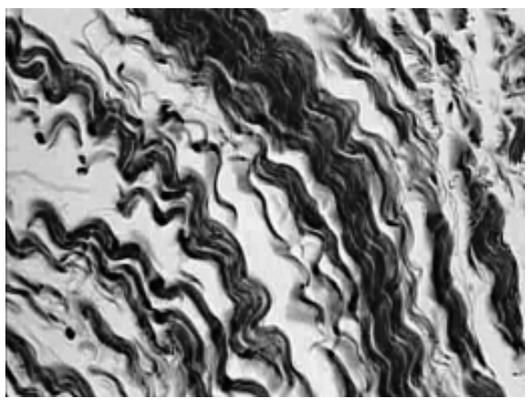

Fig. 1 - Fragment of bovine pericardium stained by Hematoxylin-Eosin and visualized magnified at 400x. The preservation of normal undulation of collagen filament and absence of cellularity were evidenced.

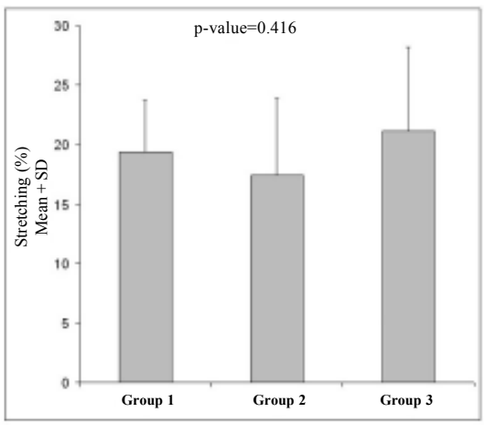

Fig. 2 – Histogram exhibiting the variations in stretching - there was no statistical difference among the four prostheses

Subcutaneous implantation in mice

Fragments of bovine pericardium from Groups I and II were washed in saline solution for about 10 minutes previous to the surgical procedure in 50 male Wistar mice (with weights of approximately 180-240 g). After anesthesia, each animal was submitted to tricotomy of the abdominal region and a medial skin incision of approximately 1 cm creating a subcutaneous bag on each side of abdomen. A pericardial fragment of each group was implanted in these bags. Five groups of 10 mice were sacrificed at 7, 14, 30, 60 and 90 post-implantation days. The removed material was sent for histologic analysis and studied using HE, TG and VH and Von Kossa stains. The results of analysis of slides were classified using a score of from zero to four crosses by one observer, indicating growing evidence of the presence of inflammatory activity. The use of animals for experimentation complied with the "Canadian Council on Animal Care".

Histological study of prostheses

After the dynamic tests, the pericardial prostheses were submitted to another histologic study to assess the integrity after mechanic stress.

Three decellularized bovine pericardial prostheses (DBPC) and a control conventional bovine pericardial prosthesis (BPC) had one of their three cusps removed and their basal (B), medial (M) and free (F) regions were studied.

The fragments were embedded in paraffin and were stained using HE, TG and VH. They were classified using a score of from zero to three crosses for the characterization of cellularity and the filament pattern of the collagen and elastin in the tissues. Again a single observer evaluated the samples.

Statistical analysis of the data

The data was described as minimum and maximum values, median, mean and standard deviations.

The pericardial groups were compared by variance analysis and multiple comparisons were achieved using the Tukey test.

The studied animal model variables were evaluated using the Wilcoxon signed rank test for matched samples.

Comparisons of the hydrodynamic variables were achieved using variance analysis and the multiple comparisons by the Wald test.

A significance level of 0.05 was adopted for all comparisons.

RESULTS

Histologic evaluation of the pericardium

After procurement, treatment and conditioning of the bovine pericardial fragments, histologic evaluation was made to confirm the effectiveness of the decellularization process. An optical microscopic was utilized to define the absence of fragments and cellular remains in the treated pericardium and to characterize the fibrillation pattern of the collagen and elastin. The effectiveness of the decellularization process that removes the matrixes of fixed cells and the cellular remains which normally remain in the pericardium after fixing with glutaraldehyde was observed (Figure 1).

Also checks were made to see that the decellularization technique did not damage the fiber network of the collagen and elastin.

Evaluation of the mechanical resistance of pericardium

From the results of the comparative tests of the groups, it is possible to conclude that there are no significant differences between the groups in respect to the thickness, stretching and temperature variables, as illustrated in Figures 2 and 3. For this last variable, only Groups 1 and 3 was considered, as there were no values greater than zero for Group 2, making its inclusion in the inferential analysis impossible. Additionally, Group 1 presented mean values of rupture tension and tenacity index significantly greater that the other two groups (p-value < 0.001) (Figures 4 and 5).

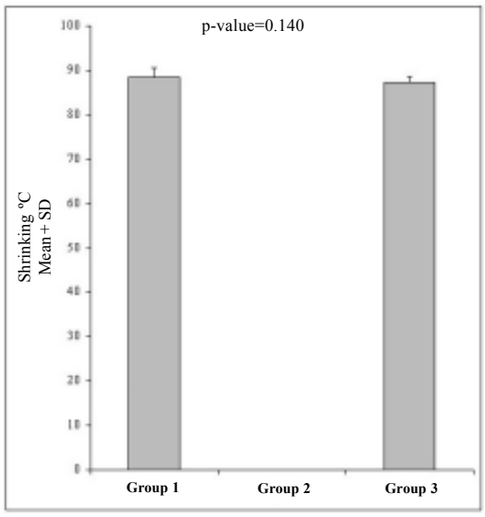

Fig. 3 – Histogram of shrinking temperature – there was no statistical difference between the Groups 1 and 3. Group 2 was not analysed

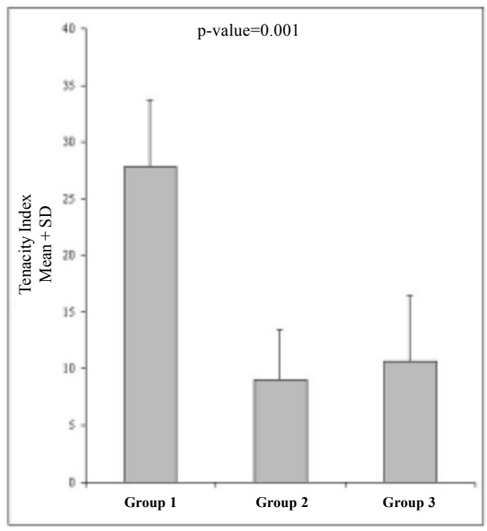

Fig. 5 – Histogram showing the variations among the tenacity index of the three groups – Group 1 demonstrated a statistically significant difference in respect to the other two groups which did not statistically differ between each other

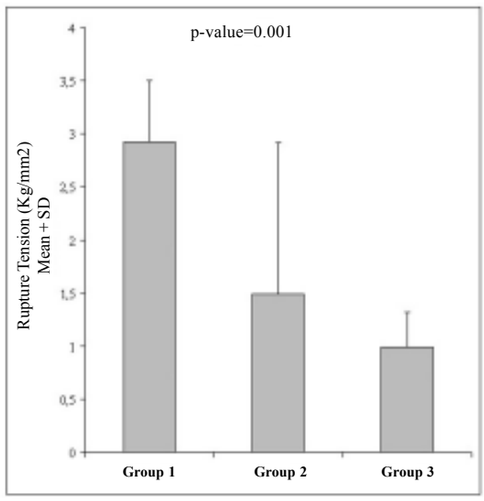

Fig. 4 – Histogram of the rupture tension there was a significant difference between Group 1 and the others

Histologic evaluation in the animal experimental model

From the results of the comparative tests of the groups, it is possible to conclude that there are no significant differences between the groups in respect to the granuloma and infiltration variables at any of the time intervals. The inferential analysis of calcification was not made as no values were different to zero.

Hydrodynamic evaluation of the bioprostheses

The global hemodynamic performance was statistically similar for all four bioprostheses studied. Statistical differences were found in relation to some analyzed measurements however, not one was seen as a difference of mechanical behavior between different prostheses and all the measurements remained within the recommended values recommended in the literature. It is important to stress that the statistical analysis for this evaluation was exploratory and it is not possible to extrapolate data (Figures 6 to 9).

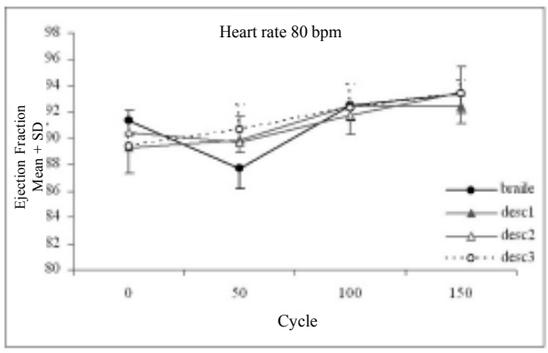

Fig. 6 – Variations in the Ejection Fractions of the four prostheses at 80 bpm

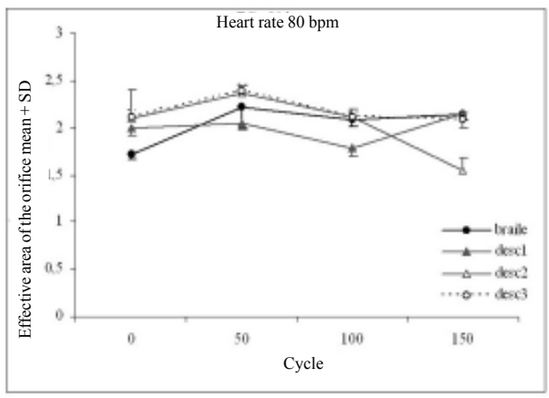

Fig. 9 – Effective area of the orifice that expresses the area that effectively is open during the valvar cycle

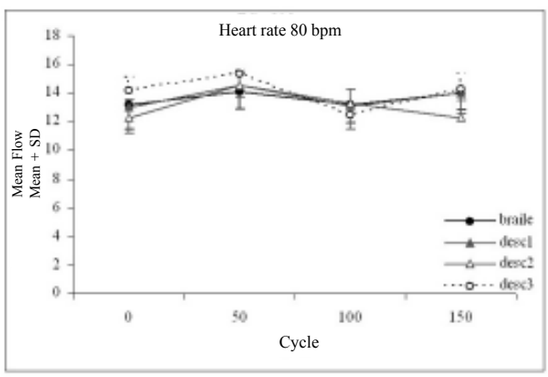

Fig. 7 – Variations in the mean transvalvar flows at 80 bpm

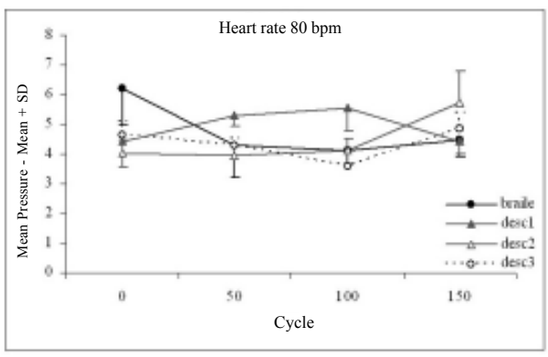

Fig. 8 - Variation in the mean transvalvar pressure in the four prostheses

Final histologic aspects of the prostheses

The final histologic examination of the pericardium removed from the bioprostheses confirmed the decellularization of the study samples and demonstrated preservation of its viscoelastic characteristics with the absence of ruptures or fragmentation.

DISCUSSION

The implantation of valvar heart prostheses has been realized for almost half a century, but a prosthesis with satisfactory criteria of durability and biocompatibility has not been developed yet. The ideal graft should present adequate resistance, be able to tolerate long-term mechanical stress; have a hemodynamic profile comparable to native valves; be immunocompatible, not trigger the defense responses of the host; be resistant to degeneration by infiltration, rupture of the fibroelastic tissue and calcium deposition; have a capacity of maintain the vitality of the proteic collagen, elastin and glycoaminnoglycan network and present a low cost.

Of all factors currently considered as being responsible for prosthetic degeneration, by far, those which are the most important and are the most studied are calcification and the toxicity of glutaraldehyde [19-23]. Among the mechanisms involved in calcification, there is evidence that the presence of dead cells and cellular debris incompletely fixed by the treatment methods, work as calcification nuclei, permitting the deposition of calcium, sodium and magnesium phosphate and carbonate crystals [23]. In addiction, treatment with glutaraldehyde can work as a triggering factor for calcium deposition. It has already been demonstrated that high levels

of glutaraldehyde incorporated in the biological tissue are related to a greater tendency of calcification in an experimental model with mice [24]. The chance relation between pre-treatment with glutaraldehyde and calcification was studied by NIMNI et al. [25], who demonstrated that the glutaraldehyde polymers and the free carbonylic residues in the collagen matrix provide an initial site for the deposition of calcium complexes.

Thus, several strategies have been evaluated with the objective of eliminating or minimizing tissue degeneration induced by these factors, in particular by using the decellularization technique. Detergents have been used, as described by COURTMANN et al. [26] and DOHMEN et al. [27]; enzymatic extraction by means of tripsin as described by STEINHOFF et al. [28], the Synergraft technique presented by O'BRIEN et al. [13]; and even cellular extraction utilized by GOISSIS et al. [16, 29] in the Institute of Chemistry of USP, São Carlos which was the method employed in this study.

In our study, complete decellularization was achieved. This was verified during the initial histological evaluation of the treated pericardium of which one fragment was removed for analysis. The same result was observed in the final histological examination of the pericardial bioprostheses in which three regions of each leaflet were tested. Other histological evaluations of decellularized bovine pericardium, which we did not include in this study, confirmed the efficacy of the method. Note that this result is not always obtained as was reported by SCHENKE-LAYLAND et al. [30] who described "almost complete" decellularization in porcine valves treated by enzymatic digestion using tripsin.

The collagen and elastin matrix was preserved. Significant fragmentation or rupture was not seen in any of evaluated specimens. Additionally, the final comparison of the pericardial bioprostheses of both groups did not demonstrate significant differences in the histologic pattern. It is important to note that the histologic evaluation has a subjective component, it depends on the assessment of an observer. Keeping this in mind, to minimize any possibility of difference in interpretation, all the samples were assessed by a single observer.

In relation to the static traction tests, we found very interesting results. The three groups of pericardium were compared as previously described. Group 2 (pericardium inappropriate for the manufacture of bioprosthesis) with the aim of assessing if the decellularized pericardial bovine had a physical behavior comparable to substandard pericardium, if it proved to be inferior to approved conventional pericardium.

The criterion "thickness" was similar among the three groups, with n = 0.117. This is the first suggestion that the chemical process of decellularization did not affect the quality of the collagen or elastin matrix. This result opposes previous reports that showed alterations in the thickness of pericardium treated by other techniques as already mentioned.

The stretching defined as deformation suffered by the sample until rupturing gave similar results in all the three groups (p-value = 0.416). This data suggests that both the quality of treated pericardium was maintained and that its elasticity was not affected by treatment.

The rupture tension and the tenacity index proved to be superior for the good quality conventional bovine pericardium, with the decellularized pericardium comparable to the rejected conventional bovine pericardium. This data is conflicting keeping in mind that the thickness and the rupture tension has a linear correlation, that is, the greater the thickness of the material, the proportionally greater is the tension necessary to rupture it. However, we believe that this variation is due to removal of the cellular components that, even incompletely fixed, improve the resistance of the tissue, without affecting the thickness. This hypothesis is corroborated by the existent tenacity index. The tenacity is the energy necessary to rupture the tissue. This was seen to be better in Group I in relation to the other two groups and Groups II and III were similar.

Finally and more importantly, the shrinking temperature was similar for Groups I and III. This variable expresses the quality of the pericardium reticulation technique and its resistance after processing. Thus, even though the traction and tenacity were better in Group I, the final resistance of pericardia was not affected.

It is important to note that these physical tests are preliminary tests in the study of the quality of a biomaterial and of the prostheses manufactured from the material. The test results show us that the pericardium presents minimum criteria of resistance and so it is possible to proceed with the physical evaluation of the studied material with laboratorial hydrodynamic tests and hemodynamic tests in animals before continuing with the possibility of clinical application.

Concluding this part of the study, the capacity of causing inflammatory response and calcification in an experimental model in mice was evaluated.

In this material, the presence of inflammatory activity was identified and granulomatous formations were already seen in seven-day explants. Similar patterns were observed in the groups of sacrificed animals in the later periods. No vestiges of calcification were found in any explanted specimens of the two groups at any time interval. Although the finding of an absence of calcification in the control material was unexpected, Marina Maizato from the Heart Institution, FMUSP reported similar results in a comparative

study between lyophilized bovine pericardium and conventional bovine pericardium (verbal communication).

In respect to the accelerated durability and hydrodynamic profile tests, we found similar performances among the four prostheses. We compared the three DBPC prostheses against one PBC control as suggested by national (NBR ISO 5840) and international norms (ANSI/AAMI/ISO 5840-1996) for heart bioprosthesis manufacture. The bioprostheses was accelerated to 60, 70, 80, 90 and 100 bpm at each 50 x 10^6 cycles to assess the performance and the test was interrupted at 150 x 10^6 cycles. However, for a question of clarity, only the results at 80 bpm (mean heart rate) are reported here. For the analyzed variables, there was no overall difference between the bioprostheses for any of the cycles and the graphically exhibited variations were randomly attributed. It is worthwhile noting that in our pilot project, we used two decellularized bovine pericardium prostheses that were submitted to accelerated durability for more than 300 x 10^6 cycles and that maintained their macro and microscopic structural integrity similar to the DBPC assessed here. Besides the resistance demonstrated, an evaluation of the performance shows us that the DBPC did not undergo alterations in the elasticity or in the movement of the leaflets, as well as maintained a good opening, which is illustrated by flow curves, the pressure and orificial opening. Thus, even though it is not possible to make any definitive affirmation about the quality of the DBPC, we consider the hydrodynamic study to be an initial exploratory evaluation whose preliminary results point to a promising area of research. In the future, these findings need of to be confirmed by experimentation using an animal model for an *in vivo* performance evaluation.

Finally, the evaluation of the leaflets removed from the bioprostheses confirmed the absence of cellularity in the DPBC samples and showed a normal pattern of collagen and elastin matrix without significant differences between the groups.

CONCLUSIONS

We evidenced significant differences between the conventional bovine pericardium and the decellularized bovine pericardium in relation to the physical resistance considering the rupture tension and tenacity index, however, there were no significant differences in relation to the thickness, stretching and the shrinking temperature.

The capacity of the decellularized pericardium to produce an *in vivo* inflammatory response did not present significant differences with respect to the conventional pericardium and the decellularized pericardium bioprostheses presented a similar hydrodynamic behavior to the conventional bovine pericardium bioprosthesis.

In light of the obtained results, even though there are some isolated discrepancies, we considered the performance of the two types of pericardium similar, both in the assessment of the tissue itself and the bioprostheses. This study is part of only one of the evaluation stages of new materials or new techniques to manufacture heart valvar prostheses. It is impossible to generalize or to extrapolate data about the decellularized bovine pericardium prosthesis just from these findings alone. Our findings verified the quality of the evaluated biomaterial and the comparable behavior of the two materials, which enable us to go to the next stage. This will consist of an *in vivo* experimental study in larger animals, for example in dogs, pigs or sheep, in which we will be able to analyze the behavior of the prostheses in contact with blood and in conditions closer to those seen in humans, even though these models are not ideal. However, this is an essential stage in the pre-clinical evaluation, without which it is not possible to continue to the final stage which is the study in *anima nobili*.

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