

# Anti-calcifying treatment of glutaraldehyde fixed bovine pericardium: comparisons and evaluation of possible synergic effects

*Tratamentos anticalcificantes do pericárdio bovino fixado com glutaraldeído: comparação e avaliação de possíveis efeitos sinérgicos*

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## Abstract

**Objective:** To evaluate a possible synergic effect of the most promising anti-calcifying agents investigated in the literature by sequential treatment.

**Method:** Glutaraldehyde-fixed bovine pericardium was treated by: heating to 50° C, 80% ether, 0.125% chitosan, heparin, NaBH<sub>4</sub> and 4% formaldehyde. Samples were evaluated by optic microscopy, shrinking and mechanical tests and implanted subcutaneously in rats. Four months later the samples were explanted and Ca<sup>2+</sup> levels measured by atomic absorption spectroscopy.

**Results:** The Ca<sup>2+</sup> levels were (in µg/mg): control: 194.45; heating to 50°C: 6.87; ether: 3.69; chitosan: 68.89; heparin: 6.81; formaldehyde: 107.34; sequential treatment: 0.17. The

mechanical characteristics changed according to treatments employed.

**Conclusion:** Sequential treatment was effective to inhibit calcification and the tissue showed an adequate mechanical structure for the manufacture of bioprosthesis.

**Descritores:** Bioprosthesis, Glutaral, Calcinosis.

## Resumo

**Objetivo:** Avaliar possível efeito sinérgico dos agentes anticalcificantes mais promissores investigados na literatura, por meio de tratamento seqüencial.

**Método:** Pericárdios bovinos fixados com glutaraldeído foram tratados com: aquecimento a 50°C, éter dietílico

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80%, quitosana 0,125%, heparina, NaBH<sub>4</sub> e formaldeído 4%. Amostras foram avaliadas pela microscopia óptica, determinação da temperatura de desnaturação do colágeno e ensaio mecânico de tração e implantadas subcutaneamente em ratos. Após quatro meses do implante, as amostras foram explantadas e o conteúdo de Ca<sup>2+</sup> determinado pela espectrometria de absorção atômica.

**Resultados:** Níveis de Ca<sup>2+</sup> (em µg/mg): Controle -194,45; aquecimento a 50°C - 6,87; éter dietílico - 3,69; quitosana

- 68,89; quitosana+heparina - 6,81; formaldeído - 107,34; tratamento seqüencial - 0,17. O comportamento mecânico variou de acordo com os tratamentos empregados.

**Conclusão:** O tratamento seqüencial foi efetivo na inibição da calcificação e o tecido apresentou comportamento mecânico adequado à confecção de biopróteses.

**Descritores:** Bioprótese. Glutaral. Calcinose.

## INTRODUCTION

Biological tissues utilized in the manufacture of cardiac bioprostheses include the porcine aortic valve and bovine pericardium. Collagen tissues, however, present with rapid degeneration and need to be stabilized with the objective of making the structure resilient and prolonging original mechanical integrity and to remove or neutralize their antigenic properties so that they can be used as biomaterials.

Several chemical agents have been used in the treatment of collagen tissues [1-6] but glutaraldehyde, introduced by Carpentier [7], at the end of the 1960s, continues to be the widest employed.

Although treatment with glutaraldehyde makes the biological tissue adequate for the manufacture of bioprostheses, late calcification after transplantation is the most common cause of failure and continues to be a great challenge until today.

A study of calcification and the means to control or impede its development have been the subject of much research over the last two decades, presenting several possible effective candidates to interrupt or delay one or more stages of the process in small animal experiments [8-30].

The aim of the current study was to evaluate a possible synergic effect of anti-calcification agents described in the literature by means of a sequential treatment protocol. By sequential, we mean successive exposure of bovine pericardium treated with glutaraldehyde and several anti-calcification substances.

## METHOD

The strategy employed in the present investigation consisted of two stages.

The first was an evaluation of the anti-calcification effects of the different agents, with different mechanisms of actions

on the bovine pericardium, that, as it had been submitted to the same fixation technique using glutaraldehyde, allowed a comparative analysis in quantitative terms, of the Ca<sup>2+</sup> levels post-implantation, as well as its mechanical behavior.

The second stage was to evaluate the possible synergic action of several anti-calcification agents by means of sequential treatment.

A flowchart of the methodology employed is shown in Figure 1.

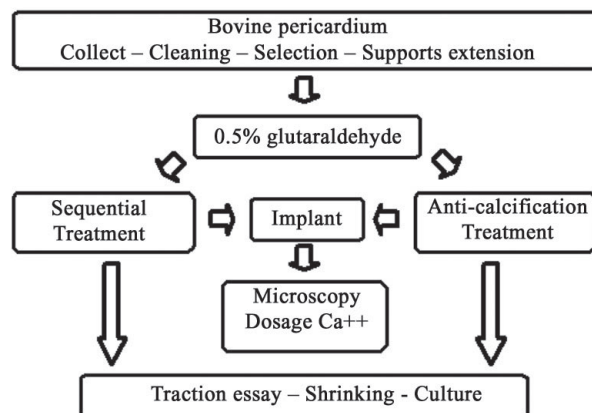


Fig. 1 - Flowchart of the method used

### Treatment of the bovine pericardium

The bovine pericardium was collected from an abattoir inspected by the Federal Inspection Service of the Ministry of Agriculture and the State Health Inspection Department immediately after the animal was slaughtered. Twenty-four membranes, with similar thicknesses and homogeneity, were stretched on plastic oval supports and fixed with a 0.5% glutaraldehyde solution at 4 °C and pH 7.4 with a phosphate buffer and kept in an illuminated atmosphere for 10 days with the solutions being exchanged after 24 and 72 hours.

The membranes were divided into eight groups according to the subsequent treatment:

- Group 1 - No treatment (Control Group);
- Group 2 - Thermal treatment at a constant temperature of 50°C in a bain marie for two days;
- Group 3 - 70% diethyl ether at pH 3.0 under continuous stirring at room temperature for 4 hours with the solution replaced four times.
- Group 4 - A 0.125% solution of chitosan at pH 6.0 and 4 °C for 72 hours;
- Group 5 - A 0.125% solution of chitosan at pH 6.0 and 4 °C for 72 hours, 0.1% solution of partially depolymerized heparin at pH 7.4 and 4 °C for 7 days and solution of 0.1M NaBH<sub>4</sub> at pH 8.8 and room temperature for 24 hours;
- Group 6 - Solution of 0.1M NaBH<sub>4</sub>;
- Group 7 - Solution of 4% formaldehyde at pH 5.0 and room temperature;
- Group 8 - Sequential treatment consisting of constant heat treatment at 50°C in a bain marie for two months, 70% diethyl ether at pH 3.0 under continuous stirring at room temperature for 4 hours with the solution replaced four times, A solution of 0.125% chitosan at pH 6.0 and 4 °C for 72 hours, 0.1% solution of partially depolymerized heparin at pH 7.4 and 4 °C for 7 days and solution of 0.1M NaBH<sub>4</sub> at pH 8.8 and room temperature for 24 hours and a solution of 4% formaldehyde.

To eliminate the chemical compound residues at the end of the treatment and between the sequential treatments, the membranes were rinsed in a solution of 0.9% NaCl under continuous stirring at room temperature for 24 hours with four successive exchanges of solution. On finishing preparation, the pericardia were kept in a 0.9% NaCl solution with exception of Group 8 which was kept in a 4% formaldehyde solution.

The formaldehyde was included in this investigation as the bovine pericardial heart valves are preserved in 4% formaldehyde after stabilization with glutaraldehyde.

#### **Partial depolarization of heparin**

The procedure utilized for the partial depolarization of

heparin was with 31,500 IU heparin and 10 mg of NaNO<sub>2</sub> in one liter of 0.9% NaCl solution; the pH was initially adjusted to 2.0 by the addition of 1M NaOH. The solution was kept at room temperature.

#### **Evaluation of the bovine pericardium**

The evaluation of the biochemical structural and functional integrity of the pre-implanted fixed pericardium was achieved by means of:

**Optical microscopy:** samples of each group were selected, stained using hematoxylin- eosin by the Von Kossa method and examined under an optical microscope;

**Shrinking test:** The abrupt shrinking temperature of the samples, which is the temperature required to free the H points of the triple helix and randomly change the configuration of the collagen, was the method employed to determine the degree of stabilization of the tissue after fixation. Temperatures greater than 82 °C assure an adequate fixation of the tissue. Five samples from each group, cut in strips of 3.0 x 1.0 cm, were immersed in a bain marie with a 0.9% NaCl saline solution and submitted to traction of 0.5g. The temperature of the bain marie was progressively increased at 4 °C/min until abrupt shrinking of the sample occurred;

**Traction trial:** Five samples of each group were submitted to a traction trial in a TNSTRON 4400R testing apparatus to determine the stretching and tension. The samples were cut in 3.0 x 0.4 cm strips fixed in the tweezers of the apparatus and immersed in a 0.9% NaCl saline solution at a constant temperature of 37 °C. An initial force of approximately 0.5g was exerted and the trial was performed at a constant velocity of displacement of 10 mm/min until the sample tore. Curves of force versus displacement were drawn. To calculate the traction tension, the initial area (width x thickness) of the samples was considered and the stretching was determined by the movement of the tweezers of the apparatus. To determine the thickness, the samples were placed between two glass slides and the measurement was taken using a digital pachymeter. Stretching was measured to the point of maximum tension of the tissue without tearing and the mechanical resistance was defined as being the value of maximum tension. The curve selected for analysis as representative of each group was the one closest to the mean value of maximum tension. Maximum tensions greater than 17.6 N/mm are considered adequate.

#### **Quantitative measurement of the Ca<sup>2+</sup>**

Five samples from each group were dehydrated in an incubator at 50 °C and mineralized in a Mufla oven at 800 °C. The mineralized samples were dissolved in 2.5M HNO<sub>3</sub>

and sent for quantitative measurement of the  $Ca^{2+}$  by atomic absorption spectrometry using a Perkin Elmer Analyst 100 spectrometer at a wavelength of 422.7 nm. The calibration curve was obtained using 1000 mg/L of standard Perkin Elmer solution with the addition of 1% lanthanum chloride. The total quantity of  $Ca^{2+}$  was expressed as mg per mg of dry tissue.

### Implant in rats

Twenty-four Wistar rats, with a mean age of 30 days, were utilized in the experiment. The animals were obtained in the animal house of the Institute of Energy and Nuclear Research, where they were treated following the norms of the Research Ethics Commission. The norms published in the Guide for the Care and Use of Laboratory Animals and the experimental principles for animal experimentation of the Brazilian College of Animal Experimentation (COBEA) were respected.

The animals were anesthetized using 70% diethyl ether, dichotomized and six samples of bovine pericardium from each group measuring 2.0 x 1.5 cm were implanted at dissected subcutaneous sites in the dorsal region of the rats (two per animal).

Four months after the implant, the animals were sacrificed and the samples explanted and washed in 0.9% NaCl saline solution after removing the host tissue.

Five samples from each group were dehydrated, mineralized and dissolved in  $HNO_3$  according to the previously described technique and sent for quantitative measurement of  $Ca^{2+}$  and the rest were prepared for optical microscope evaluation.

### Statistical analysis

The data are represented as means and standard deviations (SD). The differences between the groups were tested employing the Student t-test and the results were considered significant when the p-value < 0.05.

## RESULTS

### Optical microscopy

The structural integrity of the collagen of the pre-implant samples did not differ among the different groups; the preservation of the collagen structure was homogenous. Of the explanted samples, calcification of the tissue was assessed in relation to the treatment used. Variability in the mineral deposited was seen among the different groups, among animals of the same group and to a lesser degree among different regions of the same sample, identifying regions where the calcification process was more accentuated than others.

In samples submitted to the sequential treatment, deposits of calcium were not identified (Figure 2).

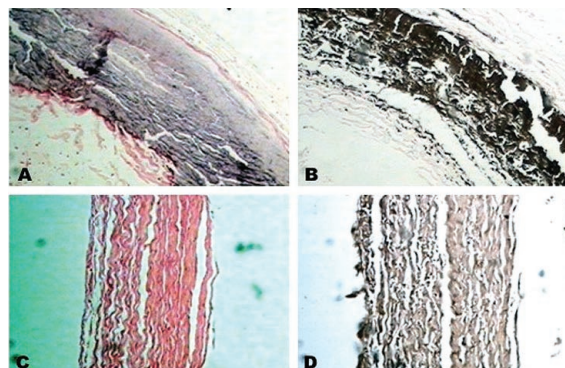


Fig. 2 – Micrographs of the explanted bovine pericardium samples of the Control and sequential treatment Groups  
A. Control Group - Hematoxylin-eosin. B. Control Group - Von Kossa. C. Sequential Treatment - Hematoxylin-eosin. D. Sequential Treatment - Von Kossa. Magnification 80x

### Shrinking test

All the samples presented with temperatures higher than the 82 °C considered adequate for fixed bovine pericardium indicating adequate fixing of the tissue.

### Mineral Analysis

The quantitative measurements of  $Ca^{2+}$  obtained from samples from the different groups after implant are presented in Table 1.

Table 1. Measurement of  $Ca^{2+}$  in the post implant samples obtained using the different treatment techniques

Treatment	$Ca^{2+}$ (µg/mg)	$\sigma$ (µg/mg)
Control	194.45	19.50
Temperature	6.87	2.35
Ether	3.69	1.26
Chitosan	68.89	57.01
Chitosan + heparin	6.81	3.17
Formaldehyde	107.34	49.88
$NaBH_4$	79.43	52.43
Sequential	0.17	0.04

### Mechanical behavior

The mechanical behavior of the samples submitted to tension (stretching and maximum tension) varied according to the treatment employed. The tension curves versus deformation after the anti-calcification treatments are presented in Figure 3.

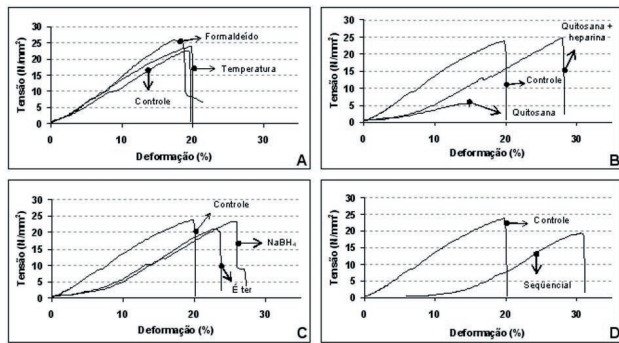


Fig. 3 - Tension x deformation curves obtained from the different treatment techniques used

## DISCUSSION

### Optic microscopy

The variability of the mineral deposit observed among the different groups, among animals implanted from the same group and, to a lesser degree, from different regions of implantation reflect the observation in medical practice where a percentage of heart valves present with intense calcification years after implantation, whilst others present with small areas of calcification or even no calcification identifiable by optical microscopy. An analysis of the results, however, is not prejudiced by this variability, as it is reflected in the standard deviation of the mean employed in the statistical analysis.

### Mineral analysis

**Temperature:** The thermal treatment at 50 °C was effective in inhibiting calcification of the implanted samples. The measurement of  $Ca^{2+}$  was  $6.87 \pm 2.35$  mg/mg vs.  $194.45 \pm 19.50$  mg/mg for the Control Group, a value very similar to that reported by Carpentier et al. [25] in samples submitted to heat treatment and explanted after three months of subcutaneous implantation in rats ( $6.7 \pm 2.3$   $\mu$ g/mg vs.  $245.4 \pm 4$   $\mu$ g/mg in the Control Group).

**Diethyl ether:** Treatment with ethylic ether was efficient in inhibiting calcification of the implanted samples. The measurement of  $Ca^{2+}$  was  $3.69 \pm 1.26$   $\mu$ g/mg versus  $194.45 \pm 19.50$   $\mu$ g/mg in the Control Group. Rossi et al. [21] studied the effects of acidified sulfuric ether in the

calcification of bovine pericardium, initially treated with 2.5% glutaraldehyde for two hours and subsequently with 0.5% glutaraldehyde buffered with 0.13M (pH 7.4) after subcutaneous implantation in rats for up to 28 days. There was a significant reduction in the calcification as seen by optical microscopy in the samples treated with sulfuric ether.

The results observed by microscopy in the current investigation were higher than those obtained by Rossi et al. [21] with absence of calcium deposits after four months of implantation.

**Chitosan:** The level of  $Ca^{2+}$  in the samples treated with chitosan were significantly lower than in the Control Group ( $68.89 \pm 57.01$  mg/mg vs.  $194.45 \pm 19.50$  mg/mg, respectively). Chanda et al. [19] employed 4% chitosan for three days at 4 °C in the treatment of bovine pericardium previously treated with 0.25% glutaraldehyde for around two months. The level of  $Ca^{2+}$  after subcutaneous implantation in rats for up to three months was  $1.10 \pm 0.27$   $\mu$ g/mg versus  $200.8 \pm 17.5$  mg/g in the Control Group.

The current investigation, although anti-calcification effects were confirmed using chitosan as previously reported, showed significantly high levels of  $Ca^{2+}$  after four months of implantation to form hydroxyapatite crystals as identified by optical microscopy. Thus, the utilization of chitosan as an anti-calcification agent in isolation with bovine pericardium did not prove to be an adequate treatment for the manufacture of heart valves.

**Heparin:** The anti-calcification effect of heparin depends on the intermediate binding substrate used as not all aminated compounds equally reduce calcification [14]. As each chitosan molecule allows the binding of multiple molecules of heparin, its use allows much incorporation of the heparin in the tissue which increases the steric effect.

As the reaction between the aldehyde groups and the amino groups of the heparin molecule leads to the formation of Schiff bases which are unstable,  $NaBH_4$  was employed to converting them into more stable secondary amino acids.

Chanda et al. [26] utilized heparin with bovine pericardium treated with increasing concentrations of glutaraldehyde (from 0.1 to 0.25%) at 37 °C for one month. The binding substrate employed was 0.1% chitosan and 0.015% gentamicin applied to the tissue for one week at room temperature. The reaction period of the 0.1% heparin with the tissue was one week at room temperature. The results obtained after subcutaneous implantation in rats for five months demonstrate a reduced level of  $Ca^{2+}$  in the group submitted to heparin ( $0.652 \pm 0.240$  mg/g) when compared to a control group ( $228.32 \pm 37.39$  mg/g).

The current investigation confirmed the anti-calcification effects of heparin using chitosan as a binding substrate. An

important confirmation was highlighted with the analysis of the results in respect to the potentialization of the anti-calcification effects exerted by the incorporation of heparin in the bovine pericardium treated with chitosan. There was a significant reduction in the levels of  $\text{Ca}^{2+}$  of the tissue treated with chitosan with the addition of heparin ( $68.89 \pm 57.01$  mg/mg vs.  $6.81 \pm 3.17$  mg/mg, respectively).

*NaBH<sub>4</sub>*: The level of  $\text{Ca}^{2+}$  with the use of  $\text{NaBH}_4$  was  $79.43 \pm 52.43$  mg/mg, thereby proving its anti-calcification effect.

In the literature, references on the use of  $\text{NaBH}_4$  are associated with the incorporation of heparin to the tissue due to its capacity to convert the unstable Schiff bases in more stable secondary amino acids. The use of  $\text{NaBH}_4$  as an anti-calcification agent in isolation, even though this has not been investigated previously, is justified by its property in converting free aldehyde groups on the surface of the tissue to hydroxyl groups, neutralizing them.

*Formaldehyde*: The level of  $\text{Ca}^{2+}$  with the use of formaldehyde was  $107.34 \pm 49.88$   $\mu\text{g}/\text{mg}$  vs.  $194.45 \pm 19.50$   $\mu\text{g}/\text{mg}$  in the Control Group. This significant reduction in the levels of  $\text{Ca}^{2+}$  was not expected considering that the inclusion of 4% formaldehyde is due not to its possible anti-calcification properties but to the fact that heart valves made from bovine pericardium are preserved in this solution after fixing with glutaraldehyde.

The preservation in 4% formaldehyde has not been duly valorized in previous studies of post-implant calcification. Although they did not report on the respective levels of  $\text{Ca}^{2+}$ , Gong et al. [30] reported less calcification with preservation of bovine pericardium treated with 0.625% glutaraldehyde and 4% formaldehyde for 24 hours when implanted in rats for 70 days. This difference, however, was not significantly different.

*Sequential treatment*: As calcification is a multifactorial process, anti-calcification agents with different mechanisms of action were used in the sequential treatment.

The post-implant levels of  $\text{Ca}^{2+}$  confirmed the synergic effect of the anti-calcification agents employed ( $0.17 \pm 0.04$   $\mu\text{g}/\text{mg}$  in the sequential treatment vs.  $194.45 \pm 19.50$   $\mu\text{g}/\text{mg}$  in the Control Group).

### **Mechanical behavior**

In the analysis of the mechanical behavior of the samples submitted to the different anti-calcification treatments, the purpose of the pericardium must be taken into account. Hence, generically, greater stretching and mechanical resistance of the tissue are preferred in the manufacture of heart valves.

Thermal treatment did not significantly change the

mechanical behavior of the tissue and is thus considered adequate. Deformation and mechanical resistance were similar to those observed for the Control Group (Figure 3A).

The sample treated with formaldehyde was also considered adequate. The samples presented with deformation similar to the Control Group and a greater mechanical resistance (Figure 3A). The sample treated with chitosan, on the other hand, did not present with an adequate mechanical structure. Although there was an increase in the deformability, there was a large reduction in the mechanical resistance when compared to the Control Group (Figure 3B).

The incorporation of heparin caused significant differences in the mechanical behavior of the tissue. The mechanical behavior was considered adequate, with increases both in the deformation and the mechanical resistance when compared with the Control Group (Figure 3B).

Thus, incorporation of heparin significantly altered the mechanical behavior of the tissue, transforming a totally inadequate tissue after being submitted to chitosan into a tissue with the best mechanical properties of all the tissues studied here.

The sample submitted to diethyl ether presented with an adequate mechanical behavior. There was an increase in the deformation and a slight reduction in the mechanical resistance when compared to the Control Group (Figure 3C).

The mechanical behavior of the sample treated with  $\text{NaBH}_4$  was considered adequate. Its use as an anti-calcification agent in isolation caused an increase in the deformation of the sample without changing the mechanical resistance when compared to the Control Group (Figure 3C).

The mechanical behavior of the sample treated by the sequential technique was considered adequate (Figure 3D). The sample presented with a greater deformation than that observed in the Control Group and the mechanical resistance was higher at  $17.6$  N/mm<sup>2</sup> (a value considered adequate for fixed bovine pericardium).

Although mineralization seen experimentally in subcutaneous implants is similar to mineralization seen clinically with explanted bioprostheses, the extrapolation of the results should be made with due care, as in this model the tissue is not subjected to dynamic stress or interactions with blood elements, a factor that has been proved to influence the calcification of cardiac bioprostheses.

### **CONCLUSIONS**

The synergism most wanted from the proposed sequential

treatment really occurred. The treatment was effective in inhibiting calcification of the bovine pericardium treated by glutaraldehyde over 4 months of subcutaneous implantation in rats and the tissue presented an adequate mechanical behavior for the manufacture of heart valves.

Another conclusion that may be deduced from the mechanical behavior of the samples is that the mechanical trials should be routinely used in the evaluation of bovine pericardium when submitted to anti-calcification treatment.

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