# Bovine pericardium for heart valve bioprostheses: *in vitro* and *in vivo* characterization of new chemical treatments

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This study is aimed at investigating the bovine pericardium treated with different chemical procedures applied to prevent dystrophic calcification; decellularization of the fresh pericardium (samples B and C); fixation of the pericardium with glutaraldehyde (samples A, B, C, D and E); detoxification with aminoacids (samples A and B) and storage in a solution of benzoic acid esters. Pericardial sacs were harvested and delivered to the laboratories to be submitted to the chemical treatments. The samples E have been treated as the samples D but before the implantation they were exposed to the surgical lamp in order to promote some drying. The samples were tested for their mechanical properties and shrinkage temperature (at 1 week and after 36 months). The *in vivo* tests were performed by means of implantation in a paravertebral subcutaneous position in rats. At the explant (2, 4 and 8 weeks) the samples were submitted to histological assay and the calcium content determined by spectroscopic atomic absorption. All the samples showed loss of tensile strength and elongation at 36 months (except for the sample A), as well as a moderate diminution of the shrinkage temperature. The histology showed that the decellularized samples (B, C) were thicker than the others and the collagen fibres were extensively homogenated. The cell colonization was macrophagic for the samples A and D while it was also composed of giant cells in the samples B, C and E at 8 weeks. The von Kossa's staining was positive only for the samples D and E after 4 weeks of implantation. The calcium content of the samples D was 285.3 mg/g at 8 weeks while in E it was 44.4 mg/g dry tissue at 4 weeks; for the remaining samples the calcium content did not increase with the time (2.1–2.3 mg/g at 8 weeks). In conclusion, the pericardium decellularization and detoxification associated with its storage in a glutaraldehyde-free solution is a promising method in order to realize more durable pericardial bioprostheses. The investigated tissue treatments applied to the bovine pericardium permit removal of the calcium nucleation sites, and hence the avoidance of the severe drawback of the aldehyde crosslink, but at the same time maintain the necessary and well known tissue stability.

## 1. Introduction

Tissue degeneration associated with aggressive calcification strongly limits the durability of bioprosthetic valves manufactured using glutaraldehyde-fixed porcine aortic valves or bovine pericardium, especially in patients with an age below 50 years. In spite of many researches performed with the aim of avoiding or reducing dystrophic tissue calcification, tissue degeneration still remains the most frequent cause of clinical failure of bioprosthetic cardiac valves. The strategies studied for the improvement of tissue durability include processes based on pre-treatment of fresh pericardium, new alternative crosslinking methods and post-fixation procedures.

Within the pre-fixation treatments on fresh tissue the detergent extraction [1–5] is the most commonly applied. It consists in a single or multiple detergent extraction with the aim of dissolving and removing cell components, such as the phospholipids of the membranes and the nuclear proteins, considered to be the site of the first calcium nucleation.

Alternative crosslinking processes to the glutaraldehyde and formaldehyde ones are classified as zero crosslinking; they include acylation [6], treatment



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with epoxy compounds [7], phosphorilization [8], photooxidation [9], glycerol impregnation [10], and others.

Finally, post-fixation treatments are based on the principle of neutralizing free aldehyde radicals remaining after the classic crosslinking method with glutaraldehyde with aminoacids [11, 12] or alpha-aminooleic acid [13, 14]. Other substances aimed at preventing calcium salts precipitation have been experimented with, including diphosphonates [15] and metal ions [16].

## 2. Materials and methods

20 bovine pericardial sacs were accurately harvested at the abattoir under veterinary control following the validated procedures already established by Sorin Biomedica Cardio for Pericarbon valve manufacturing. The pericardial sacs were immediately rinsed with sterile saline and singly packaged in plastic bags filled with 4 °C sterile saline. All the harvested material was then immediately delivered in ice to the laboratories for the treatments [17]. Before the chemical treatments the pericardial fat was removed (under laminar flow hoods) and the sacs were submitted to further inspection in order to discard areas with heavy vasculature. The preliminary selection permitted recovery of 36 rectangular patches (100 × 50 mm).

### 2.1. Chemical treatments

The selected pericardial patches were treated according to the experimental design shown in Table I. The alternative chemical treatments consist in a decellularization method performed before any chemical crosslink reaction on the fresh tissue, and a procedure to be applied only after fixation. The samples processed with this last treatment were stored before use in an aldehyde-free medium.

#### *2.1.1. Decellularization*

The method consists in a selective removal of cellular components from the pericardial stroma, such as cell and nuclear membranes and the DNA proteins [1–3]. The applied procedure involves the use of two different detergents, Triton X100 (Sigma, St. Louis USA) and SDS (sodium dodecyl sulfate) (Sigma, St. Louis USA) [4, 5]. The Triton X100 is a non-ionic surfactant used to treat the fresh pericardium at a concentration of 0.1% in saline adding 1 mM of PMSF (phenyl methyl sulphonil fluoride) (EM grade, Fluka, New York USA) as proteinase inhibitor to prevent degradation of the extra-cellular matrix. The patches are immersed in the surfactant solution and soaked overnight at room temperature. This treatment is aimed at removing the cellular membrane and proteins by disrupting lipids. After the first detergent step, distilled water washing is carried out in order to remove traces of detergent and to prevent a possible blocking action to the second detergent used.

The second detergent treatment is performed with 0.5% SDS with 1 mM PMSF in saline for 72 h at room temperature. The SDS detergent is capable of dissolving the nuclear envelope and nuclear content and it unfolds the proteins, removing them. The SDS is then completely removed by several rinsings and washings with large amounts of distilled water. The decellularization process must be immediately followed by collagen crosslinking with glutaraldehyde.

TABLE I Experimental matrix of the chemical treatments

| Sample | Decellularization | Fixation | Detoxification | Storage |
|---|---|---|---|---|
| A | | GA | GLTA | PB |
| B | TX100-SDS | GA | GLTA | PB |
| C | TX100-SDS | GA | | GA |
| D | | GA | | GA |
| E | | GA | | GA |

TX100: Triton X100; SDS: sodium dodecyl sulfate; GA: glutaraldehyde; GLTA: glutamic acid; PB: benzoic acid esters.

#### *2.1.2. Crosslink*

Tissue fixation is carried out with GA (glutaraldehyde) (EM grade, Fluka, New York USA) at a concentration of 0.2% in phosphate buffer (pH 7.4) at room temperature for 24 h. When the crosslinking procedure has been terminated some samples were used for the detoxification process and others were stored in a 0.5% GA-buffered solution.

#### *2.1.3. Detoxification*

This process has been applied with the aim of saturating the free aldehyde radicals not engaged in the collagen crosslink reaction. The masking action of the detoxificant chemical is extremely important because it reduces the collagen affinity for calcium binding and furthermore erases the residual cytotoxicity [12, 18] responsible for blood cell damage. The process is performed by immersing the pericardium patches in a solution of GLTA (glutamic acid) (Merck, Darmstadt, Germany) [11] for 8 h. After detoxification treatment the treated pericardium samples were stored in an aldehyde-free medium composed of a mixture of two different esters of the benzoic acid (PB) (Sigma, St. Louis USA).

### 2.2. Mechanical and thermal stability tests

Prior to test of the mechanical stability each specimen was washed in 0.9% saline solution. A dumbbell specimen of tissue (15.9 mm wide and 38 mm long) was cut from each sample according to the Standard ASTM D1708. The tissue thickness was measured at three points for each sample using an oleo dynamic system with a Mitutoyo gauge. Each dumbbell was subjected to a tensile test in saline solution with an Instron Universal testing machine [19, 20]. The tensile strength ($N/mm^2$) and the elongation (%) at break were recorded.

For the thermal stability tests, tissue strips 8 mm wide and 30 mm long were cut from the rectangular samples, washed and stored in saline solution at room

temperature. The specimens were loaded to 95 g and held at constant load. The temperature was then linearly increased at 1 °C/min until the pericardium collagen denatured. The elongation was plotted against the temperature, and the sharp inflection point at which shrinkage occurred was recorded as the shrinkage temperature. The mechanical and thermal stability test were respectively performed within the first week after the tissue treatment and after 6 and 36 months of storage in an appropriate environment.

### 2.3. *In vivo* evaluation

The *in vivo* test was performed by means of subcutaneous implantation in rats. 18 male Sprague Dawley rats (220–240 g, 8 weeks old) were used. All the animals were handled and maintained in accordance with the requirements of the Laboratory Animal Welfare ACT (PL89-544) and KD (PL91-579) according the guide for Care and use of Laboratory Animals (DHEW, NIH Publication No. 78-23, Revised 1978).

Each rat was anaesthetized by Ketamine injection (Abbott) and the dorsal region was shaved and scrubbed before the sample implantation. Sterile 1.5 cm diameter round samples were accurately rinsed, soaking them in 250 ml of sterile saline. Through a paravertebral incision, four subcutaneous patches in 12 rats (A, B, C, D) and five in six rats (A, B, C, D, E) were implanted, taking care to randomize the relative position of the different specimens. For the last six rats the E sample was similar to the D sample, but was exposed to the surgical lamp before implantation in order to promote some drying of the pericardium. This was done in order to simulate water evaporation from the surfaces of leaflets during valve implant in an operating theatre. All specimens were implanted with the fibrosa surface towards the rat skin. The wounds were closed with surgical thread and topical antibiotic was administered. Explants were performed at 2, 4 and 8 weeks. The rats carrying the sample E were explanted at 4 weeks. The specimens, after a preliminary gross examination, were stored in fixative medium and then processed for histological evaluation. The most suitable stainings were hematoxylin-eosin and Von Kossa's (elective for calcium deposition). The histological analysis was performed evaluating important parameters that gave indications of the tissue bioreactivity to the different treatments. These parameters were sample thickness, cellular infiltration, capsule thickness (score 1–4), vascular proliferation and calcium deposition (histological appearance).

For calcium quantitative analysis the samples were dissected free from host tissue, dried overnight at 92 °C and then weighed (calcium content was referred to the dry weight). The pericardia were then dissolved by concentrated $HNO_3$ (Sigma, St. Louis, USA), and the calcium content, expressed in mg/g dry weight, was analysed by plasma spectrophotometric emission (Plasma 400, Perkin Elmer) at the laboratory of "Camera di Commercio" (Turin).

## 3. Results and discussion

The mechanical tests clearly showed a substantial decrease of tensile strength properties in all the tested samples after 36 months of storage. In the same way it has been verified that elongation at breakage is also diminished in all the tested pericardia. The loss of mechanical properties was less evident in sample A (Table II), which maintained good mechanical characteristics and almost fully preserved elongation properties (50.4 ± 10.4% one week after the processing versus 46.0 ± 8.1% after 36 months). The data indicate that the detoxification process significantly improves the mechanical characteristics of non-decellularized pericardium, both the decellularized samples also show better mechanical properties than the untreated ones.

The shrinkage temperature (ST) did not exhibit any significant decrease in value over time for all the tested samples (Table III). Non-decellularized tissues, detoxified or not (A and D), demonstrated a very similar ST that at 36 months of storage was unchanged. In contrast, the decellularized samples (B) and the decellularized and detoxified ones (C) had a ST after the treatment very close to fresh pericardium (ST ~ 60 °C). The initially observed lower values of ST for samples B and C did not significantly change after 36 months. This indicates that the decellularization treatment induces some stable modifications in the collagen structure which reflects in the ST but not in the mechanical characteristics; this seems to indicate that the decellularization protocol used was perhaps too severe.

The rats were regularly explanted and the specimens were removed taking care to harvest the surrounding fibrotic capsule. The gross examination of the explanted samples demonstrated a relatively thicker appearance of the decellularized samples (B and C); this observation was also evident after their laboratory

TABLE II Mechanical behaviours of chemically treated pericardia

| Sample | Tensile strength (N/mm²) | | Elongation (%) | |
|---|---|---|---|---|
| | $T_0^a$ | $T_1^b$ | $T_0^a$ | $T_1^b$ |
| A | 16.8 ± 3.8 | 10.7 ± 4.8 | 50.4 ± 10.4 | 46.0 ± 8.1 |
| B | 13.3 ± 1.5 | 7.1 ± 1.7 | 45.1 ± 3.6 | 37.5 ± 4.9 |
| C | 12.1 ± 2.8 | 6.7 ± 3.2 | 42.3 ± 5.4 | 36.4 ± 8.0 |
| D & E | 12.8 ± 4.8 | 5.1 ± 1.3 | 38.6 ± 9.9 | 33.0 ± 8.6 |
| Fresh | 9.1 ± 5.4 | – | 21.9 ± 6.1 | – |

[a] $T_0$: measured within one week after the process.
[b] $T_1$: measured 36 months after the process.

TABLE III Thermal stability of chemically treated pericardia

| Sample | Shrinkage temperature (°C) | | |
|---|---|---|---|
| | $T_0$ (1 week) | $T_1$ (6 months) | $T_2$ (36 months) |
| A | 81.4 ± 1.2 | 79.3 ± 1.0 | 82.1 ± 1.1 |
| B | 62.5 ± 2.1 | 61.2 ± 2.0 | 56.6 ± 1.9 |
| C | 64.3 ± 1.0 | 63.0 ± 0.6 | 63.3 ± 1.2 |
| D & E | 85.5 ± 0.6 | 85.2 ± 0.4 | 84.9 ± 0.3 |

Shrinkage temperature of fresh pericardium is about 60 °C.

treatment. The higher sample thickness of the samples B and C is possibly due to the surfactant action of the detergents that leave a softer pericardium stroma. This aspect should be accurately controlled because it potentially affects the haemodynamic performances of the manufactured bioprostheses; this may be related to the low ST measured on these samples.

The evaluation of the tissue response demonstrated a mild inflammatory reaction in all the pericardium samples. The cell colonization of the capsule and of the pericardial stroma was characterized by macrophages infiltration in all the samples at any explant time. The presence of macrophagic elements decreased with implant time, except for samples B, C and E where the initial cellularization was, in part, substituted by a mild presence of giant cells. Lymphocytes, plasma cells and polymorphonucleates were rarely present only at short implant time (2 weeks) while later on they disappeared from the tissues close to the samples. The histological examination of pericardium stroma showed collagen fibres homogenization of the decellularized samples B (Fig. 1) and C (Fig. 2) while the samples A (Fig. 3), D and E maintained their typical aspect (well evident collagen and elastic fibres

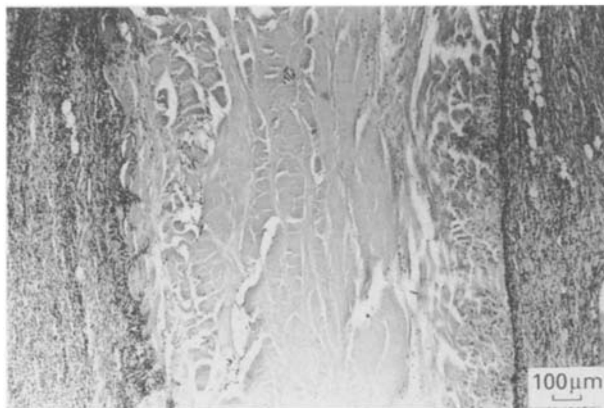


*Figure 1* Histology of sample B explanted at 2 weeks. The inflammatory reaction is evident with colonization cells inside the matrix. The collagen fibres do not show the typical crimping (von Kossa, 78X).

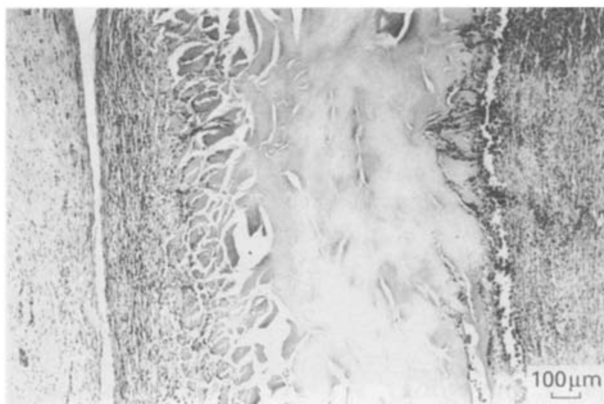


*Figure 2* Homogenization of the collagen fibres in a C sample after 2 weeks of implant. The collagen disruption is an artifact due to the histological technique (hematoxylin and eosin, 78X).

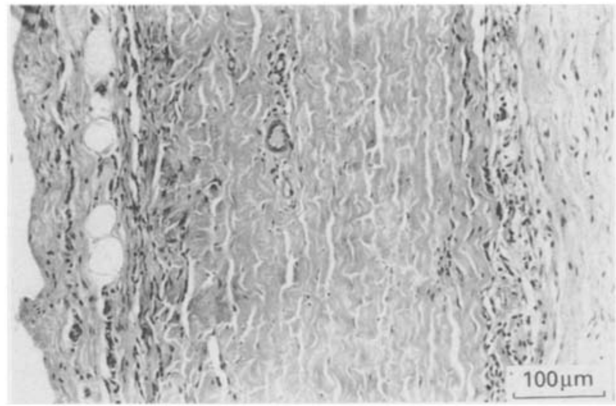


*Figure 3* Sample A after 4 weeks of implant. The pericardium stroma is fairly well preserved and the normal collagen crimping is visible (hematoxylin and eosin, 260X).

with natural crimping maintained). This fibre homogenization was also present before the implant and must be attributed to the detergent extraction (perhaps too severe). Capsule formation, although of different thickness, was evident in all the samples and it increased with implant time. Specimens A and E expressed a thickness score at 8 weeks of 1.5 and 2, respectively, while the samples B and C had a score of 3 and 3.5; the GA-treated samples D had a score of 2.5. The vascular proliferation of the fibrotic capsule and the pericardium stroma was also evaluated by means of a scoring system. Mild vascular proliferation was observed at 8 weeks for the samples A (1.3), D (1.3) and E (1.0) while for the decellularized samples B (1.5) and C (2.5) it was slightly more evident. Vascular proliferation into the decellularized pericardium stroma could be promoted by its structure; being softer it is prone to cell colonization and consequently to vascular proliferation.

The qualitative analysis of the calcium content was positive for the samples D (Fig. 4) and E. The results of von Kossa's staining was clearly positive for these samples after 4 weeks of implant while all the remaining ones were histologically negative. The elective calcium staining of samples D and E showed dystrophic calcification starting in the deep layers of the pericardium stroma without alteration of the collagen bundles. The quantitative calcium uptake (expressed as mg/g dry tissue) from the pericardial matrix at 2, 4 and 8 weeks was negligible in samples A, B and C while it became evident after 4 weeks in samples D and E (Table IV) confirming the histological findings. Samples A, B and C did not demonstrate progression of the calcium content at any implant time, while sample D increased its calcium content from 3.2 mg/g at 2 weeks to 285.3 mg/g at 8 weeks of implant. The variance analysis (ANOVA), performed for the differences in calcium content among the treatments at each implant time was statistically significant ($p < 0.05$) only at 8 weeks. The hypothesis of accelerated calcification of the pericardium due to the drying action of the surgical lamps during bioprostheses implantation has been simulated with sample E. These specimens explanted after 4 weeks have evidenced a calcium content of 4.4 mg/g dry tissue which is significantly lower ($p < 0.05$) than the corresponding not-dried

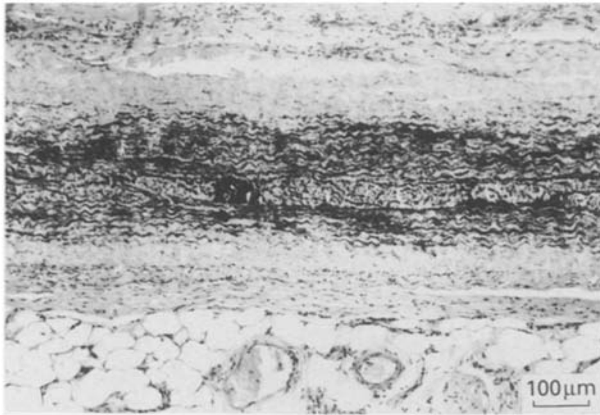


*Figure 4* Dystrophic calcium deposition in the inner layers of sample D at 8 weeks of implant. The calcification does not disrupt the collagen bundles that remain intact (von Kossa, 160X).

TABLE IV Calcium uptake from explanted pericardia (mg/g dry tissue)

| Sample | Implant time | | |
|---|---|---|---|
| | 2 weeks | 4 weeks | 8 weeks |
| A | 2.3 ± 0.7 | 2.3 ± 0.7 | 2.2 ± 0.3 |
| B | 2.0 ± 0.9 | 2.0 ± 0.6 | 2.3 ± 0.6 |
| C | 2.6 ± 0.9 | 2.2 ± 0.5 | 2.1 ± 0.2 |
| D | 3.2 ± 1.1 | 12.8 ± 20.4 | 285.3 ± 25.2 |
| E | | 4.4 ± 2.1 | |

Fresh tissue: 0.5 ± 0.47 mg/g dry tissue.

sample D at 4 weeks (12.8 mg/g). Verification of the above findings will require further investigation in a specifically designed experiment in which the degree and rate of drying and temperature will be properly taken into account.

## 4. Conclusions

The long-term storage (36 months) of crosslinked pericardium induces some loss of mechanical properties but this does not correlate with the stability and long-term resistance to fatigue of this biomaterial. In fact the durability tests performed on cardiac valves treated with the same process as sample D do not demonstrate significant differences when comparing two different storage times (1 week versus 36 months).

The decellularization method [1–5] seems promising; however, it induces some thickening and homogenization of the tissue and significantly decreases the ST; these effects, hopefully, can be avoided with a careful adjustment of the process condition. Its action in preventing dystrophic calcification is demonstrated with the results obtained from sample C.

Amino acids treatment of the pericardium as an aldehyde detoxificant [11, 12] is a very interesting approach because it inhibits the calcium intake, eliminates the residual tissue cytotoxicity and preserves, more than the other treatments, the mechanical properties of the pericardium.

The detergent extraction and detoxification procedures (sample B) can be joined together improving the long-term durability of the clinically implanted bioprostheses if an effective but milder detoxification process is developed to the point of maintaining a higher ST. The storage of detoxified samples in a solution of benzoic acid esters [11] appears to be a reliable and safe alternative to the classic aldehyde storage solution. This medium guarantees long-term sterility (confirmed at 36 months), stability and atoxicity of the treated biological tissues.

The investigated tissue treatments seem to be applicable to the bovine pericardium, allowing removal of the calcium nucleation sites, thus avoiding the most severe drawback of the aldehyde crosslink, but at the same time maintaining the necessary and well-known tissue stability.

## References


1. K. BRENDEL, R. C. DUHAMEL, J. M. MALONE and R. L. REINERT, "Vascular Graft Update: Safety and Performance, ASTM STP 898", edited by Helen E. Kambic, Adrian Kantrowitz and Pei Sung (American Society for Testing Materials, Philadelphia, 1986) p. 219.
2. J. M. MALONE, K. BRENDEL, R. C. DUHAMEL and R. L. REINERT, *J. Vasc. Surg.* **1** (1984) 181.
3. E. MEEZAN, J. T. HJELLE and K. BRENDEL, *Life Sciences* **17** (1975) 1721.
4. D. HIRSCH, J. DRADER, T. J. THOMAS, F. J. SCHOEN, J. T. LEVY and R. J. LEVY, *J. Biomed. Mater. Res.* **27** (1993) 1477.
5. N. R. JAFFE, W. D. HANCOCK, in Conference of the International Association for Cardiac Biological Implants, April 24, 1994, New York.
6. H. PETITE, I. RAULT, A. HUC, P. MENASCHE and D. HERBAGE, *J. Biomed. Mater Res.* **24** (1990) 179.
7. Y. NOISHIKI, H. SUNG, S. SHEN, C. HATA, M. ROWLAND, S. COULTER and R. QUIJANO, in Conference of the International Association for Cardiac Biological Implants, April 24, 1994, New York.
8. H. PETITE, V. FREI, A. HUC and D. HERBAGE, *J. Biomed. Mater Res.* **28** (1994) 159.
9. M. A. MOORE, I. K. BOHACHEVSKY, D. T. CHEUNG, B. D. BOYAN, WEN-MIN CHEN, R. R. BICKERS and B. K. MCILORY, *ibid.* **28** (1994) 611.
10. G. GONG, Z. LING, E. SEIFTER, S. M. FACTOR and R. W. M. FRATER, *Eur. J. Cardio–thorac. Surg.* **5** (1991) 288.
11. M. GRIMM, E. EYBL, M. GRABENWOGER, A. GRIESMACHER, U. LOSERT, P. BOCK, M. M. MULLER and E. WOLNER, *J. Thorac. Cardiovasc. Surg.* **102** (1991) 195.
12. K. LIAO, E. SEIFTER, D. HOFFMAN, E. L. YELLIN and R. W. M. FRATER, *Artif. Organs* **16** (1992) 267.
13. J. P. GOTT, PAN-CHIH, L. M. A. DORSEY, J. L. JAY, G. K. JETT, F. J. SCHOEN, J. M. GIRARDOT and R. A. GUYTON, *Ann. Thorac. Surg.* **53** (1992) 207.
14. M. N. GIRARDOT and J. M. GIRARDOT, in Conference of the International Association for Cardiac Biological Implants, April 24, 1994, New York.
15. R. J. LEVY, G. GOLOMB, J. WOLFRUM, S. A. LUND, F. J. SCHOEN and R. LANG, *Trans. Amer. Soc. Artif. Intern. Organs.* **31** (1985) 459.
16. C. L. WEBB, W. E. FLOWERS, J. BOYD, E. L. ROSENTHAL, F. J. SHOEN and R. J. LEVY, *ibid.* **36** (1990) 56.
17. A. R. MARANTO and F. J. SCHOEN, *J. Biomed. Mater. Res.* **22** (1988) 819.
18. E. GENDLER, S. GENDLER and E. NIMNI, *ibid* **18** (1984) 727.
19. R. VAN NOORT, S. YATES, T. MARTIN and M. A. BLACK, *Biomaterials* **3** (1982) 21.
20. C. A. PEREIRA, J. M. LEE and S. A. HABERER, *J. Biom. Mater. Res.* **24** (1990) 345.