

## Citric acid as a decalcifying agent for the excised calcified human heart valves

*Eksize edilmiş kalsifiye insan kalp kapaklarında dekalsifiye edici bir ajan olarak sitrik asit*

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### ABSTRACT

**Objective:** Cardiac valvular pathologies are frequently encountered as mechanical and functional disorders due to the calcification of the valves whatever the etiologies are. This pathophysiologic table usually ends up with valvular replacement. In this study we aimed to decrease/eliminate the calcium in the excised calcified human heart valves by using citric acid in vitro hence bringing about the question for possible oral treatment of calcification of the valves by citric acid ingestion.

**Methods:** Fourteen pieces of mitral and/or aortic valves excised from 12 patients undergoing valve replacement were placed in a freshly prepared phosphate buffered saline solution containing 0.625% glutaraldehyde at +4 °C for 48 h. They were rinsed with 0.9% NaCl and divided into two groups; study and control. Control tissues were further treated in a freshly prepared solution with identical properties for another 5 days. Study tissues were placed into a solution containing 3.8% citric acid (pH 7.4) and kept for 48 h at +37 °C, then rinsed with 0.9% NaCl and transferred into a fresh solution containing 0.625% glutaraldehyde with phosphate buffer at 37 °C for 3 more days. Specimens were biochemically and histopathologically evaluated and compared using Mann Whitney U test.

**Results:** Calcium and phosphate levels in the study group were lower than in the control group ( $852.5 \pm 913.41 \mu\text{g g}^{-1}$  vs  $413.05 \pm 519.53 \mu\text{g g}^{-1}$ ,  $p=0.001$  and  $207.6 \pm 321.86 \mu\text{g g}^{-1}$  vs  $124.4 \pm 289.48 \mu\text{g g}^{-1}$ ,  $p=0.035$ , respectively). Malondialdehyde and protein level values were changed insignificantly in the control and study groups. Histopathologic evaluation showed that collagen and elastin fibers were similar in both groups. In the study group, irregular and fusiform calcific formations around the collagen fibers were significantly decreased.

**Conclusions:** Decalcifying human heart valves in vitro conditions with citric acid without an adverse change to the morphology of the valvular tissue specimens is meaningful. We believe that forwarding and looking for the answer to the question "whether systemic application of citric acid could lead to the decalcification and/or reduction of calcification in the native human heart valves" would be expressive. (*Anadolu Kardiyol Derg 2008; 8: 94-8*)

**Key words:** Decalcification, citric acid, human heart valves

### ÖZET

**Amaç:** Kalp kapak hastalıkları etyolojisi ne olursa olsun kapakların büyük bölümünde kalsifik dejenerasyona bağlı mekanik ve fonksiyonel bozukluk olarak karşımıza çıkmaktadır. Bu fizyopatolojik tablo genellikle kapak değişimi ile sonlanmaktadır. Biz de bu çalışmada kapak değişimine giden hastalardan çıkartılan kapak parçalarındaki kalsifik dejenerasyonu in vitro ortamda sitrik asit ile ortadan kaldırarak veya azaltarak; oral sitrik asit kullanımıyla kalp kapaklarındaki kalsifik dejenerasyonun tedavisinin medikal yönünü gündeme getirmeyi amaçladık.

**Yöntemler:** Mitral ve/veya aort kapak replasmanı yapılan 12 hastadan elde edilen 14 parça kapak materyali 4°C'da fosfat tamponlu ve %6.25 gluteraldehid içeren taze salin solüsyonuna 48 saat süreyle muamele edildi. Kapak örnekleri salin ile yıkanıp çalışma ve kontrol gruplarına ayrıldı. Kontrol grubu aynı özellikte tazelenmiş gluteraldehit solüsyonunda 5 gün daha bekletildi. Çalışma grubu ise içinde %3.8 Sitrik asit (pH 7.4) bulunan 37°C'daki solüsyonda 48 saat bekletildikten sonra salin ile yıkandı ve 3 gün daha tazelenmiş gluteraldehit solüsyonunda 37°C'de bekletildi. Örnekler hem biyokimyasal, hem de histopatolojik olarak incelendiler. Biyokimyasal değerlendirmeler Mann Whithney U testiyle histopatolojik değerlendirmeler ise kalitatif yapılmıştır.

**Bulgular:** Çalışma grubunda kalsiyum ve fosfat seviyeleri kontrol grubundakilere göre anlamlı derecede düştü (sırası ile  $852.5 \pm 913.41 \mu\text{g g}^{-1}$ ;  $413.05 \pm 519.53 \mu\text{g g}^{-1}$ ,  $p=0.001$  ve  $207.6 \pm 321.86 \mu\text{g g}^{-1}$ ;  $124.4 \pm 289.48 \mu\text{g g}^{-1}$ ,  $p=0.035$ ). Malondialdehit seviyelerinde çalışma ve kontrol grubunda anlamlı değişiklik yoktu. Histopatolojik değerlendirme her iki gruptaki kollajen ve elastin fiberlerinin benzer yapıda olduklarını ortaya koydu. Çalışma grubunda kollajen fiberlerin etrafındaki irregular ve fuziform yapıdaki kalsifikasyonların önemli derecede azalmış olduğu gözlemlendi.

**Sonuç:** Kalsifik insan kalp kapaklarının in vitro ortamda kapak morfolojisine ters bir etki oluşturmadan sitrik asit ile dekalsifikasyonu önemli bir aşamadır. Sitrik asitin bu etkisinden yararlanılarak oral sitrik asit tedavisi ile kalp kapaklarındaki kalsifikasyonun azaltılabilip azaltılamayacağı sorusunu gündeme taşımak ve araştırılmasını sağlamak anlamlı olacaktır. (*Anadolu Kardiyol Derg 2008; 8: 94-8*)

**Anahtar kelimeler:** Dekalsifikasyon, sitrik asit, insan kalp kapakları

## Introduction

Cardiovascular diseases still constitute the major cause for morbidity and mortality. Valvular heart diseases are among the major problems encountered within cardiovascular diseases. An average of 275000 of these patients undergoes valve replacement every year worldwide (1, 2). There is still no well known and worthy of commendation method of therapy to prevent the outcome and/or progression of the disease.

Valvular disease commences with the oxido-inflammatory damage and as the process progresses many of the valves become thickened and calcified. Calcific degeneration of the valves is the final stage. When this stage is attained the functions of the valves are deteriorated to a great extent (3-5). It has been demonstrated with electron beam tomography that in some patients calcified atherosclerotic plaques have been diminished by the application of a chelating agent, ethylene diamine tetra acetic acid (EDTA), and antibiotic (tetracycline) combination (6). A great similarity between the ensuing calcification especially in the aortic valve and atherosclerosis has been estimated (7) thus this finding constitutes the starting point of our study. Apart from having a decalcifying effect, alkali citrates also have some other beneficial properties like prevention of the formation of calcium crystals (8-12), regulation of calcium metabolism and some anti-inflammatory properties (13-15) that leads us to consider alkali citrates as possible front line fighters in the fight of the removal and/or prevention of calcification on the native human heart valves.

Cardiac valvular pathologies are frequently encountered as mechanical and functional disorders due to the calcification of the valves whatever the etiologies are. This pathophysiologic table usually ends up with valvular replacement. In this study we aimed to decrease/eliminate the calcium in the excised calcified human heart valves by using citric acid (CA) in vitro hence bringing about the question for possible oral treatment of calcification of the valves by citric acid ingestion.

## Methods

This experimental cross-sectional study was conducted by the approval of the Ethics Committee of the Mersin University, Faculty of Medicine.

The resected valvular specimens during valve replacement surgery of 12 patients applied to the Department of Cardiovascular Surgery for valvular surgical intervention were divided into two groups as control and study groups according to their treatment methods i.e. treatment with or without citric acid.

Eight of 12 patients were females and 4 of them were males. Six patients had rheumatic mitral stenosis, 4 had rheumatic aortic stenosis and 2 of them had both mitral and aortic stenosis due with rheumatic etiology. Mean age of the patients was  $34.20 \pm 6.10$  years.

All the specimens were placed in freshly prepared phosphate buffered saline solution (0.1 M, pH 7.4) containing 0.625% glutaraldehyde (GA) at 4 °C for 48 h. At the end of 48 h, the valves were rinsed in 0.9% NaCl and then they were divided

into two groups as control and study. Valves in the control group were further treated in a new 0.625% GA solution (0.1 M, pH 7.4) for 5 more days (total of 7 days). Pieces in the study group were placed into a different solution containing 3.8% citric acid (pH 7.4) and kept for 48 h at 37 °C. At the end of 48 h they were rinsed with 0.9% NaCl and transferred into a fresh solution containing 0.625% GA with phosphate buffer at 37 °C (0.1 M, pH 7.4). They were kept in this solution for 3 more days (total of 7 days). All pieces in the two groups were evaluated both biochemically and histopathologically.

### Biochemical Assay

#### The determination of tissue calcium and phosphate 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 N HCl (10 mg dried tissue: 1 ml HCl) and transferred quantitatively into a 25-ml volumetric flask and diluted with 0.36 N HCl. By using a 1000 µg ml<sup>-1</sup> stock calcium standard solution (Sigma C- 5649 for atomic absorption spectrophotometer) and diluting it with 0.1% (w/v) lanthanum chloride, standard solutions of 0.5 µg ml<sup>-1</sup>, 1 µg ml<sup>-1</sup>, 1.5 µg ml<sup>-1</sup> and 2 µg ml<sup>-1</sup> were prepared. A solution of 0.1% (w/v) lanthanum chloride was used as a blank. Standards and samples were measured by using atomic absorption spectrophotometer (Terra AA240, Melbourne, Australia) at 422.7 nm. Calcium levels were expressed as µg g<sup>-1</sup> dry mass of tissue. Phosphate was analyzed by Cobas Integra 700 (Hitachi Modular Systems, Japan) biochemical analyzer and the results are expressed as µg g<sup>-1</sup> dry mass of tissue (Roche Diagnostics, GmbH, Mannheim, Germany).

#### Malondialdehyde determination

The malondialdehyde (MDA) levels, as an index of lipid peroxidation was determined by thiobarbituric acid (TBA) reaction according to Yagi (16). The principle of the method depends on measurement of the pink color produced by interaction of the barbituric acid with MDA elaborated as a result of lipid peroxidation. The colored reaction 1, 1, 3, 3 tetraethoxypropane was used as the primary standard.

#### Protein determination

Protein content was determined according to the method described by Lowry and bovine serum albumin was used as a standard (17).

#### Histopathologic Examination

The histopathologic evaluation was carried out as blinded to the examiner and performed qualitatively.

#### Light Microscopy

Heart valve samples were fixed with 10% neutral formalin, dehydrated in graded concentrations of ethanol, cleared in xylene, and embedded in paraffin. Sections in 5 µm thickness were stained with Von-Kossa stain for demonstration of calcium deposition. Photomicrographs obtained by a digital camera attached to an Olympus BX50 (Olympus GmbH, Germany) light microscope.

#### Electron Microscopy

For transmission electron microscopic evaluation heart valve samples, approximately 1 mm<sup>3</sup> in size were fixed with 2.5% glutaraldehyde, postfixed 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 Leica UCT-125 ultramicrotome (Leica Microsystems GmbH, Vienna-Austria) and contrasted with uranyl acetate and lead citrate. Sections were examined by a JEOL JEM-1011 electron microscope (Jeol Ltd. Tokyo-Japan) and photographed by a Megaview III digital camera (Olympus Soft Imaging Solutions GmbH, Münster-Germany) attached to the microscope.

#### Statistical Analysis

Statistical analyses were made using the SPSS for Windows 11.5 statistical package program (Chicago, IL, USA). The biochemical parameters were evaluated by using Mann Whitney U test and tested with  $\alpha:0.05$  significance value. P value  $<0.05$  was accepted to be significant.

### Results

#### Biochemical Evaluation

The calcium and phosphate levels estimated in the control group valves were  $852.5 \pm 913.41 \mu\text{g g}^{-1}$  tissue and  $207.6 \pm 321.86 \mu\text{g g}^{-1}$  tissue whereas in the study group valves calcium and phosphate levels were found out to be  $413.05 \pm 519.53 \mu\text{g g}^{-1}$  tissue and  $124.4 \pm 289.48 \mu\text{g g}^{-1}$  tissue, respectively. The degree of decrease in the calcium ( $p=0.001$ ) and phosphate ( $p=0.035$ ) levels were statistically significant. The MDA levels, an indicator of lipid peroxidation in the cell membrane, were  $0.133 \pm 0.006 \text{ mg g}^{-1}$  tissue in the control group specimens and  $0.186 \pm 0.009 \text{ mg g}^{-1}$  tissue in the study group specimens. The difference between the groups in terms of MDA was statistically not significant ( $p>0.05$ ). Total protein level, another indicator of cell integrity, was found to be  $0.380 \pm 0.049 \text{ mg g}^{-1}$  tissue in the control group specimens and  $0.365 \pm 0.443 \text{ mg g}^{-1}$  tissue in the study group specimens. This difference was also not significant ( $p>0.05$ ) (Table 1).

#### Histopathologic Evaluation

Connective tissue cells and fibers were evaluated by Von-Kossa staining at light microscopic level. An obvious calcification pattern was observed in the control group of the aortic and mitral valvular specimens (Fig. 1A), whereas some calcified cells and other connective tissue components were present in study group (Fig. 1B). Connective tissue cells and fibers were examined in both aortic (Fig. 2-3) and mitral (Fig. 4-5) valve specimens at electron microscopic level. There were some calcified collagen fibers and membrane-bound structures of cells both in the control and the study groups but the calcified components were found to be more prominent in the control group (Fig. 2-5).

### Discussion

In most of the cardiac valvular diseases, calcium regulation is deteriorated due to oxido-inflammatory damage and the exposed calcium binds to phospholipids that lead to the formation of calcium phosphate foci. In time, these foci develop and coalesce turning into broad calcific nodules. As these changes take place, the valvular tissue becomes thicker and weaker which clinically means losses in the functions of the valves (3-5). The corruptive changes taking place in the valves are together with increases in calcium levels with calcium being the cause rather than the result (18).

In review of these undesirable effects of calcification on the human heart valves we recently have reported retardation, if not inhibition, of calcification by the use of a range of chelating agents; EDTA, CA and N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), in the bovine pericardium (19-22). As part of our ongoing research program we are now reporting the beneficial effect of CA on the excised calcified human heart valves.

Citric acid is a readily available, commonly used chelating agent with a very high affinity to calcium. It is also widely found in the living systems (23, 24). It has a great advantage of not damaging the tissues morphology in a neutral pH environment. Chemically CA chelates calcium from the outer shell of the hydroxyapatite crystal ( $\text{Ca}_5[(\text{OH})(\text{PO}_4)_3]$ ) and reduces the physical dimensions of the crystal leading to decalcification (23-26).

In study group specimens the significant reduction in the calcium and the phosphate levels without any harm to the cell membranes and collagen content was observed, that are reflected by the insignificant changes in the MDA and the protein content respectively, and the supporting findings in histopathologic evaluation showing decrease in calcific nodules without changing the collagen fibers arrangement compared to the Control group specimens, lead us to consider CA a favorable choice for the decalcification of human heart valves without affecting the native valvular structure.

By applying 3.75% EDTA, that carries similar properties with CA, for 15-45 minutes on the corneas of the patients with calcific band keratopathy, Najjar et al. (27) obtained complete or partial recovery in patients visual perception. This application has taken its place in the treatment protocol of the Wills Eye Hospital (Philadelphia, USA) (27). In this application calcium hydroxyapatite crystals become soluble and removed. This salutary result takes place in a short time period and it supports our experimental protocol in terms of adequacy of the concentration of CA we used and of the time we treated the

**Table 1. Biochemical results of calcium, phosphate, MDA and total protein levels in Control and Study groups**

| Parameters                                                                                             | n  | Control group      | Study group         | p*    |
|--------------------------------------------------------------------------------------------------------|----|--------------------|---------------------|-------|
| Ca <sup>2+</sup> , $\mu\text{gr/gr}$                                                                   | 14 | 852.5 $\pm$ 913.41 | 413.05 $\pm$ 519.53 | 0.001 |
| Phosphate, $\mu\text{gr/gr}$                                                                           | 14 | 207.6 $\pm$ 321.86 | 124.4 $\pm$ 289.48  | 0.035 |
| MDA, mg/gr                                                                                             | 14 | 0.133 $\pm$ 0.006  | 0.186 $\pm$ 0.009   | 0.089 |
| Total protein, mg/gr                                                                                   | 14 | 0.380 $\pm$ 0.049  | 0.365 $\pm$ 0.443   | 0.230 |
| Data are represented as Mean $\pm$ standard deviation, * - Mann Whitney U test<br>MDA- malondialdehyde |    |                    |                     |       |

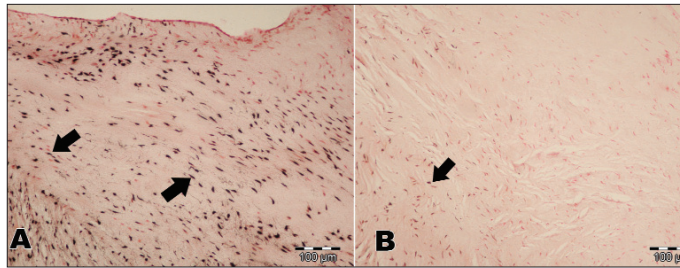


**Figure 1.** Light microscopic images of the aortic valvular specimens. Calcified structures (arrows). A - Control group, B - Study group (Von-Kossa staining X300)

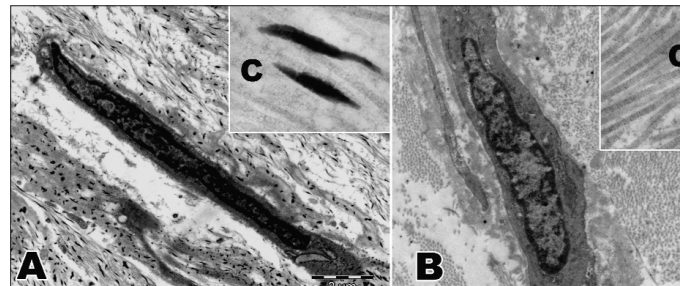


**Figure 2.** Ultrastructural photomicrographs of the aortic valve of a patient. Collagen fibers (C). A - Control group (X 10000, Inset X 100000), B - Study group (X 10000, Inset X 50000) (Uranyl acetate X Lead citrate)

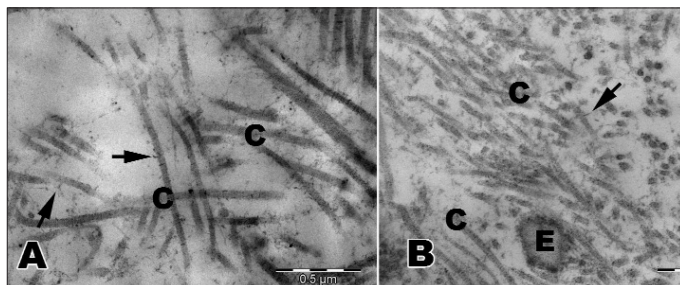


**Figure 3.** Ultrastructural photomicrographs of the aortic valve of another patient. Collagen fibers (C), Elastic fibers (E), Calcifications (arrows). A - Control group (X60000), B - Study group (X60000) (Uranyl acetate X Lead citrate)

valvular specimens for decalcification. On the other hand, as for the atherosclerotic coronary arterial disease, EDTA rendered the nanobacteria sensitive to tetracycline by breaking down their protective shells that are composed of calcium phosphate, thus leading to a decrease in the calcium score and a regress in the atherosclerotic plaque. Partial or complete healing in the symptoms of 16 of 19 patients with stable angina pectoris has been reported with this study by Maniscalco et al. (6). Although with present information, the results of EDTA application in atherosclerotic coronary artery disease and peripheral arterial disease are controversial, it has found a widespread use in the USA but, however, the situation does not look to be that bright in the use of CA for this purpose (28-31). In a study on the effect of CA on atherosclerosis, it has been reported that CA salts had diminished the calcified areas in the tunicae media of the calcified rat aortas which were embedded in the abdomens of the rats. Furthermore, maturation and accumulation of the hidroxiapatite crystals were also found to be retarded (32). In this context, alkaline citrates, having a preventive property in the formation of

hydroxiapatite crystals, found a place in the clinical treatment of patients having idiopathic calcium urolithiasis (8-12). Citrates regulate calcium metabolism and have some antiinflammatory effects hence there is a growing interest in citrate salts (13-15).

The similarities between atherosclerosis and calcification, especially of the aortic valve and the defined properties and effects of the chelates on the soft tissues formed the basis of our study. The limited biochemical and histopathologic parameters used in this study were, we think, sufficient to demonstrate the harmless effect of CA on tissue integrity as it decalcified the valvular tissue.

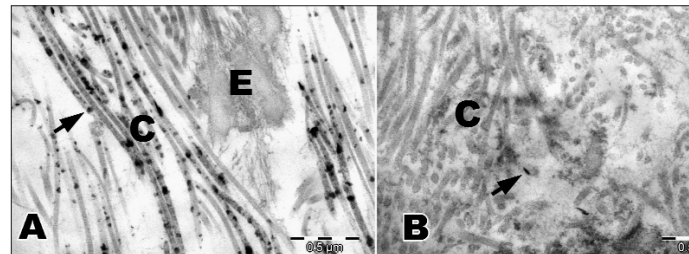


**Figure 4.** Ultrastructural photomicrographs of the mitral valve of a patient. Collagen fibers (C), Elastic fibers (E), Calcifications (arrows). A - Control group (X50000), B - Study group (X50000) (Uranyl acetate X Lead citrate)

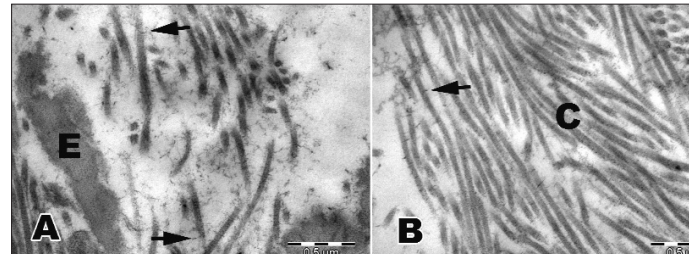


**Figure 5.** Ultrastructural photomicrographs of the mitral valve of another patient. Collagen fibers (C), Elastic fibers (E), Calcifications (arrows). A - Control group (X50000), B - Study group (X50000) (Uranyl acetate X Lead citrate)

## Conclusions

As a conclusion, the decalcification of calcified human heart valves in vitro conditions with citric acid without giving rise to any evident adverse change in the morphology of the valvular tissue specimens is meaningful. We believe that forwarding and looking for the answer to the question "whether systemic application of citric acid could lead to the decalcification and/or reduction of calcification in the native human heart valves" would be expressive.

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