In search of the best xenogeneic material for a paediatric conduit: an experimental study

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Abstract

OBJECTIVES: The development of calcification-resistant bioprosthetic materials is a very important challenge for paediatric surgery. The subcutaneous implantation in rats is the well-known first-stage model for this kind of research. Using this model, we aimed to compare calcification of the porcine aortic wall and bovine pericardium and jugular vein wall cross-linked with glutaraldehyde (GA) and ethylene glycol diglycidyl ether (DE). We also determined the efficacy of DE-preserved tissue modification with 2-(2-carboxyethylamino)ethylidene-1,1-bisphosphonic acid (CEABA).

METHODS: Three groups of each biomaterial were evaluated: GA-treated, DE-treated and DE + CEABA-treated. The microstructure of non-implanted biomaterials was assessed by light microscopy after Picro Mallory staining; the phosphorus content of the DE and DE + CEABA samples was assessed by atomic absorption spectrophotometry. Samples were implanted subcutaneously into young rats for 10 and 60 days. The explant end-point included quantitative calcification assessment by atomic absorption spectrophotometry and light microscopy examination after von Kossa staining.

RESULTS: All GA-treated biomaterials had a high calcium-binding capacity (>100 µg/mg dry tissue). DE preservation decreased the vein wall and pericardium calcification content by 4- and 40-fold, respectively, but was ineffective for the aortic wall. The calculated CEABA content was almost equal in the vein wall and pericardium (17.7 and 18.5 µg/mg) but had no effect in the DE vein wall. Mineralization in the GA- and DE-treated aortic and vein walls was predominantly associated with elastin. CEABA modification decreased elastin calcification but did not block it completely.

CONCLUSIONS: Each xenogeneic material requires individual anticalcification strategy. DE + CEABA pretreatment demonstrates a high mineralization-blocking efficacy for the bovine pericardium and should be employed to further develop the paediatric pericardial conduit. Aortic wall calcification cannot be blocked completely using this strategy.

Keywords: Xenograft • Paediatric conduit • Calcification • Glutaraldehyde • Ethylene glycol diglycidyl ether • Bisphosphonic acid

INTRODUCTION

Valved conduits made from different xenogeneic biomaterials are widely used for right ventricle outflow tract reconstruction [1]. Children are the main patient cohort undergoing this kind of operation. It is well known that xenogeneic materials treated with glutaraldehyde (GA) calcify faster in children than they do in adults [2–4]. Calcification is the main cause of xenograft dysfunction in children and young adults, leading to suboptimal long-term clinical results.

Further significant developments in right-sided valved conduits are likely connected with tissue-engineered grafts; however, the search for the optimal conduit material and preservation technique is still of great importance.

The most frequent xenogeneic material components of conduits are the porcine aortic root (leaflets with aortic wall sinuses),...
bovine pericardium and the bovine jugular vein (BJV) [1, 5]. These materials differ in their microstructure, fibrillar protein ratio and amino acid composition. We hypothesized that the calcium-binding activity of these biomaterials varies with the same anticalcification strategy. One strategy lies in the substitution of the aldehyde cross-linking agent for epoxy compounds. Our clinic has around 20-year experience with various xenogeneic right-sided valved conduit types in adults; the most reliable calcification resistance was shown for diepoxide-treated pericardial xenografts [6, 7]. In children, experience in the use of epoxide-treated pericardial conduits is limited and its durability remains poorly studied, and prevention of calcium accumulation in epoxide-treated conduits in paediatric patients remains a challenge. An additional form of anticalcium treatment could be to try covalent amino bisphosphonic acid immobilization on the reactive epoxy groups that remain available after cross-linkage due to the masking effect. When immobilized, bisphosphonic acids are capable of blocking the formation and growth of calcium phosphate crystals by a strong chemiadsorption to hydroxyapatite, which is known as the ‘crystal poisoning effect’ [8].

It has been reported that not all phosphonic compounds are equally capable of mitigating cardiovascular implant calcification in vivo. In this study, we chose to use 2-(2-carboxyethylamino)ethylidene-1,1-bisphosphonic acid (CEABA), synthesized in the early 1990s [9], which has been shown to have several noteworthy biological effects.

The first tests of biomaterial calcium-binding properties are always performed in subcutaneous rat model [10–12]. It is well known that 2- to 3-month calcification of an implant in young growing rats is equal to calcium amount in clinical bioprostheses implanted in humans for 7–10 years. The lesser is the subcutaneous implant calcification the more promising is the biomaterial for further development and potential clinical use [13, 14]. Hence, the subcutaneous rat model allows to reveal and to reject ineffective treatment methods immediately.

This experimental study aimed to compare the calcification of the porcine aortic wall, bovine pericardium and BJV wall, cross-linked with GA and ethylene glycol diglycidyl ether (DE), in a subcutaneous rat model. We intended to weigh the efficacy of the DE-preserved tissue modification with the novel bisphosphonate CEABA.

**MATERIALS AND METHODS**

**Materials**

DE with a purity of 97% was received as a commercial product from the N. Vorozhtsov Novosibirsk Institute of Organic Chemistry, SB RAS (Novosibirsk, Russian Federation). CEABA was synthesized at the same institution by reacting vinylidene-1,1-diphosphonic acid with β-alanine (Fig. 1). GA and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline were obtained from Sigma-Aldrich (St Louis, MO, USA). Biomaterials [porcine aortic wall (sinotubular area), bovine pericardium and BJV wall] were obtained from the local slaughterhouse. Contegra (Medtronic, Minneapolis, MN, USA) wall samples were used as the control group for comparisons with experimental BJV wall samples because long-term clinical results (calcification rate, in particular) with Contegra are well known.

**Biomaterial preparation**

Fresh biomaterials (porcine aortic root, bovine pericardium and BJV) were selected from healthy animals immediately after slaughter and were rinsed several times with 0.9% sodium chloride. The surrounding connective tissue was thoroughly removed. Each biomaterial was preserved (within 6 h post-mortem) using the following 3 methods:

1. Treatment with 0.625% GA (0.1 M HEPES buffer, pH 7.4, at room temperature, with 2 changes on the 2nd and the 7th day) for 21 days (GA samples).
2. Treatment with 5% DE (0.1 M HEPES buffer, pH 7.4, at room temperature with 1 change on the 3rd day) for 14 days (DE samples).
3. Rinsing of the DE cross-linked biomaterial with sterile 0.9% sodium chloride and further treatment with 1.2% aqueous CEABA solution (pH 6.0, 37˚C) for 12 h (DE + CEABA samples).

After completion of each method, the biomaterial was thoroughly rinsed 3 times for 20 min and stored in a complex alcohol solution [mixture of 1, 2-octanediol, phenoxyethanol and 2,4-hexadienoic acid (1%) and ethanol (20%)] [15].

**Quantitative bisphosphonate assessment in DE + CEABA samples**

CEABA content (μM/g dry tissue) was calculated from the difference between the phosphorus content in the modified and non-modified DE-treated biomaterials:

\[
\frac{[\text{CEABA}]}{\text{mass}} = \frac{([P]_m - [P]_n)}{62} 
\]

where \([P]_m\) and \([P]_n\) represent phosphorus content (μg/mg) in the modified and non-modified biomaterials, respectively.

The phosphorus content was evaluated in the non-implanted DE and DE + CEABA samples. Samples were dried, weighed and hydrolyzed at 100°C in 5 ml 14 M nitric acid mixed with 0.2 ml 17 M perchloric acid and then were fully dried in an air jet. The hydrolysates were reconstituted in 5 ml 10% nitric acid. Phosphorus quantification was carried out using an IRIS Advantage (Thermo Scientific ‘TJA Solution’, Waltham, MA, USA) atomic emission spectrometer.

**Subcutaneous implantation of biomaterial in rats**

All experimental procedures maintained the applicable standards set in the ethics of the animal experimentation and research integrity and was approved by our local ethics committee.

Seventy 4-week-old male Wistar rats (80–100 g) were used for the subcutaneous implantation studies. The rats were anaesthetized...
with sevofluorane and shaved before the surgical procedure. Six incisions were made on the dorsal surface to prepare subdermal pouches for biomaterial samples. Six samples of different types were implanted in 1 animal. Each pouch was filled with 1 sample and then closed with 1 stitch. Two samples of each type were explanted on postoperative Days 10 and 60; they were rinsed with 0.9% sodium chloride and fixed in 10% neutral buffered formalin for further histological studies. The remaining 60-day explants were dried at 60°C for calcium analysis.

**Histological study**

All non-implanted and explanted samples fixed in formalin were dehydrated and embedded in paraffin. The 6-μm-thick sections of the non-implanted samples were stained using a Picro Mallory Trichrome Stain Kit (Bio-Optica Milano SpA, Milan, Italy) to visualize collagen and elastin fibres. A Von Kossa stain was applied to determine the presence of calcium phosphate in the explanted samples. The sections were treated with 5% silver nitrate for 30 min, then counterstained with eosin (pink staining) and examined using a light microscope Axioskop 40 (Carl Zeiss Microscopy GmbH, Gottingen, Germany).

**Calcification analysis**

The explanted 60-day biomaterial samples were dried, weighed and hydrolyzed in 5 ml 14 M nitric acid at 100°C and then were fully dried in an air jet. The hydrolysates were reconstituted in 1 ml 12 M hydrochloric acid mixed with 0.1 ml 0.25% buffered lanthanum chloride. Calcium quantification was done using the Thermo Solaar M6 (Thermo Fisher Scientific, Waltham, MA, USA) atomic absorption spectrophotometer.

**Statistical analysis**

Continuous data were reported as the median and interquartile range (25–75%). The Mann–Whitney U-test was used to compare 2 groups; the Kruskal–Wallis non-parametric analysis of variance test was employed to compare 3 or more groups. P-values <0.05 were considered as statistically significant. Data analysis was performed using STATISTICA version 10.0 (StatSoft Inc, Tulsa, OK, USA).

**RESULTS**

**Features of non-implanted tissue**

The preservation methods used in this study did not affect the microstructure of the biomaterial. All biomaterials differed in their collagen/elastin ratio, which can be clearly seen after the Picro-Mallory staining (Fig. 2). The pericardial tissue was mainly collagen, and only a small number of cells were found in the interfibrillar space. The BJV wall contained both collagen and elastin fibres. Thick elastin fibres were directed longitudinally and circular to the vessel axis; their density was roughly equal in all samples. Moreover, the structure of the BJV wall did not depend on the territorial origin of the biomaterial; the vein wall from the local slaughterhouse was similar in structure to that of the Contegra wall. The aortic wall contained just a small amount of collagen and a dense network of elastin fibres, and the tunica media contained a lot of smooth muscle cells (SMCs).

Initially, each of the biomaterials contained a different amount of phosphorus (Table 1). The smallest amount was revealed in the pericardium and the largest in the aortic wall, which is apparently due to the mass of SMCs in the tunica media. After modification, the calculated content of CEABA was almost...
equal in both the BJV wall and the pericardium ($P = 0.81$); however, it was lower ($P = 0.011$) in the aortic wall.

**Biomaterial calcification**

All GA-treated biomaterials displayed high calcium-binding activity; the bovine pericardium demonstrated the largest calcium deposits (Fig. 3). The calcium amount in the porcine aortic wall did not vary with the preservation method. In contrast, the calcium accumulation in the DE-treated BJV wall was 4 times lower than in the GA-treated one and 2.5 times lower than in the Contegra wall. As for the bovine pericardium, the calcium content was 40 times lower in the DE-treated samples compared with the GA-treated samples. The effectiveness of the CEABA modification varied in each of the DE-treated biomaterials; the calcium-binding capacity of the aortic wall and pericardium was indicated to be 10 and 3.5 times lower, respectively, while the modified venous wall demonstrated only a 33% calcium content reduction ($P = 0.11$).

In the elastin-containing materials (aortic and BJV walls), the calcification process mainly began on the elastin fibres (Figs 4A–C and 5A and B). Small initial calcium deposits in the

**DISCUSSION**

The findings of this study demonstrate the variations in the calcification onset and progress, which are dependent on the microstructure of the biomaterial, the cross-linking agent and the bisphosphonate fixed on the tissue. It has been previously shown that conduits containing the porcine aortic root usually demonstrate low freedom from reoperation and an increased calcification rate, regardless of the treatment [1, 6, 12, 16]. In this experimental study, we also show that the calcium-binding capacity of the porcine aortic wall is not associated with the type of preserving agent (GA or DE); it remains high with both agents. Similar results were obtained in earlier studies with other epoxide compounds [10, 17].

We speculate that the cause of this lies in the microstructure of the material, i.e. a lot of elastin fibres and SMCs. Because of its amino acid profile, elastin contains a small number of reactive groups that bind with aldehyde and epoxide reagents, but a greater number of calcium-binding sites; therefore, the more elastin a biomaterial contains, the faster it gets calcified [11, 18–20]. According to our data, it is elastin fibres that primarily accumulate calcium in the first 10 days after subcutaneous implantation. In addition, small dust-like crystals are found between the elastin fibres. It is likely that they form in the remnants of SMCs, which are known to be strong calcium nucleators [21]. The CEABA modification significantly reduced the aortic wall mineralization but did not fully suppress it, which can be seen in the 60-day samples with the von Kossa stain. We hypothesize that the bifunctional cross-linking agent reacts intensely with the aortic
wall cell proteins. The remaining groups of the cross-linker are then bound by bisphosphonate. Hence, calcium phosphate deposits in the 10-day CEABA samples are present only in the elastic fibres; however, the results of the 60-day implantation demonstrate the unreliability of this aortic wall anticalcium treatment in the long term. Therefore, we consider the porcine aortic root as an unsuitable material for paediatric conduits, at least before a reliable calcification-preventing treatment is on hand. Recent studies have suggested that either procyanidine cross-linking [12] or neomycin and pentagalloyl glucose treatment [22] may have the potential.

The BJV wall tended to amass calcium intensively after GA treatment; the calcium amount after 60 days was even bigger than in the aortic wall. By the 10th day, calcium deposits are clearly shown in both the elastin and collagen fibres, which develop into large calcium phosphate crystals over time. Notably, the Contegra wall accumulated 33% less calcium ($P = 0.037$) compared with the GA-treated samples. We speculate the cause of this to be in the specific effect of the solution that the Contegra wall is stored in. It contains 20% isopropanol, which may cause a reduction in the residual aldehyde groups participating in the calcification process [23, 24]. Preservation with the DE provides an enhanced mitigation of BJV wall calcification, in comparison with the GA samples and the Contegra, which is presumably due to the suppression of calcium binding in the collagenous parts of the biomaterial. However, we observed elastin mineralization in the DE samples on the 10th day after subcutaneous implantation. The extra CEABA modification defers calcium binding but does not block it completely. Thus, no calcium phosphate was found in the DE + CEABA samples on the 10th day (Fig. 5C); however, the calcium content only slightly differed from the DE samples on the 60th day ($P = 0.11$) (Figs 3 and 5F). Notably, immobilized CEABA amount was higher in the BJV wall than in the aortic wall but was not more effective for anticalcification. This suggests that the nature of the immobilized bisphosphonate effect is not dose dependent.

Comparison of our results with the earlier clinical data [5, 25] inclines us to conclude that calcification is not the major problem with BJV conduits; vein tissue mineralization can be further reduced using epoxy cross-linkage, blocking collagen calcification. Modification by CEABA does not significantly affect calcium accumulation in this biomaterial. Therefore, further bisphosphonate screening as well as new approaches in the blocking of elastin-calcium-binding sites are necessary.

Bioprosthetic materials containing collagen are known to calcify otherwise. Collagen predominates in porcine aortic valve leaflets and in the bovine pericardium. The experimental investigations of collagenous tissues began with a study by Schoen et al. [13], which was devoted to the chemical and morphological features of mineralization in GA-treated porcine aortic valve leaflets implanted subcutaneously in rats and orthotopically in calves. At present, the calcification process in GA-treated collagen materials has been fully defined. The calcium accumulation in the bovine pericardium is known to begin in the region between the outside serosa and middle fibrosa layers. Calcium-binding centres are not solely cell elements, they are predominantly collagen fibres [26]. We provide evidence for the effective blocking of calcification in the collagenous pericardium tissue via the substitution of the common GA for a diepoxy compound. Additional modification with CEABA maintains the calcium content in the pericardium samples at the level observed in the non-implanted tissue (0.95 µg/mg) during the 60 days of experiment. The efficacy of the combined approach, which employed epoxy cross-linking and additional bisphosphonate modification of the collagen-containing material, has been corroborated by other studies [10, 21]. The obtained data suggest that DE-treated, CEABA-modified bovine pericardium is suitable for further xenograft development.

Interpreting the obtained results, it is necessary to take into consideration that the mineralization process has 2 stages: calcium phosphate nucleation (the formation of the calcium phosphate crystal nucleus) and further growth of crystal (qualitative growth of the crystal mass) [8]. The GA-treated collagen and SMCs are strong calcium nucleators. Collagen calcification can be blocked by replacement of cross-linking agent. It is blocking at the first stage, because calcium-binding sites do not appear. For this reason, DE-preserved pericardium does not calcify, the DE vein calcifies much lower and only due to elastin. Elastin is not linked by either GA or DE and calcifies actively due to calcium-binding sites of the protein itself. Devitalized SMCs also have much calcium-binding sites. Elastin and SMCs are predominant.
components of the aortic wall. CEABA delays elastin mineralization process, mitigating the second calcification stage (growth of the crystal mass), but does not block it completely.

It remains difficult to screen bisphosphonates to determine the compound that suppresses calcification of a cardiovascular implant while fixed on the tissue. In the 1980s and 1990s, several studies exploring the anticalcification effect of immobilized bisphosphonates on GA-treated bioprosthetic materials were published. The major immobilizing agent was (3-amino-1-hydroxypropyldiene)-1,1-bisphosphonic acid [27, 28]. This compound, featuring a short carbon chain, contains a hydroxyl on the gem carbon. It is the hydroxyl that is responsible for the enhanced hydroxyapatite crystallization-blocking capability [8]. The efficacy of the approach has been corroborated for GA-treated porcine aortic leaflets and the bovine pericardium; however, it proved ineffective for the GA aortic wall. Rapoport et al. [10] reported that calcium accumulation in the aortic wall could be minimized by complex treatment with epoxy cross-linker triglycidyl amine and calcium binding of epoxy-treated collagen, leaving elastin calcification of xenografts could be partially for the bovine pericardium and can be employed to improve pericardial xenografts [12]. This model is not orthotopic, it does not involve a natural blood environment and haemodynamic loading, which are crucial factors of calcification.

Mitigation of biomaterial calcification in a small rodent model is not an obligatory predictor of calcium resistance in humans. This limits the extent to which the results mimic the conditions in human cardiovascular implants; therefore, direct conclusions cannot be drawn.

**CONCLUSION**

In conclusion, each type of xenogeneic material requires an individual anticalcification strategy. The strategy of cross-linking with DE and additional modification with CEABA is the most effective for the bovine pericardium and can be employed to improve pericardial xenografts. Calcification of the BJV wall could be partially reduced by substitution of GA for a DE, which should block calcium binding of epoxy-treated collagen, leaving elastin calcification unaffected. Additional CEABA treatment decreases, without completely suppressing, calcification of the xenogenous graft wall. The aortic wall consisting mainly of elastin and SMCs undergoes calcification that cannot be blocked completely using this strategy.

**ACKNOWLEDGEMENTS**

The authors thank Lidia N. Bukreeva (V. Sobolev Institute of Geology and Mineralogy SB RAS, Novosibirsk) for the atomic emission phosphorus analysis.

**Funding**

This work was supported by the Russian Science Foundation [grant number 16-15-10315].

**Conflict of interest:** none declared.

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