Prevention of calcification with TPEN in pericardial bioprosthetic heart valve material

Biyoprostetik kalp kapağı materyalinde kalsifikasyonun TPEN ile önlenmesi

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ABSTRACT

Objective: Calcification is a frequent cause of the clinical failure of bioprosthetic heart valves fabricated from glutaraldehyde pretreated bovine pericardium. The major object of the present study is to prevent calcification of pericardial bioprosthetic heart valve materials with TPEN.

Methods: Bovine pericardium was cut into 2-cm^2 pieces, rinsed in phosphate-buffered saline solution, transferred into +4°C phosphate-buffered saline containing 0.625% glutaraldehyde for initial fixation for 48 h, and allocated into two groups. Control samples were treated in an identical fresh solution for five more days. Others underwent additional fixation in phosphate-buffered saline 2µM TPEN for 48 h. They were then transferred into phosphate-buffered saline + 0.625% glutaraldehyde solution at 37°C (pH 7.4) for three more days. Pericardial patches were inserted into the dorsal pouches of 18 juvenile male Wistar rats as control and study groups. Rats were divided into two groups and sacrificed consecutively by the end of 9th and 12th weeks. The biomechanical properties and calcium contents of explanted tissues were tested and were also assessed histopathologically.

Results: The difference in the calcium contents of the control and study groups' pericardial tissues at the 9th, and 12th weeks were statistically significant (p=0.0001, p=0.0001). The comparison of calcium contents between controls of 9th and 12th weeks and study groups' of the 9th and 12th weeks pericardial tissues were also significant (p=0.0001 and p=0.0001). Histopathologic and biomechanical assessment also supported these findings.

Conclusion: Calcific degeneration of glutaraldehyde-fixed bovine pericardium can be reduced by using TPEN without any effect on durability. *(Anadolu Kardiyol Derg 2007; 7: 365-70)*

Key words: Calcification, bioprosthetic heart valve, glutaraldehyde, TPEN

ÖZET

Amaç: Kalsifikasyon, sığır perikardından glutaraldehid ile muamele edilerek elde edilen biyoprostetik kalp kapaklarında görülen klinik yetmezliğin en sık rastlanan nedenidir. Bu çalışmanın temel amacı biyoprostetik kalp kapaklarında görülen kalsifikasyonun TPEN ile önlenmesinin araştırılmasıdır.

Yöntemler: İki santimetrekarelik parçalara ayrılmış sığır perikardından elde edilen parçalar, fosfat tamponlu salin solüsyonuyla yıkandıktan sonra, ilk fiksasyon için +4 C°'de bulunan %0.625 glutaraldehid içeren fosfat tamponlu salin solüsyonunda 48 saat bekletildi ve 2 gruba ayrıldı. Kontrol grubu örnekleri, aynı özellikteki yeni hazırlanmış solüsyonla 5 gün muamele edildi. Diğerlerine 2µM TPEN içeren fosfat tamponlu salin solüsyon 48 saat ek fiksasyon için uygulandı. Bu parçalar +37 C°'de bulunan %0.625 glutaraldehid içeren fosfat tamponlu salin solüsyonda (pH 7.4) 3 gün için nakledildi. Perikart parçaları juvenil 18 adet erkek rat'ın (Wistar) sırtına iki taraflı çalışma ve kontrol grubu olarak yerleştirildi. Sıçanlar iki gruba ayrıldı ve 9. ve 12. haftaların sonlarında ardı sıra sakrifiye edildiler. Çıkartılan perikart dokularının kalsiyum içerikleri ve biyomekanik özellikleri ölçüldü ve histopatolojik olarak araştırıldı.

Bulgular: Dokuzuncu ve 12. haftalardaki kontrol ve çalışma grupları karşılaştırıldığında kalsiyum içerikleri istatistiksel olarak anlamlıydı p=0.0001, p=0.0001). Kontrol grubunun perikart örneklerinin ve çalışma grubunun perikart örneklerinin 9 ve 12. hafta kalsiyum içeriklerinde farklıydı (p=0.0001, p=0.0001). Biyomekanik ve histopatolojik incelemeler de bu bulguları destekliyordu.

Sonuç: Glutaraldehid ile fikse edilmiş sığır perikardiyumundaki kalsifik dejenerasyon dokunun dayanıklılığını etkilemeksizin TPEN ile azaltılabilir. *(Anadolu Kardiyol Derg 2007; 7: 365-70)*

Anahtar kelimeler: Kalsifikasyon, biyoprostetik kalp kapağı, glutaraldehid, TPEN

Introduction

The main clinical limitation in the use of heart valve bioprosthesis is calcification that eventually leads to device failure (1, 2). Despite the clinical importance of the problem, the pathogenesis of calcification is incompletely understood. The earliest events in calcification of bioprosthetic tissues are hypothesized to result from glutaraldehyde (GA) induced cellular devitalization, leading to disruption of cellular calcium regulation (3). Calcium ions may concentrate on the interior aspect of membrane by binding to acidic phospholipids and calcium-binding proteins. Inorganic phosphate ions, produced by phosphatases, combine with calcium ions onto the cell membrane and serve as nucleators. Initial calcification deposits eventually enlarge and coalesce,

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resulting in grossly mineralized nodules that stiffen and weaken the tissue and thereby give rise to an improperly functioning prosthesis (3, 4). As an ongoing effort to find a proper strategy to prevent bioprosthetic tissue calcification, various agents have been tested for their anticalcification activity. The most promising preventive strategies have included binding of calcification inhibitors to GA fixed tissue, removal or modification of calcifiable components, modification of GA fixation and use of tissue cross linking agents other than GA (5-12). As a part of our continuing research program investigating the anticalcification methods for bioprosthetic heart valve materials, we have recently reported the effects of the chelating agents ethylenediaminetetraacetic acid (EDTA) and citric acid (CA) that, under appropriate conditions, diminished calcification from the pericardial tissues (13, 14). We now report a novel lipid-soluble divalent metal chelator N,N,N',N'-Tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), that crosses artificial and natural membranes, and can be used as an anti-calcification agent similarly to EDTA but at significantly lower concentrations (15, 16).

Thus, by using a rat subdermal implantation model TPEN was used to treat pericardial tissues after fixation with the GA and the amount of calcific material that accumulated within the tissues was investigated biochemically and histopathologically. Biomechanical tests were also applied to the tissues to find out TPEN's effects on the durability as well.

The aim of the present study is to prevent calcification of pericardial bioprosthetic heart valve materials with TPEN.

Methods

Tissue preservation

Conventionally preserved pericardium, consisting of freshly excised bovine pericardium, had been dissected free from a dhering fat tissue and cut into 1 cm^2 pieces. They were then rinsed in a phosphate-buffered saline solution (PBS; 0.1 M, pH=7.4) and then transferred into +4°C PBS containing 0.625% GA for an initial fixation period of two days. The GA solution was prepared using a standard 25% commercially available solution (Merck Darmstadt, Germany). Pericardial samples were subsequently transferred into appropriate solutions for further fixation. The weight: volume ratio was 1 g per 30 ml fixation solution. The different groups were defined as follows:

Control group (CON)

After the initial (two-day) fixation, tissues were placed into fresh solutions with the same properties and temperature. Fixation was allowed to continue for five more days at +4°C (for a total of 7 days).

Study group (STU)

After the initial (two-day) fixation, tissues were placed into PBS solution at 37°C containing 2μM TPEN (to which 1 M NaOH was added to maintain the pH at 7.4) for a period of 48 h and then transferred into freshly prepared PBS + 0.625% GA solution at 37°C for further three days (for a total of 7 days).

Subcutaneous implantation of samples in rats

All animal studies conducted were in strict accordance with the Helsinki Declaration, and approved by the Ethics Committee of our institution. Eighteen male Wistar rats (body weight 50-75 g) were kept in standard laboratory cages for 9-12 weeks with free access to standard laboratory diet and water before the beginning of the experiment. The light/dark cycle was 12 hours, relative humidity was between 45-55% and the temperature was kept between 22-25°C. In order to investigate calcification rate, pericardial patches were inserted into the dorsal pouches of 18 anesthetized Wistar rats. Pericardial pieces were randomly assigned into two groups (18 pieces per group). All specimens were rinsed (3 x 10 min for each piece) in saline by agitating prior to implantation, and each animal received both types of patch. By the end of nine weeks after implantation, nine of the animals were sacrificed with an overdose of sodium pentobarbital (100mg/kg). The implants were explanted and rinsed with saline solution. Each explanted tissue sample was cut into three equal pieces; one piece was further processed for the measurement of calcium content using atomic absorption spectrophotometry (AAS), the second piece was prepared for histopathological examination and the last piece was prepared for biomechanical testing. These groups of pericardial tissues were named as CON9 and STU9. The same procedures were applied to the rest of the rats by the end of the 12th weeks and the prepared pericardial tissues were named as CON12, and STU12.

Measurement of tissue calcium levels

Following storage at -20°C, tissues were dried at 104°C for 24 h, weighed, ashed in a muffle furnace at 200°C for 12 h, and dissolved in 3 M HCl (10 mg dried tissue/ml HCl). The solution was transferred quantitatively to a 25-ml volumetric flask and diluted with 0.36 M HCl. For the determination of calcium content, the sample was diluted 1:50 with 0.1% (w/v) lanthanum chloride. A 1000 μg/ml stock calcium standard solution (Sigma C-5649 for atomic absorption spectrophotometer) was diluted with 0.1% (w/v) lanthanum chloride to prepare standard calcium solutions of 0.5, 1.0, 1.5 and 2.0 μg/ml. A solution of 0.1% (w/v) lanthanum chloride was used as a blank. Standards and samples were measured using AAS, with detection at 422.7 nm (17). Calcium levels were expressed as mg per g tissue dry mass.

Measurement of pericardium biomechanics

The biomechanical properties of pericardial tissues were investigated using a tensile testing machine (MAY03, USA) equipped with a 50 kg load cell. The tensile loading speed in all tests was 1mm/s. Data were transferred to the computers translating to the numerical signals by 16 bit A/D converter for off line analysis. The sampling rate was chosen as 1000 sample/s. Load-displacement data were recorded using BIOPAC MP 100 Acquisition System Version 3.5.7 (Santa Barbara, USA). These recordings were normalized by cross-sectional area and this curve was converted to a stress-strain curve. Stress/strain curves for each specimen were generated and the maximum tensile stress, maximum strain and toughness were determined. The stress (Megapascal=MPa) was calculated by dividing the failure load by the cross-sectional area of the specimen. The maximum strain was calculated as the displacement of the specimen divided by the initial gauge length (in mm/mm). The area under the stress-strain curve is defined as toughness, which is the energy required to cause breaking of the pericardium (MPa).

Morphological assessment of tissue calcification

For transmission electron microscopic evaluation rest of the pericardial samples were fixed with 2.5% glutaraldehyde, post fixed with 1% osmium tetroxide, dehydrated in graded alcohol series, cleared with propylene oxide and embedded in epon. Thin sections (50-70 nm) were cut by a microtome (Leica UCT-125) and contrasted

with uranyl acetate and lead citrate. Sections were examined and photographed by an electron microscope (JEOL JEM-1011).

Statistical Analysis

SPSS for Windows version 9.0.0" (SPSS Inc, 1989–1999, Chicago, Il, USA), a package program was used for all statistical analyses. Student's t test was used to calculate the differences obtained in tissue calcium levels and biomechanical tests. P-values <0.05 were accepted to be significant. The results are expressed in terms of mean±SD.

Results

Calcification of the implanted pericardial tissues

The calcium content of the TPEN treated pericardial tissues was significantly lower than the controls in all groups (Table 1). The difference between each control and study group within the same time interval was statistically significant (p=0.0001 and p=0.0001 respectively). The comparison of calcium contents between CON9 and CON12 and STU9 and STU12 showed that the difference between 9th weeks against 12th weeks were significant (p=0.0001 and p=0.0001 respectively).

Biomechanical properties

Biomechanical properties of CON and STU groups are listed in Table 2. No significant differences in maximum stress, maximum strain and toughness were observed in STU and CON groups in 9th weeks (p=0.950, p=0.460 and p=0.999, respectively) and in 12th weeks (p=0.999, p=0.994 and p=0.999, respectively). The comparison of maximum stress, maximum strain and toughness between CON9 and CON12 (p=0.986, p=0.077 and p=0.874, respectively) and STU9 and STU12 (p=0.999, p=0.655 and p=0.812, respectively) showed that there were no statistical differences between 9th weeks and 12th weeks.

Results of the morphological analysis

STU 9: There were structurally well preserved fibroblasts among collagen fibers. In the transverse sections of these collagen fibers, mild calcification areas were observed (Fig. 1A). In some fibers, calcification was particularly segmental. In these calcified areas striation of collagen fibers was visible (Fig. 1B).

CON 9: There were fibroblasts displaying irregular cell contours and they had abnormally dilated GER cisternae with amorphous dense materials in them. The surrounding collagen fibers were calcified and fragmented. Dense arrangement of collagen fibers had disappeared and loosened. The amount of calcification also increased (Fig. 2A). Fibrils which were considered to form due to disaggregation of the three dimensional structure of the collagen fibers were found between collagen fibers (Fig. 2B).

STU 12: Thinning occurred especially in the calcified collagen fibers (Fig. 3A). Cell degeneration, decondensation of the nuclear chromatin structures, disappearing of the cell contours and abundant cell debris were also found. However, there was no evident calcification in the cell structures. The majority of the cell organelles were extremely degenerated (Fig. 3B).

CON 12: More severe collagen fiber calcification than other groups and collagen fiber irregularities were observed. There were fragmentations in the calcified areas (data not shown) and thinner fibrils which were thought to form due to disaggregation of the three dimensional structure of some collagen fibers than previous groups (Fig. 4A). The existence of excessive degenerated cells, decondensated and scattered nuclear chromatin and the calcifications on membrane structures of these cells were observed (Fig. 4B).

Table 1. Calcium contents of the explanted pericardial tissues for both control and study groups at 9th and 12th weeks

Calcium Content, µg/mg Tissue		
	9. week	12. week
CON	126.42±17.21	171.71±23.70
STU	60.88±14.97	85.78±18.95
CON- control group, STU- study group		

Table 2. Presentation of the data regarding stress, strain and toughness for both control and study groups at 9th and 12th weeks

Variables	9. week		12. week	
	CON	STU	CON	STU
Maximum stress, MPa	7.65±3.06	10.28±3.68	9.53±3.84	9.23±2.97
Maximum strain, %	21.0±8.0	15.0±5.7	12.0±0.60	10.0±0.50
Toughness, MPa	0.84±0.30	0.86±0.55	0.50±0.12	0.51±0.25
CON- control group, STU- study group				

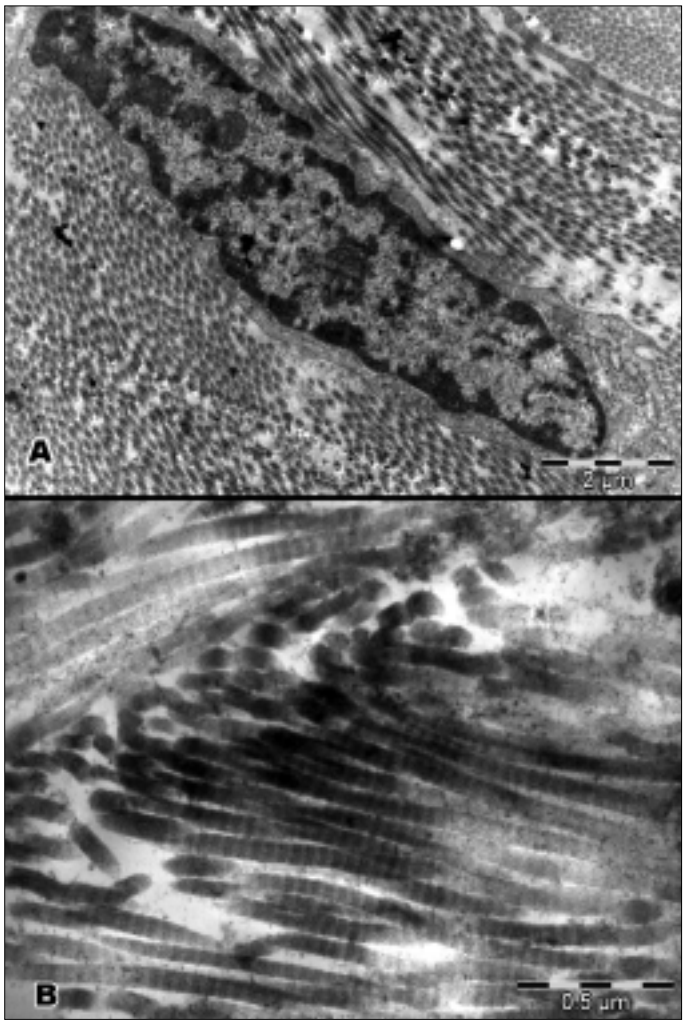

Figure 1. A. Well preserved fibroblast and, slightly calcified collagen fibers (X12000). B. Some collagen fibers showing segmental calcification (X60000)

Discussion

On the one hand, freed calcium in bioprosthetic materials fixed with GA, forms the calcium phosphate hydroxyapatite crystals (3). On the other hand, it leads to disintegration and destruction of the cells by activating matrix metalloproteinases and oxidative system (18-21). In time, the clinical outcome of this process is the calcific degeneration, insufficient and improper functioning of bioprosthetic bovine pericardial valvular prosthesis (1, 2). The same mechanisms are applicable for the calcification encountered in peripheral or coronary arterial atherosclerotic diseases. Chelates, especially EDTA, remove calcium from the atheromatous plaques by binding the calcium in the plaque and by blocking the proteolytic enzymes and the oxidative system (22, 23). In this aspect, it has been demonstrated to be effective in the treatment of cardiovascular system's atherosclerotic diseases and has been used in the USA (24). In an experimental study by Schick et al. (25) oral alkaline citrates have been shown to be effective in the reduction of calcification areas in the media layers of the abdominal aortas of the rats and impaired the maturation of the hydroxyapatite and their deposition in the arterial tissues.

In our previous studies, we managed to decrease the calcific degeneration in bovine pericardium by treating the tissues with EDTA and citric acid (13, 14). An advantage of TPEN over EDTA and CA, TPEN is a lipid-soluble material; we therefore hypothesized that we would end up with better results in the anticalcification of the pericardial tissues. However in this study, as time passed calcification was observed in both the study and the control groups. Calcium levels in the study group were significantly lower than in the control group by the end of the 9th and 12th weeks but this decrease in calcification in the study group, contrary to our anticipation, reached only to the amount of 50% reduction of calcium compared to the control group. In conjunction with less calcification in the study group, collagen fibers preserved their structures by the 9th week, but there were breakdown of the fibers in the calcified areas. The cells and their organelles were observed in well preserved to be preserving their integrity (Fig. 1A). Although fibrillary and cellular structures were relatively well preserved in the study group, fragmentation and disintegration in calcified collagen fibers, severe membrane degeneration and calcification in cells of the control group were observed (Fig. 1B). By the 12th week in the study group, thinning in some

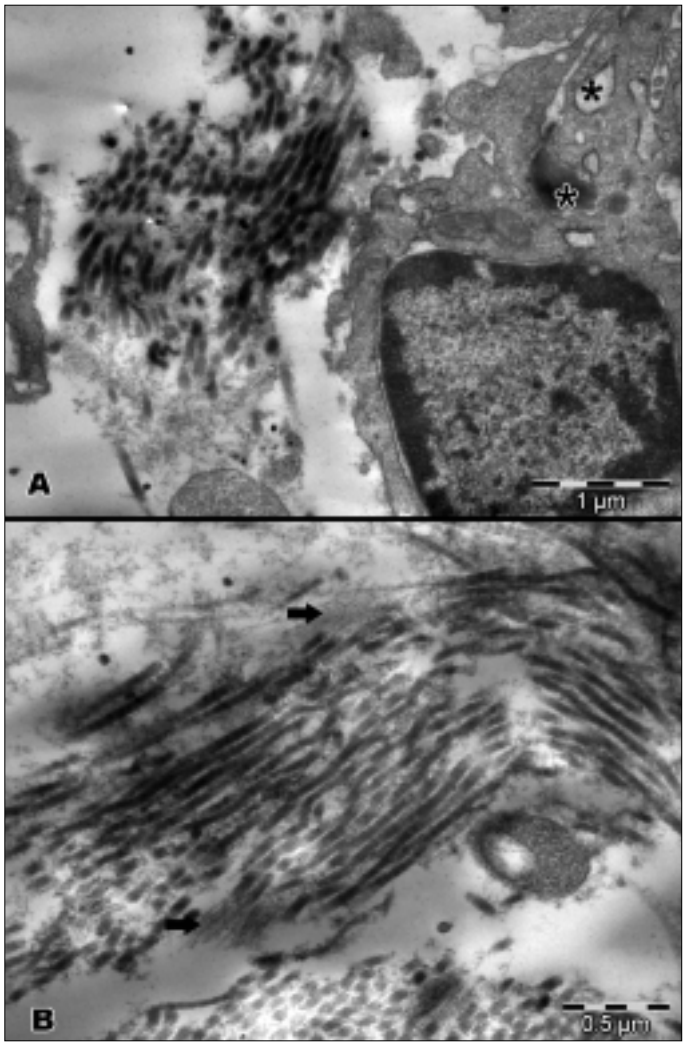

Figure 2. A. An irregularly contoured fibroblast and its dilated GER cisternae (asterisk) (X25000). B. Fibrils formed by disaggregation of calcified collagen (arrow) (X40000)

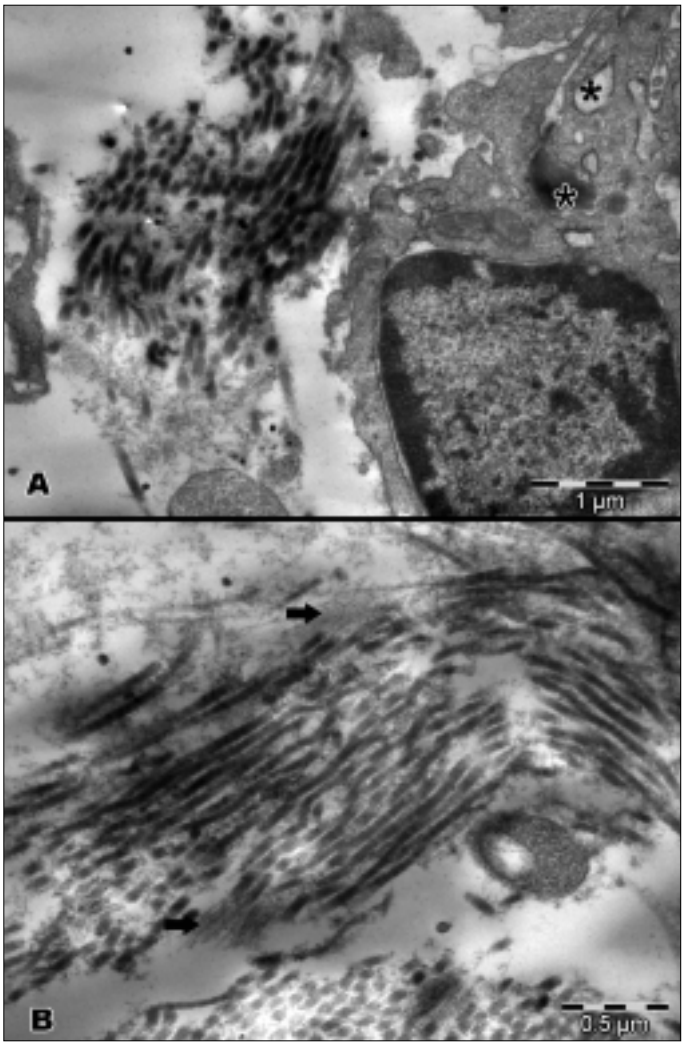

Figure 3. A. Thinning of calcified collagen fibers (arrow) (X30000). B. Degenerated cell, cell debris and decondensated nuclear chromatin (asterisk) (X5000)

calcified collagen fibers structure disappeared and severe degeneration in the cells and their organelles were also observed, and the cells and cells' organelles displayed deformation. In contrast to this, there were no calcifications in the cellular structures (Fig. 2A). In comparison with the study group at the 12th week, we noted heavier calcification and disintegration in the collagen fibers and more severe decondensation with scattering of nuclear chromatin (Fig. 2B). As to the control group specimens, we observed disintegration in the collagen fibers and the cells and noted the commencement of heavier calcification by the 9th week. In addition to these pathologic findings, nuclear chromatin integrity was also compromised by the 12th week.

The function of pericardial bioprostheses and their performance are known to depend on the mechanical properties of the pericardium. Strain, stress and toughness are important biomechanical parameters on pericardium strength and they give a clue about collagen integrity. In spite of obtaining a 50% decrease in calcification and better preservation of the integrity of the collagen fibers and the cells, contrary to our anticipation, we failed to demonstrate an improvement in the biomechanical tests in the study group, however, there was no worsening either.

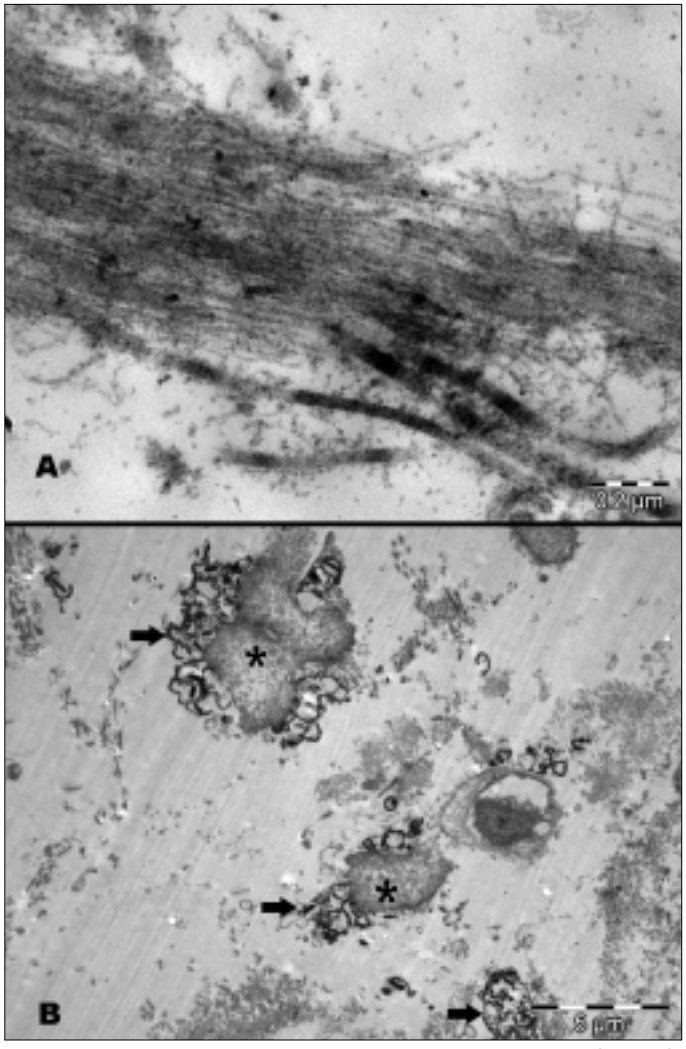

Figure 4. A. Increased disaggregation of collagen fibers (X75000). B. Severe degenerated cells and calcifications on membrane structures (arrow) (X5000)

According to these data, we can put forward that TPEN managed to retard calcification and partially preserved the collagen fibers and the cells.

The TPEN has an affinity to several heavy metals such as $Zn^{2}+$, $Cu^{2}+$, $Fe^{2}+$, $Mn^{2}+$, $Ca^{2}+$ (26). This compound have been used to inhibit the neurotoxic effects of $Zn^{2}+$ in vivo (27, 28), $Zn^{2}+$ -dependent ubiquitin-protein ligases (29-31) and ligand binding to certain G protein-coupled receptors (28). It has been suggested that TPEN might be useful in prevention and treatment of Alzheimer's disease via selectively chelates $Cu^{2}+$ and $Zn^{2}+$ and reduce ß-amyloid deposition in vivo (32, 33). The TPEN has also been used to improve myocardial protection during prolonged ischemia by modulation of intracellular Ca2+ homeostasis (34). It has been reported that the affinity of TPEN for calcium is low, but TPEN activates $Ca+^{2}$ channels and pumps to remove calcium from the cytosol (16).

In experimental studies it was demonstrated that TPEN reduced metabolic damage after prolonged global ischemia. Reperfusion injury and arrhythmias related to it were also shown to be decreased. In an in vivo and in vitro study performed by Shmist et al. the results show that the activation of sarcolemmal $Na+/Ca+^{2}$ exchanger by TPEN increases $Ca+^{2}$ extrusion from the cytoplasm of cardiomyocytes, preventing cytosolic $Ca+^{2}$ overload which explains the beneficial effects of TPEN on postischemic cardiac status (16). In tissue samples treated by GA, calcium regulation is altered and hence changes commence to end up with calcific degeneration therefore by keeping in mind the indicated properties of TPEN we anticipated a better performance in the down regulation of calcium but unfortunately, we only managed to reduce calcification by 50% in both study groups. Although pericardial tissue injury was retarded, we failed to improve its properties in the biomechanical tests.

It must be kept in mind that beneficial effects of TPEN in the cells are available only in a narrow concentration range between 100nM and 2mM therefore we did not use it in higher concentrations.

As a conclusion, with the results derived from this study we can hypothesize that after GA fixation, application of TPEN as an adjuvant together with other detoxifying agents for the reduction of calcification in bioprosthetic materials might prove better results.

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