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An anti-calcifying system. An option against calcification of bioprostheses

Un sistema anticalcificante. Una opción contra la calcificación de las bioprótesis

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ABSTRACT

Bioprosthetic heart valves are currently the best option for replacing a damaged heart valve. It has been known for several years that calcification (mineralization) represents the main drawback of bioprosthesis. Accordingly, to address this issue, our institution –over 35-year experience in bioprosthesis production– performed research and developed an anticalcification system for bioprosthesis. Our bioprostheses were initially made of duramater and, afterwards, of bovine pericardium. The latter was treated for preservation with glutaraldehyde, a solution that optimally prepares the biological tissue; however, it attracts calcium. The proposed and tested system consists of adding glycine, a simple amino acid, to the general treatment. This treatment prevents calcium from adhering to the biological tissue, which results in a longer functional lifetime than that achieved with traditional treatment. Following satisfactory *in vitro* and *in vivo* studies, we proceeded to the clinical phase with 1,362 prostheses successfully implanted so far in all positions within a 10-year period.

Keywords: anticalcification treatment, cardiac bioprosthesis, bovine pericardium, duramater, glutaraldehyde, glycine.

RESUMEN

Las bioprótesis cardiacas son en la actualidad la mejor opción para sustituir una válvula cardiaca enferma. Desde hace varios años se sabe que el principal problema de las bioprótesis es la calcificación (mineralización). Por tanto, en nuestra institución, donde se tiene ya experiencia de 35 años en la elaboración de bioprótesis, se investigó y desarrolló un sistema para tratar de proteger nuestras bioprótesis de este problema. Nuestras prótesis originalmente se hicieron con duramadre y posteriormente con pericardio bovino preservadas con glutaraldehído, solución que prepara bien al tejido biológico, pero que tiene el inconveniente de atraer calcio. El sistema propuesto y probado fue el de agregar un aminoácido sencillo, glicina, al tratamiento general. Este tratamiento impide la adhesión de calcio al tejido biológico, permitiéndole un tiempo de vida funcional más prolongado que con el tratamiento convencional. A partir de estudios in vitro e in vivo muy satisfactorios, se pasó a la etapa clínica, en donde hasta el momento se han implantado 1,362 prótesis en todas las posiciones con excelentes resultados a 10 años.

Palabras clave: *tratamiento anticalcificante, bioprótesis cardiacas, pericardio bovino, duramadre, glutaraldehído, glicina.*

In general terms, the use of cardiac prostheses can be considered relatively new, when compared to other types of surgeries. It also applies for cardiac bioprostheses. Since the 1970's, given the multiple problems of mechanical prostheses, homografts were considered as an option in the treatment of some heart valve diseases.[1,2] Over the years, from a hemodynamic point of view, bioprostheses have shown superior qualities than mechanical prostheses. The latter ones

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have a series of drawbacks and limitations, such as the fact that, because of not operating with a single and central outlet flow, they cause exaggerated shear stress (turbulence),[3-7] which finally may be the origin of thrombus formation, with inherent consequences such as impingement on the prosthetic tilting discs, and systemic embolism.[8,9]

On the other hand, anticoagulation can lead to the opposite effect, resulting in risk of hemorrhages.[10-12] These problems can sometimes be a small one, such as those called strands,[13-16] a phenomenon that involves the formation of threads or strands of thrombi that may become emboli and migrate toward the brain. This is particularly important since it has been shown that a large clot is not mandatory at all to produce brain injury. Lifelong anticoagulation has been used in order to avoid this serious problem. In our socio-economic environment, all the above mentioned represents a serious drawback. Not all patients who receive this type of prosthesis have the economic capacity and knowledge to be disciplined in the use of oral anticoagulation. By the same token, a large number of patients with cardiac valve prostheses live in remote locations, with difficult access to medications, in addition to poor INR monitoring.

Furthermore, mechanical prostheses that were initially reputed to be "eternal" have shown important weaknesses in their structure and functioning. In addition to the foregoing, the prosthetic discs do not open and close simultaneously, but often move at different times, increasing the degree of turbulence and therefore the possibility of thrombosis.

Opposed to normal heart valves, mechanical valves do not have a single flow channel, but rather have several and this favors turbulence. Hence, the possibility of thrombosis with this type of prosthesis is notably increased.

To complicate the matter, they may suffer a structural deterioration called cavitation,[17-20] where the coating of the discs is damaged, causing an irregularity in that place that, in turn, is the focus of platelet adhesion and ultimately of thrombi.

There also exists another abnormal phenomenon which is the growth of a new tissue adherent to the ring of the prosthesis with extension towards the mobile discs of the mechanical prostheses, called pannus.[21-25] Limited and even impeded excursions of the tilting discs may result in collapse of the valve function and fatal consequences.

On the contrary, cardiac bioprostheses practically maintains the normal flow of the natural leaflets, providing a single, central, and Newtonian-type flow. All the above prevents all the consequences noted for mechanical prostheses. Furthermore, in cases of structural failure, the possibility for acute failure is somehow minimal, and it allows the patient to have a period of several days to go to a medical center to be treated. Anticoagulation is not essential in patients who are carriers of biological prosthesis. Thus, the quality of life is better than those with mechanical prosthesis.

Much has been written about the durability of both types of prostheses. It was previously suggested that mechanical prostheses were for the whole life of the patient, and biological prostheses had a very short useful life, mainly due to the calcification of the leaflets. However, advancement in bioprosthesis design, by having better preparation, by using new materials for their support apparatus, and especially by adding a protection system, has ended up in a longer lifespan, as much as or even better than mechanical prosthesis. It might as well be the main reason why nowadays mechanical prostheses have been replaced by biological ones practically all over the world.

A few countries, including Mexico unfortunately, cling to the outdated idea that mechanical prostheses are better and more durable. However, experience has shown the opposite. In fact, mechanical prostheses have been replaced by the undeniable advantages of biological prostheses, to the extent that very serious entities such as the American College of Cardiology (ACC) and the American Heart Association (AHA) accept that the most recommended and judicious is to use bioprostheses in the majority of cases, even in young people,[26] as demonstrated by the very recent work of a series of hospitals in England and Ireland, currently being used in almost 80% biological versus 20% mechanical. According to AHA/ACC, in the period spanning from 1999 to 2002, the proportion of implanted mechanical prosthetic decreased from 41 to 33%, with a consequent increase in biological prosthesis from 50% to 65% in the same time frame.[27]

Another rigorous demonstration about this change from mechanical to biological prostheses is represented by Starr's work in 2007. Between 2003 and 2005, the trends reached 80% for the use of bioprostheses in aortic position.[28] Unfortunately, in our country, the situation regarding the use of bioprostheses is exactly the opposite, something totally unexplainable and unjustifiable. An important exception to the above can be found in our institution, where the proportion in recent times for biological prostheses usage is roughly 80%.

In the history of cardiac prostheses, various stages were-described as a part of the development of biological prostheses; namely, heterografts (porcine),[29,30] homografts,[31,32] duramater,[33-35] fascia lata,[36-39] antibiotic-based preparations,[40] and other substances such as chromium mercury, formalin, glycerol[41,42] with somewhat discouraging results. In the 70's, Dr. Alain Carpentier used glutaraldehyde (GA) as a mechanism to more safely prepare these biomaterials.[43,44]

Bovine pericardium is the biological raw material most frequently used for the manufacture of cardiac bioprostheses. The main component of the pericardium is type I collagen, an extracellular matrix protein belonging to a large family of proteins, closely related to each other, known as the collagen superfamily, of which to date have been described in detail at least 17 different genetic types. In the particular

case of type I collagen, there is a generic composition that can be described as the repetition of the Gli X-Y sequence, where the X position is almost always occupied by a proline or hydroxyproline residue, and the Y position is frequently occupied by lysine or hydroxylysine; although this Y position is more variable and can be occupied by other amino acids. Of these 17 types of collagen, 6 are the most important and frequent, and of them, type I is the most numerous and frequent one. Given the evidence that the biological tissues in bioprostheses end up calcifying, many researchers have taken on the task of investigating the causes of the above as well as the shorter duration of bioprostheses. These investigations have clarified several points; nonetheless, not all the mechanisms leading to the aforementioned calcification are totally known. Even so, much progress has been made in this field.[45,46]

GA has been identified as a good tissue preparer. It helps to fix and align collagen fibers, making it less antigenic, more resistant and maintaining elasticity.[47] Additionally, it sterilizes biological tissue.

At the same time, alongside these beneficial effects of GA, it is now known that its molecules that remain in the tissues attract calcium that ends up fixing to the bioprosthesis.[48,49] The GA solution aims to fix the tissue, so this solution acts mainly on lysine, hydroxyline, proline and hydroxyproline residues, forming Schiff bases and causing cross-linking of collagen molecules (chemicals bridges) which gives it greater resistance. However, the aldehyde groups that did not react remain free and can react very easily with primary amino groups present in practically all proteins. The secondary amino groups are predominantly found in cationic form at neutral pH and therefore the changes in the net charge of the protein are considerably smaller, making them less reactive.

With this preparation, which guarantees that the collagen fibers are aligned in the tissue and chemical bridges are formed between the various layers of it in the pericardium, it is ensured that the tissue prepared with GA is ductile and resistant, in addition to being sterilized in prostheses manufactured with this treated tissue, but they will always have the threat of calcification.

Due to the process currently followed for the conservation of the pericardium used for bioprostheses, the presence of reactive aldehyde groups, at the time of application, is imminent. For these reasons, these groups function as centers in which molecules present in the plasma, including some cations, can be easily deposited. Therefore, one of the main problems that bioimplants currently present is their tendency to calcify relatively easily and out of control. This calcification could be due, at least partially, to the mechanism explained above.

On the other hand, the use of GA as a protein coupler is widely known and used in a wide variety of biochemical and immunological techniques. A prime example is the method described by Avrameas to conjugate enzymes to antibodies, or that described by Kishida to couple ferritin or that used by Nicholson to also conjugate hemocyanin to antibody molecules. In all these processes it is essential to eliminate the functional aldehyde groups that remain after conjugating the proteins, given that in most cases, these conjugates will be placed in the presence of other proteins that are potentially reactive due to the presence of free amino groups, chiefly primary aminos. To avoid this undesirable reaction, the aldehyde groups must be blocked. This can be achieved by means of using various amino acids, proteins or substances containing free primary amino groups. This is possible thanks to the presence of amino groups in high concentrations, which interact and completely exhaust the reactive aldehyde groups.

With this preparation, which guarantees that the collagen fibers are aligned in the tissue and bridges are formed between the various layers of it in the pericardium, it is guaranteed that the tissue prepared with GA is ductile and resistant in the prostheses manufactured with this treated tissue, but they will always have the threat of calcification. For this reason, we focused on searching for a protective mechanism by experimenting both *in vitro* and *in vivo* by adding to the tissue after preparation, an amino acid, glycine, which is the simplest of the amino acids since it only has one free aldehyde, and by covalent substitution with the GA of the bovine pericardium, eliminates the possibility that its free aldehydes can bind to other substances as they are saturated and neutralized with glycine and there are no free aldehydes in the pericardium where calcium or other substances could be fixed (calcium, cholesterol, iron, lipids, etc.). Therefore, it inhibits calcification, thereby prolonging the lifetime of the prostheses treated in this way.

In light of these data and with the previous clinical experience that bioprostheses do indeed calcify, it exists a current consensus that these prostheses must be protected against calcification. Structural valve deterioration conditions, such as leaflet thickening, failure in movement, and even rupture are factors that may promote the need for reoperation. *Figure 1* shows a bioprosthesis with thickened leaflets, calcified and even with rupture of one of them. Thus, it is clear that since biological prostheses are not protected against calcification, they will inevitably end up suffering structural deterioration and shorter lifespan. When some type of protection is used, the clinical follow-up of bioprostheses treated with some anti-calcifying system has shown good results. The durability of bioprostheses with this treatment has significantly improved. In the 80's, when bioprostheses had fewer years of implantation and no anti-calcification system was included, the average survival of bioprostheses was 8-10 years. By improving the designs, materials and, by adding an anti-calcification system, this survival is spanning today

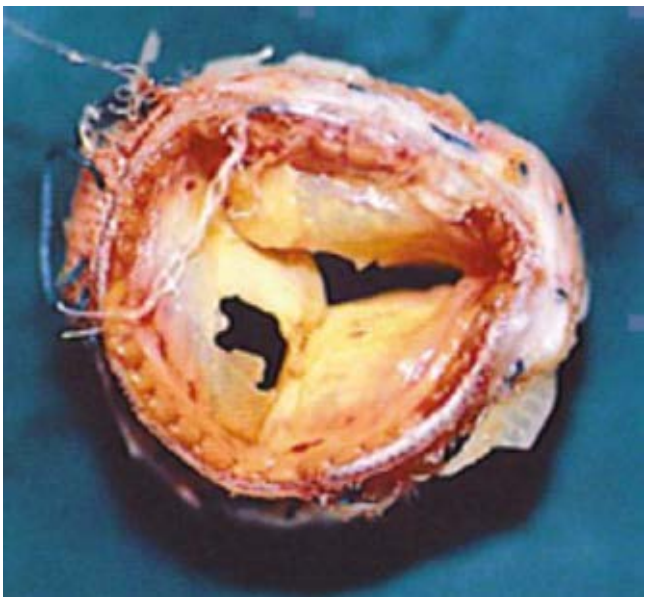

Figure 1: Dysfunctional bioprosthesis.

between 15 and 18 years after implantation, almost 2-fold as a result of the application of all these systems.

At our institution, with the application of this method and 1,362 prostheses placed in 10 years, none of these prostheses has suffered clinical deterioration requiring reoperation. Our results are similar to those previously reported in the literature. Up to date, several techniques have been developed and used to mitigate, reduce or avoid the calcification of bioprostheses. In clinical practice, four major ideas and methods have been applied for this purpose. These methods can be grouped as follows:

1. Removal of lipids (phospholipids, cholesterol, etcétera).
2. Covalent substitution (INC).
3. Detergents.
4. Removal of GA (INC investigation).

The first of them (A) is important because the normal lipid components in the pericardium and practically any tissue can attract calcium from the bloodstream, initiating the first stage of calcification, nucleation. The substances most used for this purpose are ethanol, methanol and chloroform.

The second one (B) is also highly important, since the GA with which the pericardium is impregnated, through a chemical reaction, can unite its free amino groups with those of the circulating calcium, thus causing the beginning (nucleation and continuation) of the phenomenon of dystrophic calcification. If these possible unions are covered with other substances (in this case an anti-calcifying system), they can no longer be used by calcium. The most used substances for this purpose are iron, aluminum and some amino acids. This mechanism is possibly the best and most effective to avoid calcification.

The third of them (C) has a similar purpose to the previous one and the most used substances are sodium dodecyl sulfate, aminoleic acid and L-glutamic acid. Its effect is not as comprehensive as the previous one.

The fourth one (D) is another mechanism to remove practically all the GA from the beginning, seeking to ensure that the possibility of calcium adhesion is minimal. This method implies i) leaving the GA in the tissue for the shortest possible time and therefore the GA concentration will be lower, which means that the possibility of calcium adhesion is minimal, ii) use glycine from the beginning, and iii) do not store the bioprosthesis in aldehyde until its clinical use (which can last months or years and therefore redeposit more aldehyde in the tissue) a method that as a whole protects it from having aldehyde on its surface again and therefore less possibility of calcification. Currently, all the commercial brands that use any of the anticalcification methods, even if they apply it in a professional and strict manner, all end up storing their product in some aldehyde until its clinical use. However, when the prostheses are placed again for months or years in aldehyde, the amount of this that adheres to the tissue easily exceeds the level of protection it previously had, and again there are many suitable amino sites to bind calcium. Finally, the protective effect turns out to be no longer effective.

MATERIAL AND METHODS

The method to follow was to carry out the process for this protocol *in vitro* and also *in vivo*. In the *in vitro* process, the tissue used (bovine pericardium) was placed in a 0.5% glutaraldehyde solution at a pH of 7.2 at time intervals that were 24 hours, 7 and 15 days; once prepared with GA, the pericardium was washed with 0.9% physiological solution and then treated with a 5% glycine solution for 24 hours at a temperature of 4 ºC. The pericardium was cut into segments of 1 cm square and was stored in a solution with 50% glycerin at room temperature until subcutaneous implantation in guinea pigs *(in vivo)*, which were divided into four groups of six animals each with the treatment shown in *Table 1*.

Table 1: Treatment of the pericardium in subcutaneous implantation in Guinea pigs.

Group	Treated with 0.5% glutaraldehyde	Treated with 0.5% glycine, (hours)
A	24 hours	24
B	07 days	24
C	15 days	24

Two groups of experimental animals were formed. The first of them included 3 subgroups (A, B, C) in which all the samples that were implanted were treated with GA: group A was in it for 24 hours, group B for seven days and group C for 15 days. The twenty-four female Hartley strain guinea pigs were six-week-old with a weight of 375 g on average. On the same day, all animals were anesthetized with ether and an incision was made in the mid-dorsal part of approximately 1 cm, through which the sample was placed subcutaneously; A mark was made for each group of animals according to the implant. The permanence period of the implant was 30, 90 and 180 days, sacrificing two animals from each group in each of these periods and thus removing the implant for its corresponding study.

RESULTS

The following is shown in *Figure 2*: in *Figure 2A* a rectangular segment of bovine pericardium treated only with GA is observed. *Figure 2B and C* show how the glycine-based treatment completely eliminates the amount of glutaraldehyde that can subsequently be a potential source of calcium fixation.

The analysis of these *in vitro* tests showed that the amount of GA molecules that remain in the tissue thus treated and that can potentially attract other calcium molecules once placed in the patient, practically disappear with the glycine-based treatment. As shown in *Figure 2A*, the amount of GA accumulated in the tissue is very important and the staining demonstrates this. Exposure time of this pericardium was 24 hours, treated only with GA. In *Figure 2B*, exposure time to glutaraldehyde was 7 days, but when it was also treated with glycine for 24 hours, it is shown how the amount of residual glutaraldehyde has decreased considerably. In *Figure 2C*, where the time of exposure to GA was 15 days, when it is subjected to the anti-calcifying treatment with glycine, the residual GA is practically zero.

Next step was to extract the pericardium segments implanted in the guinea pigs in order to analyze whether calcium had been deposited in them. This is done in equipment (atomic absorption photometer) with a special preparation, which reports the amount of calcium that has accumulated in each pericardium sample. The results were reported in mg/g of dry tissue, based on the previously found basis that the pericardium contains, per se, 2.83 mg/g, as shown in *Table 2*.

The results in *Table 2* demonstrate that in group A, treated only with GA but without the base of the anti-calcifying system (glycine) from the beginning, despite the short permanence time of the tissue in GA, the amount of accumulated calcium is already a little higher than that of the pericardium (2.98 versus 2.83) and these quantities increase after 14 days to 9.35 in the animals that had the sample for a month. From group B, the pericardium was also treated only with GA for thirty days, the samples showed even higher figures, 3.96, 8.12 and 12.15. In group C sacrificed at six months, these measurements of the amount of accumulated calcium were 4.83, 15.25 and 22.05, which is much greater than the original of the tissue, an unequivocal sign of the attraction mechanism of both the tissue itself and the GA remaining in the pericardium.

On the contrary, in the pericardium group treated with GA, but with the addition of the anti-calcifying system (glycine), these figures were surprisingly lower. In the first group (A), sacrificed after a month, the calcium report was only 0.399, 0.670 and 1.03, figures that, as can be seen, are even below normal. At three months, these figures have increased slightly to 0.61, 0.927, and 1.20, being this observation the same as for the one-month group. And finally, with the group sacrificed at six months, the samples report calcium quantities of 0.69, 1.01 and 1.52, respectively.

Figure 2: A) (24 hours) shows a rectangular segment of bovine pericardium treated only with 0.5% glutaraldehyde. B) 7 days. C) 15 days with the treatment based on 0.5% glycine for 24 hours. Residual glutaraldehyde is practically zero.

Table 2: Calcium concentrations*.

Guinea pigs sacrificed at	Exposure time to glutaraldehyde	Pericardium treated with glutaraldehyde without glycine	Pericardium treated with glutaraldehyde with glycine
One month	24 hours	2.98	0.399
	7 days	4.03	0.67
	14 days	9.35	1.03
Three months	24 hours	3.96	0.61
	7 days	8.12	0.927
	14 days	12.15	1.26
Six months	24 hours	4.83	0.69
	7 days	15.25	1.01
	14 days	22.05	1.52

* Calcium amounts are expressed in mg/g of dry tissue.

DISCUSSION

There is no doubt about the preponderant role played by GA in the preparation of biological tissues. On the one hand, it allows the fixation of collagen, making the tissue strong and ductile enough to be used in cardiac bioprostheses. In addition, it sterilizes the tissue, a discovery by Carpentier in porcine bioprostheses, and later asserted by many others in bovine pericardium.[43,44] At the same time, while ensuring these properties, GA entails the peril of becoming a pole of attraction for calcium, and this will translate into rigidity of the prosthesis, even rupture, which considerably reduces the useful life of the biological prostheses *(Figure 1)*.

Beyond a shadow of a doubt, bioprostheses have excellent hemodynamics, due to their ability to maintain a central and unique flow, which leads to a quality of life very close to normal, without noise that disturbs the patient (as happens with mechanical prostheses), without the mechanical gradient, without the absolute need for anticoagulation (which is one of the several serious drawbacks that make mechanical prostheses undesirable, or at least riskier). On the other hand, as formerly mentioned, there is the always latent danger for bioprosthesis, especially in young patients, of the aforementioned calcification.

Therefore, finding a mechanism that ensures that bioprostheses do not calcify, or at least that it happens as late as possible, is a premise today. With this procedure that we present here, it is clearly shown, both *in vitro* and *in vivo*, how glutaraldehyde, in addition to fulfilling its purpose of preparing and sterilizing biological tissue, is practically eliminated. In this way, it prevents calcification of biological prostheses. Thus, the real possibility opens up for the use of this type of prosthesis in both young as well as in older patients, with the certainty that the feared phenomenon of calcification has decreased dramatically.

CONCLUSION

We can say that bioprostheses are hemodynamically superior than mechanical prostheses. Nevertheless, their useful life can be shortened due to the calcification process caused by the attraction of calcium ions due, in turn, to the use of glutaraldehyde for their preparation and deposited into their collagen. This system developed at our institution (INC) and tested *in vitro* and *in vivo*, reduces the possibility of calcium aggregation in such a way that calcification is minimal or at least considerably stopped, thereby lengthening the useful life of the bioprostheses. Notably important is to highlight the fact that this system described herein makes bioprosthesis available and appropriate even for young patients. This procedure has now moved into the clinical stage. In the last 10 years, 1,362 prostheses treated with this system have been placed, without any reoperation due to calcification having been reported so far. This will be the subject of another further report.

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