

# Picric Acid: Can it be Useful in the Decalcification of Pericardial Biomaterials?

## *Pikrik Asit: Perikardiyal Biyomateryallerin Dekalsifikasyonunda Yararlı Olabilir mi?*

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### BACKGROUND

The calcification of pericardial bioprosthetic biomaterials is a clinical concern. The aim of this study was to investigate the effect of a decalcifying agent, picric acid, on decalcification in bovine pericardial tissue.

### METHODS

Bovine pericardium, cut into 1 cm<sup>2</sup> pieces were transferred into +4°C phosphate buffered saline solution containing 0.625% glutaraldehyde for initial fixation, then allocated into two groups. Group I received identical treatment in refreshed solution for five more days. Group II underwent further fixation in phosphate buffered saline + 2% picric acid solution (pH=7.4, 37°C) for a period of 48 h and then transferred into a fresh phosphate buffered saline + 0.625% glutaraldehyde solution at 37°C for three more days. Pericardial patches were implanted into dorsal pouches of 20 juvenile male Wistar rats for 42 days.

### RESULTS

Group II demonstrated a decrease in calcification (130±25.3 µg/mg of tissue). Value estimated in Group I was 262.4±51.5 µg/mg of tissue (p=0.000). Histopathologic evaluation revealed higher calcification in group I than in group II (p=0.023).

### CONCLUSIONS

Picric acid, once being a destructive explosive, might be a constructive component by decalcifying prosthetic heart valve materials.

Key words: Bovine pericardium; decalcification; picric acid.

### AMAÇ

Perikardiyal biyoprostetik materyallerin kalsifikasyonu klinik bir sorundur. Bu çalışmada, bir dekalifikasyon ajanı olarak pikrik asitin sıgır perikardında oluşan kalsifikasyon üzerindeki etkileri araştırıldı.

### YÖNTEM

Sıgır perikardı 1 cm<sup>2</sup> büyüklüğünde kesildi, içerisinde %0.625 glutaraldehit bulunan fosfatla tamponlandı, +4°C soğuklukta salin solüsyona konularak inisiyel tespit yapıldı ve parçalar iki gruba ayrıldı. Grup I parçalar işlem aynı özelliklerdeki taze solüsyonla beş gün sürdürüldü. Grup II parçalar fosfatlı tamponlanan salin + %2 pikrik asit solüsyonu içeren çözeltide (pH 7.4, 37°C) 48 saat süreyle ek tespit işlemine tabii tutuldu. Daha sonra fosfat ile tamponlanmış salin + %0.625 glutaraldehit içeren solüsyona transfer edildi, üç gün burada tutuldu. Tüm perikardiyal parçalar erkek Wistar cinsi sıçanların sırt boşluklarına yerleştirildi ve 42 gün burada tutuldu.

### BULGULAR

Grup II dokularda kalsifikasyonun daha az olduğu gözlemlendi (130±25.3 µg/mg). Grup I dokularda saptanan değer 262.4±51.5 µg/mg idi (p=0.000). Histopatolojik çalışmalar grup I dokularda daha yüksek kalsifikasyon oranlarını ortaya koymuştur (p=0.023).

### SONUÇ

Bir zamanlar patlayıcı ve yıkıcı olarak kullanılan pikrik asit biyoprostetik materyallerin dekalifikasyonunda yapıcı bir rol üstlenebilir.

Anahtar sözcükler: Dekalsifikasyon; pikrik asit; sıgır perikardı.

Valve replacement is sometimes inevitable in an attempt to repair a functionally and morphologically deteriorated heart valve in approximately 30% of valve sparing procedures.<sup>(1)</sup> Some authors consider mechanical valve implantation in such instances as “trading one disease for another” as patients are committed to a life long anticoagulation therapy and to its potential hazards.<sup>(1-3)</sup> Hence interest was raised in the use of glutaraldehyde (GA) treated collagen based biomaterials for this purpose.<sup>(4,5)</sup> Today about 30% of the heart valve market is constituted by bioprosthetic valves of several designs.<sup>(2)</sup> GA was first introduced by Carpentier and colleagues as a compound to modify heterograft collagen chemically and make it immunologically acceptable in the human host.<sup>(6)</sup> GA pretreated porcine valves have been used for heart valve replacement since 1968.<sup>(5)</sup> Nowadays it is widely accepted that GA pretreatment constitutes the basics of dystrophic calcification that finally leads to complete structural and functional deterioration.<sup>(1,2,4,6-8)</sup> This eventually results in re-operations and their associated morbidity and mortality in approximately 20%-30% of the recipients by the tenth postoperative year.<sup>(8)</sup>

In the development of alternative tissue preservation procedures, particular attention is paid to various pre and post-fixation tissue treatments to offset the effects of GA.<sup>(6)</sup> Taking this factor as a starting point, we were curious to establish whether picric acid (PA), once being a destructive explosive, could be a constructive hope in the people’s lives, serving as a decalcifying agent in bioprosthetic heart valve materials in the long run. Our review of the literature failed to establish research related to the use of PA for decalcification in biomaterials. Consequently, this preliminary study describes our experience in a sub dermal bovine pericardium implantation in a rat model.

## METHODS

### Tissue Preservation

Conventionally preserved pericardium, consisting of freshly excised bovine pericardium, was dissected free from adhering fat tissue and cut into 1 cm<sup>2</sup> pieces. They were then rinsed in a phosphate buffered saline (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 - to volume ratio was 1 gram per 30 ml fixation solution.<sup>(9)</sup> The different groups were defined as follows.

### Group I (Control Group):

After the initial fixation for 48 hours with PBS+0.625% GA at +4°C, tissues were placed into fresh solutions with the same properties and temperature. Fixation was allowed to continue for further five days at +4°C (for a total of 7 days).

### Group II (Study Group):

After the initial two days of fixation with PBS+0.625% GA at +4°C, tissues were placed into PBS solution at 37°C containing 2% PA (in which 1 N NaOH was added to keep pH at 7.4,) for a period of 48 hours (30 ml/g tissue) and transferred into freshly prepared PBS+0.625% GA solution at 37°C for another three days (for a total of 7 days).

### Subcutaneous Implantation of Samples in Rats

All animal procedures used were in strict accordance with the National Research Council’s guide on the Care and Use of Laboratory Animals. The study was approved by the Ethics Committee of our institution (22 September, 2004 - 20/2) before study.

To investigate calcification rate, pericardial patches were inserted into the dorsal pouches of 20 juvenile anaesthetized male Wistar rats (weighing 100 to 125 g). Pericardial pieces were randomly assigned into two groups. All specimens were rinsed (3x10 minutes for each piece) in saline by agitating prior to implantation and each animal received both types of patches. After 42 days, the animals were sacrificed by an overdose of sodium pentobarbital. The implants were explanted and rinsed with saline solution. Each explanted tissue sample was cut into two equal halves. One half was further processed for atomic absorption spectrophotometry, and the other half was fixed in 10% formalin and prepared for histo-pathologic study.

#### The Determination of Tissue Calcium Levels

Following storage at  $-20^{\circ}\text{C}$ , tissues were dried at  $104^{\circ}\text{C}$  for 24 h, weighed, ashed in a muffle furnace at  $200^{\circ}\text{C}$  for 12 hours, and dissolved in 3 N HCl (10 mg dried tissue: 1 ml HCl) and transferred quantitatively to a 25-mL volumetric flask and diluted to 0.36 N HCl. For the determination of calcium content, the sample was diluted 1:50 with 0.1% (w/v) lanthanum chloride. A standard solution was also prepared by diluting 0.1% (w/v) lanthanum chloride. A solution of 0.1% (w/v) lanthanum chloride was used as a blank. Standards and samples were measured by using atomic absorption spectrophotometer at 422.7 nm.<sup>(10)</sup> Calcium levels were expressed as  $\mu\text{g/g}$  dry mass of tissue.

#### Preparation of Tissues for Histo-pathologic Analysis

Fixed pericardial samples were dehydrated in graded concentrations of ethanol, cleared in xylene, and embedded in paraffin. Sections in 5 mm thickness were stained with haematoxylin-eosin, and Von-Kossa stain for routine histo-pathological examination and demonstration of calcium deposition. The degree of pericardial calcification was evaluated semiquantitatively as

follows; none (0); a few small and punctuate calcific deposition (1); larger and more distinct calcification (2); heavy calcification as a solid mineral deposition (3).

#### Statistical Analysis

SPSS 9.0 for Windows (SPSS Inc, 1989-1999) package program was used for statistical analyses. The results are expressed in terms of mean  $\pm$  SD. The differences of biochemical results between the groups were analyzed by Student t test. Type I error was accepted as 0.05. The difference in histo-pathologic evaluation was assessed by using Mann-Whitney U-test. A p value  $<0.05$  was accepted to be significant.

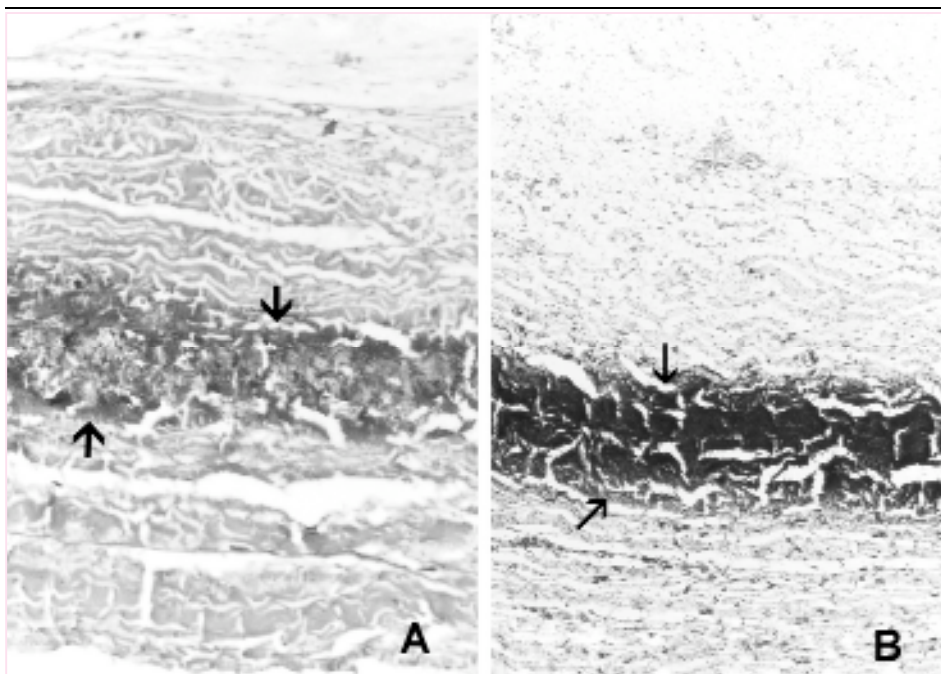
## RESULTS

#### Biochemical Assessment

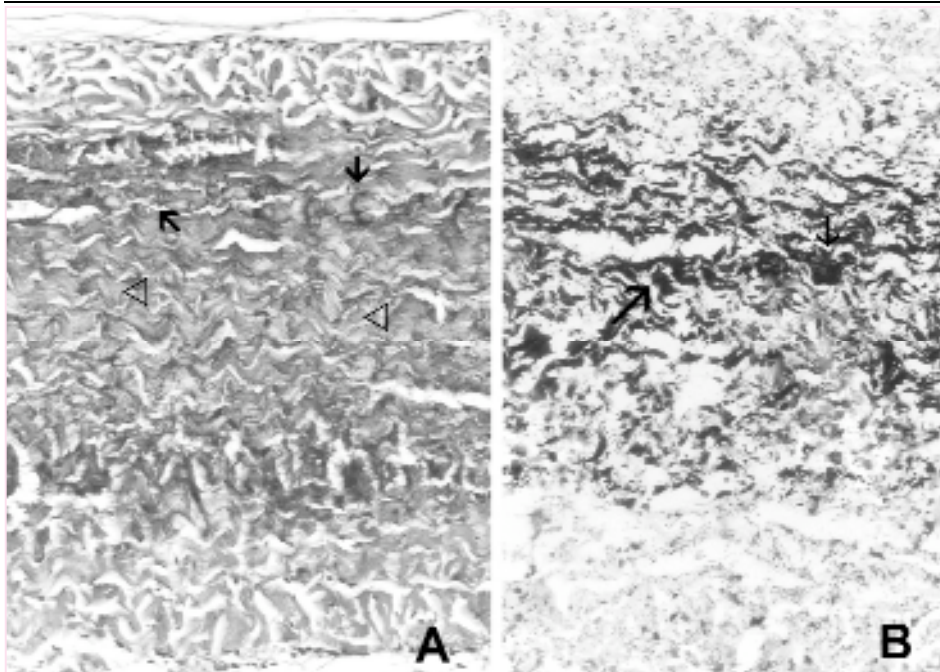
The biochemical analysis showed a decrease in the amount of calcification in group II ( $130 \pm 25.3 \mu\text{g/mg}$ ), whereas the value found in group I was  $262.4 \pm 51.5 \mu\text{g/mg}$  ( $p < 0.001$ ).

#### Morphological Assessment of Tissue Calcification

The assessment of the degree of calcification in



**Fig. 1.** Light photomicrograph of pericardium in group I. (A) Confluent calcification pattern among collagen fibers with Hematoxylin-eosin stain (arrow). (B) Intensively black staining of calcification in the same area with Von-Kossa stain (arrow). (Original magnifications: A, x400; B, x400).



**Fig. 2.** Light photomicrography of pericardium in group II. (A) Multifocal fine granular grainy microcalcific depositions (arrow) among the regular texture collagen fibers (arrow head) with Hematoxylin-eosin stain. (B) Black stained spots of the same area with Von-Kossa stain (arrow). (Original magnifications: A, x400; B, x400).

histological sections generally agreed with the results of biochemical analyses. The value calculated for calcification for group I specimens were  $2.73 \pm 0.47$  and for group II specimens  $1.72 \pm 0.64$  respectively. Fig. 1 (A and B) illustrates confluent calcium deposition with destruction of collagen bundles in group I pericardium specimens with haematoxylin-eosin and Von-Kossa stains, whereas Fig. 2 (A and B) illustrates the regular texture of collagen fibers with little calcific deposits within group II specimens with the same stains ( $p=0.023$ ).

#### DISCUSSION

As far as valve replacement is concerned, the major hindrance in the long-term use of GA-fixed bioprosthetic heart valves in human hosts is their dystrophic calcification.<sup>(2,7,11)</sup> Many studies, since the introduction of GA as a fixative by Carpentier and colleagues, have pointed out that the major factor limiting clinical use of the commercially available GA pretreated bioprosthetic valves is their late structural deterioration. This commonly occurs as calcific mineralization and degenerati-

The biological valve prostheses display superior hemodynamics and lower thrombogenicity.<sup>(7,8,16)</sup> This protects the patients from the potential and sometimes life threatening hazards of the anticoagulation therapy.<sup>(1,8)</sup> Augmentation of mechanical resistance and attenuation of antigenicity of biomaterials are additional advantages of GA treatment.<sup>(17,18)</sup> Today, it is very well agreed that although being a universal and almost unique tissue fixative, GA is also the accused compound to be responsible in the formation of most of the calcifications in bioprosthetic valves.<sup>(2,6,7,11,19)</sup> It can be said that for the use of biomaterials in human hosts, GA is a remedy and, in some ways, is considered the villain of tissue mineralization. Although dynamic stress is also accused in the acceleration of mineralization, the haemodynamic component should be accepted as a part of the phenomenon as calcification also occurs in subcutaneous implants.<sup>(17)</sup> Research to date has demonstrated various anticalcification strategies in animal models, including pretreatment of the valves with either metallic salts, detergent, ethanol or aminodiphosphonates, coimplants of controlled release drug delivery systems, and covalently

bound anticalcifying agents, post-fixation detoxification treatment with various amino acid solutions and all were shown to be effective by binding the residual aldehyde groups or by affecting cell morphology.<sup>(16,17,20-24)</sup>

As part of our research program in finding out a novel decalcifying agent in bioprosthetic materials, we recently reported the effect of ethylenediaminetetraacetic acid (EDTA)<sup>(7)</sup> as a chelating agent. In this study we aimed to find out the effect of a weak decalcifying acid, PA, in mitigating calcification in GA treated bovine pericardium, via a two stage fixation process. PA (2,4,6, trinitrophenol) once found application as a true military type explosive.<sup>(24)</sup> Today it is primarily used in the manufacture of explosives and as an intermediate in dye manufacturing. It is also present in many laboratories, for use as a chemical reagent and tissue fixative.<sup>(25)</sup> PA can also be used as a decalcifying agent in various fields.<sup>(26)</sup>

Traditionally decalcifying agents are divided into two groups; mineral acids and chelating agents. Mineral acids are further divided into two groups as weak acids and strong acids. Examples of weak acids are acetic acid and PA. Occasionally these components may be incorporated into the chemical formulae of certain fixatives (including Bouin's and Carnoy's fluid), so that they have simultaneous roles of fixation and decalcification. Weak acid decalcifiers are relatively slower and more gentle than most others and are best used when only a small amount of mineralization is known or suspected to be present.<sup>(26,27)</sup> Taking these properties of weak acids into consideration, we planned to use PA as a decalcifying agent in an attempt to set aside the unwanted effects of GA.

Although the tissue fixation and decalcification mechanism of PA is not very well known, in our

study, PA successfully retarded calcification in group II pericardial tissues. This is demonstrated by biochemical and histo-pathological findings, showing regular texture of collagen fibers preserved with very little calcium foci in histologic sections (Fig. 2A and 2B). As very well known, calcific deposits begin as small and punctuate formations and gets distributed usually diffusely and randomly within the tissue.<sup>(28)</sup> Our findings on histo-pathologic specimens are concordant with this as PA significantly retarded calcification. Since review of the literature revealed no similar studies with this acid, to our knowledge this is the first trial on this concept using this compound.

This study constitutes a part of the efforts in reducing calcification and degeneration in GA treated bovine pericardial biomaterials with a decalcifying and fixative weak acid, PA, which was not tested for this purpose. The compounds that were necessary for the present investigation were commercially available and were easily obtained.

As a result we raise the question: "Can yesterday's destructive explosive be tomorrow's constructive hope in decalcification of pericardial bioprosthetic materials in the long run?" Preliminary data looks inspiring but further studies are needed to evaluate and assess the optimum concentration and duration of treatment with PA that is needed to treat the tissues after GA pretreatment. The shrinkage temperatures, *in vitro* - *in vivo* properties of durability after PA post-treatment in the short and long term are yet to be investigated.

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