LYOPHILIZED GLUTARALDEHYDE-PRESERVED BOVINE PERICARDIUM FOR EXPERIMENTAL ATRIAL SEPTAL DEFECT CLOSURE

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Abstract

A variety of patch materials has been used to close large atrial septal defects (ASD). Autologous pericardium and glutaraldehyde-preserved bovine pericardium are the most used. Lyophilized bovine pericardium has not been tested inside the cardiovascular system. The aim of this work was to study the behaviour and effectiveness of lyophilized glutaraldehyde-preserved bovine pericardium in ASD closure. Sixteen mongrel dogs were operated on. A 3 cm diameter atrial septal defect was created, and closed with: Group I (n=8): Lyophilized glutaraldehyde preserved bovine pericardium (LGPBP). Group II (n=8): Vascular Dacron patch. The animals were evaluated clinically, by echocardiography, macroscopically, and microscopically. Statistical analysis was done with analysis of variance (ANOVA) and Student's *t*-test. All the animals survived the surgical procedure and study time (6 months). Clinically all the animals displayed normal physical activity, with normal cardiac sounds. Echocardiography showed that both groups had a normal heart without intracardiac shunts, no thrombus formation, and no vegetations. Macroscopically all the animals showed good integration of the lyophilized bioprosthesis and Dacron patch. All group I animals presented a decrease of the area of the ASD in the left atrium ($p<0.001$ by ANOVA and Student's *t*-test). Microscopically, group I presented dense and well-organized collagenous tissue, areas of cartilaginous metaplasia and remnants of the lyophilized bioprosthesis ($p<0.001$ by ANOVA and Student's *t*-test). Group II showed encapsulated Dacron patch covered with collagenous tissue and cartilaginous metaplasia. In conclusion, the new lyophilized bioprosthesis is well integrated into the atrial septum, without complications and is effective for ASD closure.

Keywords: *In vivo* biocompatibility, biological biomaterials, scaffolds, cardiovascular tissue, animal models, lyophilizated bioprostheses, atrial septal defect, bovine pericardium.

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Introduction

Glutaraldehyde-preserved bovine pericardium (GPBP) is widely used in cardiovascular surgery with good results and is one of the most used biological patches (Crawford *et al.,* 1986; David, 1998). However, it is well known that GPBP is subjected to degenerative processes involving rigidity, calcium deposition and structural changes after implantation in humans with the subsequent bioprosthesis failure, mainly due to glutaraldehyde residues and host's immune system (Schmidt and Baier, 2000; Gendler *et al*., 1984; Beauchamp *et al*., 1992; Grimm *et al*., 1992).

Many studies have been published in order to understand, to prevent or to treat the degenerative process (Franks, 1998; Chandy *et al*., 1996; Golomb, 1987). Lyophilization has been used to decrease aldehyde residues in GPBP, hence decreasing cytotoxicity and enhancing resistance to calcification (Maizato *et al*., 2003). The lyophilized glutaraldehyde preserved bovine pericardium (LGPBP) has been tested *in vitro* (Maizato *et al*., 2003), in non anatomical lung resection (Olmos-Zúñiga *et al*., 2001), as a dural substitute (Laun *et al.,* 1990), abdominal wall repair (Olmos-Zúñiga *et al*., 1996, Zuki *et al*., 2007) and subcutaneous implantation (Maizato *et al*., 2008), with good results, nevertheless in all these works the LGPBP was implanted outside the cardiovascular system, and the main factor for GPBP degradation is the contact with host blood, as well as the site of implantation and function (Pires *et al*., 1999; Gabbay *et al*., 1984).

We have a good experience working with GPBP (Santillan-Doherty *et al*., 1996) and LGPBP (Olmos-Zúñiga *et al*., 1996), thus we though that it could be used safely in cardiovascular surgery. After a thorough literature review, we did not find any report about the use of LGPBP in cardiac surgery. We evaluated the clinical, echocardiographic, macroscopic and microscopic behavior of LGPBP in an intracardiac position, as experimental ASD closure.

Material and Methods

This protocol was reviewed and approved by the Ethics Committee of the INER (*Instituto Nacional de Enfermedades Respiratorias "Ismael Cosio Villegas"*), and carried out under the Technical Specification for the Care and Use of Laboratory Animals of the Official Mexican Norm (*Especificaciones Técnicas para el Cuidado y Uso de Animales de Laboratorio de la Norma*

Oficial Mexicana 1999) and the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health (National Institutes of Health Publication No 86 to 23, revised 1996).

Glutaraldehyde-preserved bovine pericardium preparation

Twenty pericardia were obtained from 6 to 18 months old cows at the local abattoir after euthanasia with a penetrating captive-bolt pistol. Local mechanical cleansing was performed manually by dissecting off excess pericardial fat. The pericardium was submerged in 4ºC saline solution in order to be transported. Further cleansing was performed in the laboratory to remove all fatty and connective tissue by dissecting it off the pericardium with clean dry gauze; clean pericardium was further washed with 4ºC Hanks solution in a container with an electromagnetic agitator for 6 h. The pericardium was then mounted on 15 cm diameter plastic frames and submerged in 0.5% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4) at 4ºC for at least 15 days before testing. After the initial preservation period, samples were taken both from the preservation solution and the pericardium for microbiological cultures, which all were negative.

Lyophilization

The GPBP was cut in 15 cm x 15 cm squares and washed in saline solution for 1 h in order to remove all the glutaraldehyde excess. They were placed in crystal containers, frozen at -70ºC during 1 h, and lyophilized at 10 mBar of vacuum at -55ºC for 4 h. Finally the lyophilized glutaraldehyde preserved bovine pericardium (LGPBP) was cut in pieces of 10 cm x 10 cm, sealed in airtight double-layered polyethylene bags, sterilized with sterrad (low-temperature hydrogen peroxide gas plasma sterilizing process, Johnson & Johnson Medical, Inc., New Brunswick, NJ, USA) and stored at room temperature before surgical application. Small squares of 5 mm x 5 mm were taken for microbiological culture just before the surgical implantation. All the cultures were negative.

Surgical technique

Sixteen healthy 2 years old mongrel dogs regardless of sex, weighing between 25-35 kg underwent surgery as follows: The animals were prepared before surgery with a 24 h fast for solids and a 12 h fast for liquids.

Anesthesia. Initial anesthesia was induced by intravenous (i.v.) hydrochloric Xylazine, 0.1 mg/kg, (Rompum, Bayer, Leverkusen, Germany) and propofol 6 mg/kg (Diprivan, Astra Zeneca. Edo. México, México) followed by immediate endotracheal intubation (Endotracheal tube, Rush. Kamunting, Malaysia) connected to a volume ventilator (Harvard Apparatus, Boston, MA, USA). Anaesthesia was maintained by giving a mixture of 98.5 % oxygen and 1.5 % isofluorane (Isotec 3, Ohmeda Anaesthesia Machine, GE Healthcare, Chalfont St. Giles, U.K.). They were medicated with IV Enrofloxacine (5 mg/kg Baytril, Bayer, Leverkusen, Germany), and 30 mg/kg Metilprednisolone (Metilprednisolona, Pisa, Guadalajara, Jal, Mexico).

Cardiopulmonary bypass. A Gambro roller pump and a paediatric membrane oxygenator and tubing set (Terumo Cardiovascular Systems Corporation, Ashland, MA, USA) were used for the cardiopulmonary bypass (CPB). The system was primed with 600-800 ml Ringer solution, 0.5 ml/kg mannitol (Osmorol 20, Pisa, Guadalajara, Jal., Mexico) and 500 IU/L heparin (Inhepar, Pisa, Guadalajara, Jal, México).

Surgical procedure. The anaesthetized dog was positioned in the left lateral *decubitus* position. Through a right thoracotomy in the 4th intercostal space, routine total CPB for ASD closure was installed. Intravenous 70 IU/kg heparin (Inhepar, Pisa) was given. An activated coagulation time >400 sec was approached. A 16-20 Fr aorta cannula for the aorta and separate cannulae (20-22 Fr) were used for each *vena cava*. The CPB flow was 1.8-2.5 L/min and conducted at 32ºC. The aorta was cross-clamped, 600 ml cold cardioplegia solution (St. Thomas solution) at 4ºC was given through the aortic root. With a quiescent heart the caval veins were snared; through a right atriotomy we created a circular 3 cm diameter atrial septum defect on the *fossa ovalis*. No dog had a native ASD. The defect was closed with: Group I (n=8) Lyophilized glutaraldehyde preserved bovine pericardium patch (LGPBP), the wrinkled surface facing the right atrial chamber, and Group II (n=8) Dacron patch (Hemashield Gold™, Boston Scientific, Wayne, NJ, USA) (control group), with running polypropylene 4-0 (Prolene, Ethicon, Johnson & Johnson). The lungs were inflated and the left atrium de-aired. The right atrium was closed with running polypropylene 4-0. The aortic clamp was released while the dogs were re-warmed on the pump to normal body temperature (36.5ºC-38ºC) (Bistner *et al*., 2002). Three dogs were defibrillated with one 20J charge. De-airing of the left ventricle was accomplished through the cardioplegic cannula placed on the aortic root. The animals were successfully weaned off of CPBP. All the blood in the oxygenator was returned, if possible, through the aortic cannula. The aorta was decannulated and Protamine Chlorydrate (Protamina 1000, Grossman Laboratories. México D.F., México) infusion started. After haemostasis was completed, a 24 Fr chest tube was placed in the right pleural cavity connected to a water seal. The thoracotomy was closed in layers with Polydioxanone 2-0 (Polydioxanone monofilament, Atramat, México D.F., México). The chest tube was withdrawn after 15 min of suction. The animals were allowed to awake on the surgical table and they were extubated. All the animals received postoperative IM Enrofloxacine 5 mg/kg (Baytril, Bayer), and Dipiron 28 mg/Kg (Sodic Metamizol, Parafarm, México, D.F., Mexico) during one week. We did not use postoperative anticoagulation or antiplatelet treatment.

The operated dogs were kept in metabolic cages. They were subjected to continuous clinical observation looking for any clinical complication (heart failure or general health problem). We performed heart auscultation with a Littmann Cardiology III stethoscope for the detection of any post-surgical cardiac murmur. The dogs were allowed to run freely outside the cage during 15 minutes, followed by 15 minutes rest to measure heart rate and respiratory rate (daily

during the first postoperative week, and every other day through out the 6 months of study time)

Echocardiographic evaluation
The animals were anaesthetized with IV Propofol (6mg/kg, Diprivan, Astra Zeneca) in order to be subjected to transthoracic echocardiographic evaluation a week after the surgical procedure and a week before euthanasia, with the Echocardiography machine Vivid FiVe™ (cardiovascular ultrasound systems, General Electric, Schenectady, NY, USA). Ejection fraction, heart rate, cardiac output, and patch thickness were evaluated as well as intracardiac shunt, thrombi or vegetations.

Macroscopic evaluation
The dogs were euthanized 6 months after the surgical procedure with an overdose of sodium pentobarbital (Anestesal, Pfizer, Estado de México, México). The ASD area was examined carefully for the detection of postoperative healing complications. The atrial septum including the operated area was cut, examined carefully on both atrial sides for the development of endothelium, fibrous tissue, calcification, and for the detection of dehiscence, vegetations, thrombus formation, implant shrinkage, or graft loss.

Morphometric analysis
The thickness of the explanted atrial septum and patch were measured with a Vernier caliper. The excised atrial septum (basal measure) and explanted patch were placed on a reference scale, graded in millimeters in order to evaluate the area. The image digitalization software program IMAGE (Pro plus 4.0, Media Cybernetics, Bethesda, MD, USA) was used in order to obtain the area. The patch morphometric analysis was done by tracing the final visible patch area and comparing it with the basal area before implantation, to get the relative final area.

Microscopic evaluation
The tissue samples collected from the operated area were fixed in 10% buffered formalin for 24 h. They were dehydrated in ascending grades of ethanol, cleared in xylol, embedded and blocked in paraffin wax, and then cut into 4 μm thick sections with a rotating microtome. The sections were mounted on glass slides and stained with haematoxylin and eosin for general histology and Masson's trichrome for the demonstration of collagenous tissues.

Statistical analysis
Numerical data is expressed as mean ± standard deviation (SD)/± standard error (SE). Analysis of variance (ANOVA) and Student's *t*-test were used to compare clinical, macroscopic and histological findings between the two groups. Differences were considered to be significant when $p<0.05$.

Results

All animals tolerated the surgical procedure and finished the study period. Clinically, all the animals showed normal physical activity from the first postoperative day. All animals were allowed to run freely and they ran effortlessly during 15 minutes without showing dyspnea or any heart failure symptom. After 15 minutes rest the heart rate and respiratory rate were 5-15% above standard physiological dog parameters (Bistner *et al.*, 2002) since it was impossible to calm down the animals. These signs were normal while the dog was under general anesthesia: heart rate (HR) 110.12 bpm (± 14.58/ ± 5.15); RR 22.94/min (± 2.74/± 0.68). The animals presented normal heart sounds and no heart block. None of the dogs showed any sign of cardiac failure.

Echocardiographic evaluation
In the echocardiographic study performed a week after the surgical procedure, all 16 dogs showed a normal ejection fraction, normal cardiac output, normal heart rate (Table 1), normal cardiac chambers, normal contractility, no intracardiac shunt and no evidence of vegetation or thrombus formation. The pre-euthanasia evaluation showed the same results as the first echocardiographic study (Table 2); nevertheless, the interatrial septum was thicker in all 16 animals (Fig. 1), ($p<0.001$, ANOVA).

Macroscopic evaluation
No shunt was detected in any animal. All the animals in both groups showed good healing at the suture line of

Table 1. Echocardiography performed a week after the surgical procedure.

Parameter	Group I	Group II
Ejection fraction	66.6% (± 2.4/± 0.9)	65.6% (± 2.9/± 0.01)
Cardiac output (l/min)	6.00 (± 0.34/± 0.12)	6.13 (± 0.29/± 0.10)
Heart rate (bpm)	110.12 (± 14.58/± 5.15)	109.12 (± 12.21/± 4.31)

Values given as mean (±SD/±SE).

Table 2. Echocardiography performed pre-euthanasia.

Parameter	Group I	Group II
Ejection fraction	66.4% (± 2.1/± 0.8)	66.5% (± 3.0/± 1.1)
Cardiac output (l/min)	5.88 (± 0.34/± 0.12)	5.91 (± 0.32/± 0.11)
Heart rate (bpm)	106.62 (± 15.22/± 5.38)	108.00 (± 12.87/± 4.55)

Values given as mean (±SD/±SE).

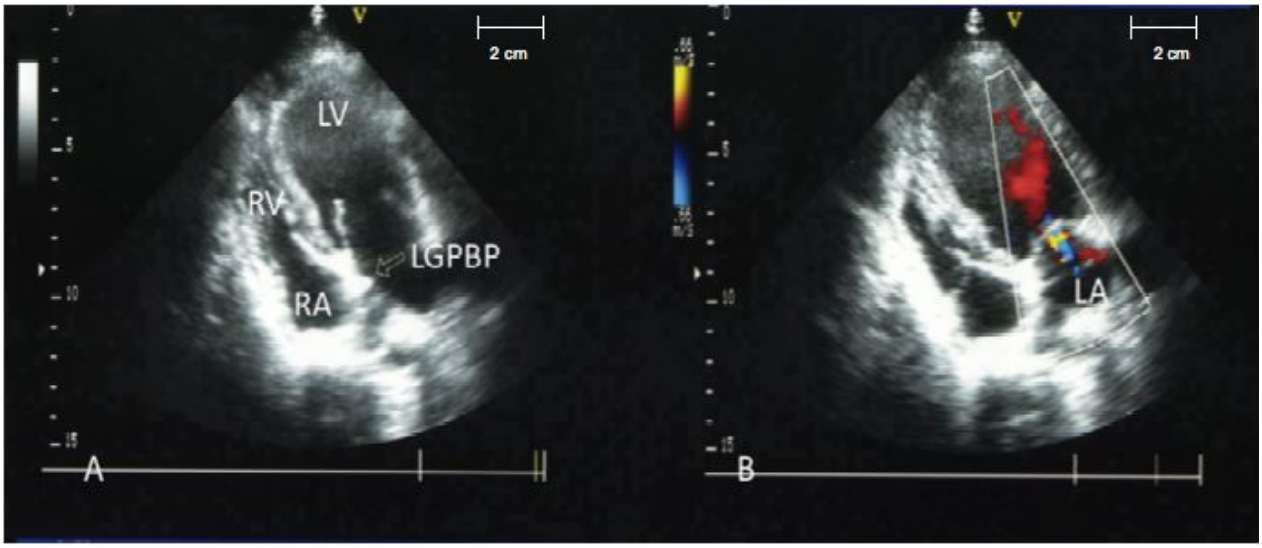

Fig. 1. Echocardiographic image. Four chambers projection. **(A)** The arrow shows a thick LGPBP patch. **(B)** Color doppler flow shows an intact interatrial septum. LA: Left Atrium. LV: Left ventricle. LGPBP: Lyophilized glutaraldehyde preserved bovine pericardium. RA: Right atrium. RV: Right ventricle.

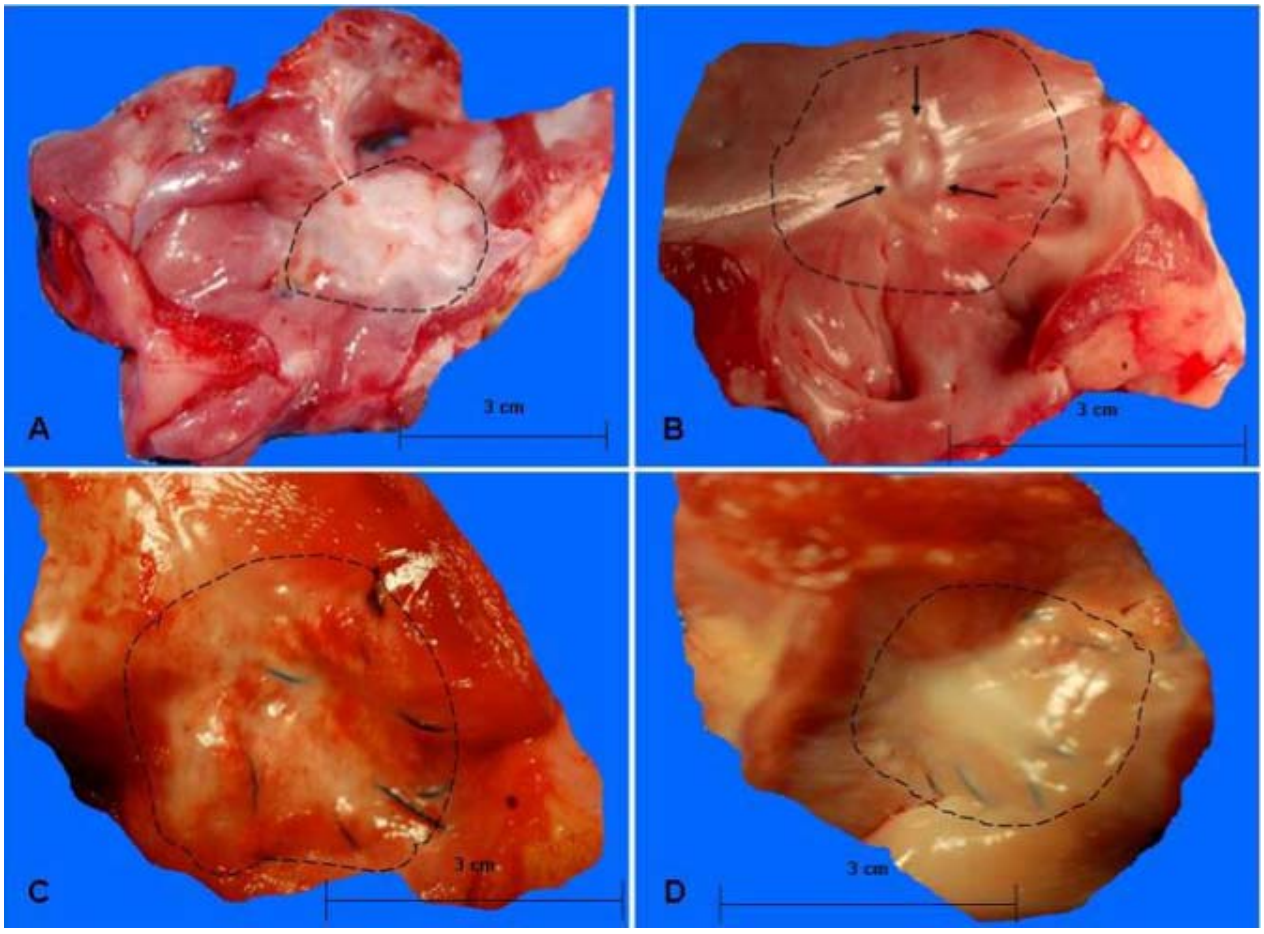

Fig. 2. The dotted circles in **A** and **B** show the area of the created atrial septum defect, closed with lyophilized glutaraldehyde preserved bovine pericardium (LGPBP). The lyophilized bioprosthesis is well integrated into the septum, covered with endothelium: **A** – Right atrium view; **B** – Left atrium view. The 3 arrows point out the decreased area of the LGBP patch, which was covered with atrial muscle

The dotted circles in **C** and **D** show the area of the created atrial septum defect, closed with Dacron: **C** – Right atrium view; **D** – Left atrium view. The Dacron patch is well integrated into the atrial septum. It remains distinguishable, covered with endothelium, without shrinkage.

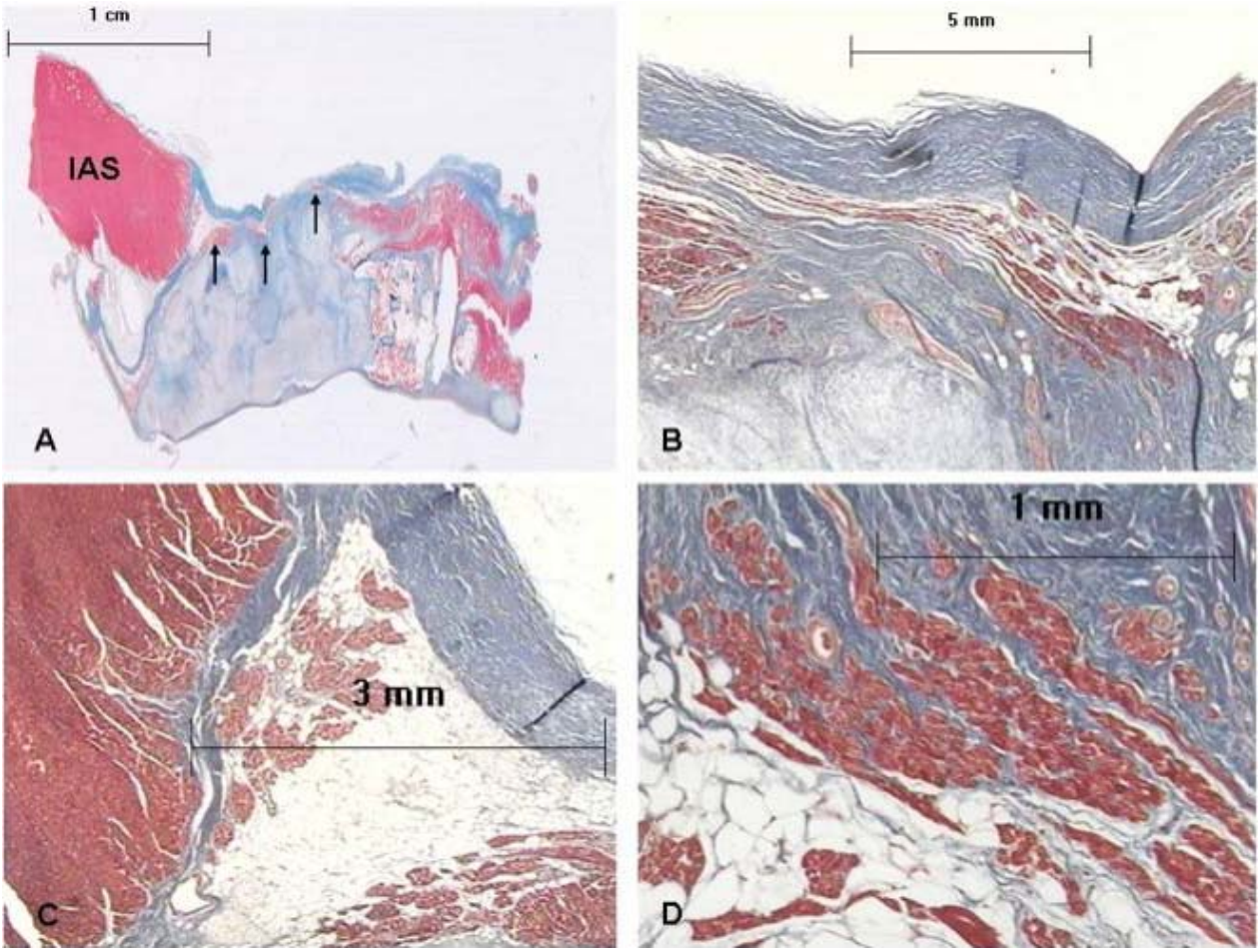

Fig. 3. (A) The glass slide view of the interatrial septum (IAS). The arrows point out atrial muscle nests embedded in the collagenous fibres of LGPBP. Masson's trichrome staining. (B, C and D) show the atrial muscles nests and projections embedded in the collagenous fibres of LGPBP and in the fatty tissue. Masson's trichrome staining.

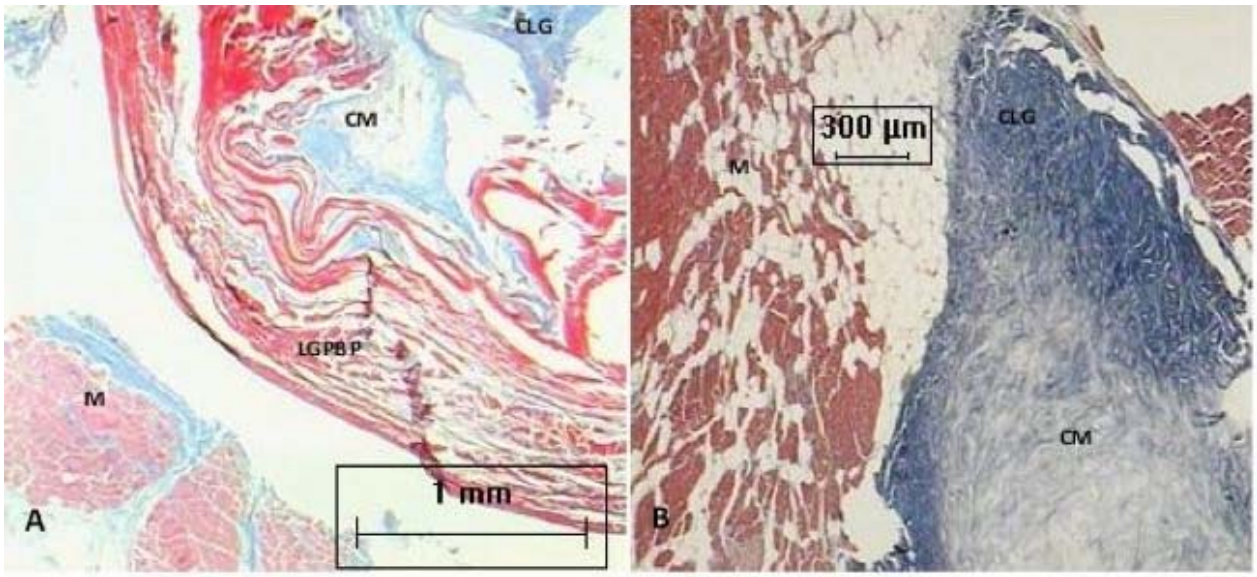

Fig. 4. Lyophilized glutaraldehyde preserved bovine pericardium (A) and Dacron (B) photomicrographs. Masson's trichrome staining, CLG: Collagen fibres. CM: Cartilaginous metaplasia. LGPBP: Lyophilized glutaraldehyde preserved bovine pericardium fibres. M: Myocardial cells.

LGPBP and the Dacron patch, they were well integrated into the atrial septum. The surface of all patches was smooth, covered with thickened fibrous tissue, with no evidence of dehiscence, no vegetation, no thrombus formation. The suture line and the LGPBP were covered by tissue that was indistinguishable from normal atrial muscle on the left side of the atrium. The right atrium patch area in group I decreased 8.2% (± 2.4/± 0.9); and in group II 7.7% (± 1.9/± 0.7). In group II it was possible to recognize the suture line and the Dacron patch. In group I, the visible patch area decreased 86.6% (± 9.2/± 3.3) on the left side of the atrial septum ($p<0.001$, ANOVA, Student's *t*-test) due to the fact that it was covered by atrial muscle (Fig. 2 A,B), in contrast with group II dogs, where the total area of the Dacron patch was visible (Fig. 2 C,D). The patch thickness in group I was 3.26 mm (± 0.13/± 0.04), and in group II 3.38 mm (± 0.18/±0.06).

Histological evaluation

All animals showed endothelial cells on the LGPBP. Group I dogs showed nests and projections of atrial muscle, facing the left atrial chamber, embedded in the collagenous and adipose tissue of LGPBP, beyond the atrial septum border (Fig. 3A,B,C,D). All animals in group I showed scattered bundles of LGPBP, dense and well organized collagenous tissue as well as areas of cartilaginous metaplasia, which replaced the bioprosthesis (Fig. 4A). All animals in group II showed an encapsulated Dacron patch with well organized collagenous tissue and areas of cartilaginous metaplasia, (Fig. 4B).

Discussion

The application of biological materials and prostheses for medical use is a widespread practice. Autologous pericardium is usually available and inexpensive but it is difficult to handle, since it has a propensity to roll at the edges rendering the procedure unduly tedious and time consuming. In addition, uncommon complications like residual shunt due to lack of coaptation, shrinkage, aneurysm formation and calcification have been reported (Mohri *et al*., 1970). Synthetic patches can develop thromboembolic complications, hemolysis, endocarditis, shrinkage, and calcification (Mohri *et al*., 1970; McGoon, 1982; Alelhan *et al*., 2001; Shrivastava and Radhakrishnan, 1989; Di Eusanio and Schepens, 2002; Hayabuchi *et al*., 2007; Us *et al*., 2004). Glutaraldehyde preserved bovine pericardium (GPBP) has been used to construct heart valve prostheses, repair patches, and shape conduits with good results, nevertheless is not free from complications (David, 1998; Pires *et al*., 1999; Gabbay *et al*., 1984). Glutaraldehyde is used on bovine pericardium to improve mechanical and immunogenic properties, it reduces thrombogenicity, increases resistance to enzymatic degradation and allow a longer period of storage (Schmidt and Baier, 2000; Gendler *et al*., 1984; Beauchamp *et al*., 1992; Grimm *et al*., 1992). This bioprosthesis has been lyophilized in order to reduce glutaraldehyde residues and to ease storage and transportation conditions (eliminating the maintenance of a constant 4ºC temperature and pH of 7.4.) without changing its molecular structure and behaviour (Olmos-Zúñiga *et al*., 2002; Olmos-Zúñiga *et al*., 1996). Its biocompatibility and safety inside the cardiovascular system have not been established. Our experience working with GPBP (Santillan-Doherty *et al*., 1996) and LGPBP (Olmos-Zúñiga *et al*., 1996), prompted us to think that LGPBP can be used safely as an intracardiac patch. We did not find any study evaluating the intracardiac use of LGPBP in the global literature. The aim of this work was to evaluate the behaviour and biocompatibility of LGPBP as a cardiac patch for ASD closure.

In the present study, the results of LGPBP patching are good, since there were no operative deaths, due to the characteristics of this bioprosthesis, which is easier to handle and to shape than autologous pericardium, GPBP and Dacron. There was no late mortality or clinical complications.

The finding of decreased ASD area in the left atrial septum of LGPBP patch could be explained as a result of healing contraction. The LGPBP is a biological prosthesis, it is subjected to wound healing process starting with absorption by the immune cells. The formation of fibrotic tissue is finally subjected to contraction. We can also establish the hypothesis that ASD area decreased as a result of atrial tissue growth or migration, using the LGPBP as scaffold, since our microscopic findings of heart muscle cells beneath the bioprosthesis as well as nests and "digitations" of atrial muscle beyond the atrial septum borders suggest that there is a migration or proliferation of the atrial muscle. This is different from pannus, since pannus formation is due to persistent neoinitimal development by protracted inflammatory response and is commonly seen after prosthetic valve replacement. Microscopically, pannus is formed by endothelial cells, chronic inflammatory cells (neutrophils, lymphocytes, plasma cells, macrophages, foreign body giant cells and mast cells) (Teshima *et al*., 2003). In contrast with Dacron, which is a synthetic material, it is not subjected to contraction during the wound healing process, and it is not used as scaffold. The shrinkage of a patch is of little or no importance in closure of simple defects – like ASD – that lie in a single plane (Crawford *et al*., 1986). We wonder if with longer study time in LGPBP group we could find a completed closed ASD area with own's patient atrial tissue, from the hypothesis that LGPBP can be used as scaffold for atrium tissue growth or migration. There are no reports of this finding in world's literature, and this subject deserves more research.

It is also important the finding that both patches suffered cartilaginous metaplasia. The LGPBP was replaced with dense well organized collagenous tissue and cartilagenous metaplasia, after 6 months of implantation. This is the result of wound healing process in which the collagenases produced by inflammatory cells and fibroblast play a key role in the degradation of collagenous implants (Wolley, 1984; Schlosser *et al*., 2005), like bovine pericardium. The fibroblasts form new collagenous tissue and suffer cartilaginous metaplasia. The Dacron patch is not absorbed, however induces an immunological response

by the host. The finding of cartilaginous metaplasia can be explained as follows: Dacron patch is a compound vascular graft constructed of woven polyester and bovine collagen. The woven polyester can not be digested by inflammatory cells, remaining unabsorbed. The bovine collagen part, which is biological in its origin, can be digested by inflammatory cells and collagenases, like other collagenous tissues, and with time suffers cartilaginous metaplasia (Wolley, 1984; Schlosser *et al*., 2005).

The degradation of bovine pericardium is retarded by glutaraldehyde treatment, and it is not changed by lyophilization, (Laun *et al*., 1990; Maizato *et al*., 2003; Zuki *et al*., 2007; Maizato *et al*., 2008). They also found that lyophilization decreases the inflammatory response. These are the reasons of finding LGPBP remnants after six months of implantation, which it is also subjected to collagenases action produced by inflammatory cells. In contrast, James *et al*. (1991) reported that lyophilized bovine pericardium without glutaraldehyde treatment, increases absorption rate, supporting the fact that glutaraldehyde treatment, decreases its antigenicity, delaying the digestion by inflammatory cells (Huang *et al*., 1990).

In spite that our microscopic findings did not shown any calcification area, we believe that cartilaginous metaplasia is the initial step to calcification. Bioprosthesis calcification can be a negative issue, but for single plane defects can be useful, since this calcified tissue gives support to hold low atrial pressures (McGoon, 1982). Calcification is not the *sine qua non* for bioprosthesis, since there are reports about calcification with autologous pericardium and synthetic vascular prosthesis when are used for patching (McGoon, 1982; Di Eusanio and Schepens, 2002; Hayabuchi *et al*., 2007; Us *et al*., 2004). Thus, every patient with a patch made with autologous pericardium, biological or synthetic prosthesis must be subjected to a close follow-up.

LGPBP is well incorporated into the atrial septum, covered with endothelium and lyophilization did not change the antithrombogenicity of GPBP, since we did not find any sign of thrombus formation, in spite of the avoidance of anticoagulation or antiplatelet treatment.

Conclusions

We can conclude that lyophilized glutaraldehyde preserved bovine pericardium provides excellent post-operative results, is effective for ASD closure, since it is well integrated into the atrial septum, decreases ASD area, develops a strong fibrous tissue and it tends to be non-thrombogenic. It posses excellent handling characteristics, is easy to prepare, to store, to transport and it is sterilizable.

The LGPBP awaits a prospective randomized study with long-term follow-up in order to prove that can be used as an scaffold by the native atrial muscle to close the ASD, as well as its superiority as a patch.

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Discussion with Reviewer

Reviewer I: Do you think that other biomaterials can be tested for comparison?

Authors: Yes, we are going to use autologous pericardium and glutaraldehyde-preserved bovine pericardium to compare them with LGPBP. We have already operated on two dogs using autologous pericardium.