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Neomycin binding preserves extracellular matrix in bioprosthetic heart valves during in vitro cyclic fatigue and storage

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Abstract

Bioprosthetic heart valve (BHV) cusps have a complex architecture consisting of an anisotropic arrangement of collagen, glycosaminoglycans (GAGs) and elastin. Glutaraldehyde (GLUT) is used as a fixative for all clinical BHV implants; however, it only stabilizes the collagen component of the tissue, and other components such as GAGs and elastin are lost from the tissue during processing, storage or after implantation. We have shown previously that the effectiveness of the chemical crosslinking can be increased by incorporating neomycin trisulfate, a hyaluronidase inhibitor, to prevent the enzyme-mediated GAG degradation. In the present study, we optimized carbodiimide-based GAG-targeted chemistry to incorporate neomycin into BHV cusps prior to conventional GLUT crosslinking. This crosslinking leads to enhanced preservation of GAGs during in vitro cyclic fatigue and storage. The neomycin group showed greater GAG retention after both 10 and 50 million accelerated fatigue cycles and after 1 year of storage in GLUT solution. Thus, additional binding of neomycin to the cusps prior to standard GLUT crosslinking could enhance tissue stability and thus heart valve durability.

Keywords: Glycosaminoglycans; Elastin; Glutaraldehyde; Heart valve

1. Introduction

Bioprosthetic heart valves (BHVs) have been used since the early 1970s in valve-replacement surgeries. The use of bioprosthetic valves has increased from 20% in 1995 to 40% in 2000 and is currently 60–70%. Bioprosthetic valve xenografts are obtained either from porcine aortic valve or bovine pericardium [1–4].

Typically, biological tissues are chemically fixed to prevent immune rejection and tissue degeneration. Glutaraldehyde (GLUT), a water-soluble crosslinker, is the chemical of choice to crosslink the tissue because it almost completely reduces tissue antigenicity. GLUT has been used for crosslinking xenografts since 1969 [5]. GLUT devitalizes the tissue, crosslinks the majority of proteins thereby preventing enzymatic degradation, and sterilizes the tissue for implantation [6]. However, GLUT crosslinking has various shortcomings: residual or unstable GLUT in the interstices of the crosslinked tissue has been implicated in inflammatory response reactions, cytotoxicity, calcification and lack of endothelialization [7]. Another drawback of GLUT is its inability to stabilize glycosaminoglycans (GAGs) and elastin present in the bioprosthetic valves fabricated from porcine aortic valves [8,9]. In native heart valves, GAGs provide hydration and minimize the stresses acting on the valves. Loss of GAGs from BHVs has been reported during preparation, fixation, storage, in vitro fatigue cycling and in vivo implantation [10–13], and this loss may in part be responsible for the reduced durability of GLUT-treated BHVs.

We have shown that other fixatives such as 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and sodium metaperiodate are only partially effective in preventing GAG loss against GAG-degrading enzymes [11,14]. Neomycin trisulfate, a hyaluronidase inhibitor, has been

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incorporated in the tissue with carbodiimide fixation chemistry to prevent the enzyme-mediated GAG degradation. It was found to be very effective at stabilizing tissue against both in vitro and in vivo GAG-degrading enzymes [15]. GAG retention in neomycin-crosslinked valves led to reduced tissue buckling [16].

In the present study, we show that neomycin-mediated GAG-targeted crosslinking of the porcine aortic valves preserved GAGs during both in vitro accelerated fatigue cycling as well as after storage for 1 year. In addition, such crosslinking also stabilized elastin, another important extracellular matrix component. These findings, along with our previous data, indicate a mechanistic pathway for increasing the durability of heart valve bioprostheses.

2. Materials and methods

2.1. Materials

Ammonium acetate, neomycin trisulfate hydrate, (D+)-glucosamine HCl, hyaluronidase type VI-s from bovine testes (3000 units), chondroitinase ABC from *Proteus vulgaris* affinity purified (10 units), 1,9-dimethylmethylene blue (DMMB), calcium chloride, type VII collagenase (7500 units) from *Clostridium histolyticum* were all purchased from Sigma Aldrich Corporation (St. Louis, MO). GLUT (50 wt.% in H_2O) was obtained from Polysciences, Inc. (Warrington, PA), elastase from porcine pancreas (135 units mg^{-1}) was purchased from Elastin Products Company (Owensville, MO), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *n*-hydroxysulfosuccinimide (NHS) were obtained from Pierce Biotech (Rockford, IL). P-Dimethyl aminobenzaldehyde, acetyl acetone, Tris buffer, sodium azide and HEPES were purchased from Fisher Scientific (Fair Lawn, NJ), MES hydrate was obtained from Acros Organics (Somerville, NJ). Rabbit polyclonal antibody to elastin (ab21610) was obtained from Abcam (Cambridge, MA). Rabbit IgG Vectastain Elite ABC kit (PK6101) was obtained from Vector Laboratories (Burlingame, CA).

2.2. Harvest and fixation of heart valves

Porcine aortic heart valves were obtained at the time of slaughter from a local abattoir (Snow Creek Meat Processing, Seneca, SC). The aortic root was cut along the cuspal commissures and the cusps were left attached to the base of the aortic sinuses. For valves obtained for accelerated fatigue testing the aortic valves were kept intact. The aortic valves were transported to the laboratory in saline on ice. The valves were rinsed in buffered saline for three rinses of 10 min each in an orbital shaker. The aortic valves and the cusps were chemically crosslinked within 3–4 h of harvesting in order to minimize the amount of GAGs lost during collection and transportation. They were fixed in different groups as described in Table 1. For storage purposes and for storage studies, the valves and cusps fixed in three different groups were stored in 0.2% GLUT depending on the timeframe of the study. For all studies six samples per group ($n = 6$) were used unless otherwise mentioned.

2.3. Optimization of neomycin concentration

To determine the optimum concentration of neomycin for GAG fixation, cusps were incubated in 1 mM, 500 μM, 100 μM and 10 μM concentrations of neomycin in MES hydrate (pH 5.5) for 1 h followed by standard EDC/NHS and GLUT fixation. GLUT crosslinking alone was used as control. Samples were subjected to hexosamine and DMMB assays prior to or after GAG digestion using chondroitinase/hyaluronidase mixture to determine the efficacy of GAG fixation.

2.4. Deoxystreptamine as a control for neomycin

2-Deoxystreptamine dihydrobromide (DOS) was used as a control for neomycin. It has similar structure and amine functionalities for carbodiimide-based linking to the tissue but it lacks the GAGase enzyme inhibition activity of neomycin. For this study, 1 mM of DOS was used followed by EDC/NHS and GLUT similar to neomycin. Hexosamine assay and DMMB assays were performed to determine the amount of GAGs present in the cusps and GAGs released into the enzyme/buffer liquid, respectively.

2.5. GAG stability studies

Stability of the GAGs present in the valves was determined by treating valves with GAG-degrading enzymes. Briefly, the valve cusps were excised from the aortic sinuses and the cusps were washed in 100 mM ammonium acetate buffer (pH 7.0) three times, for 5 min each rinse. The cusps were cut into two halves along the radial direction and one half was treated in 1.2 ml of 5 U ml^{-1} of hyaluronidase and 0.1 U ml^{-1} of chondroitinase ABC in 100 mM ammonium acetate buffer at 37 °C under vigorous shaking at 650 rpm for 24 h. The corresponding other halves were treated with 100 mM ammonium acetate buffer alone as control. In studies where whole cusps were used, the enzyme concentrations were doubled. These optimum enzyme concentrations were chosen based on previous study [10].

2.6. Elastin and collagen stability studies

The effectiveness of elastin and collagen stabilization in the cusps was determined by treating cusps against elastase and collagenase, respectively. The cusps were rinsed, lyophilized and weighed to measure the initial dry weight. The lyophilized cusps were then treated with porcine pancreatic elastase or type VII collagenase as described previously [17–19]. Briefly, the cusps were treated with 1.2 ml of elastase (5 U ml^{-1}) in 100 mM Tris buffer, 1 mM $CaCl_2$, 0.02% NaN_3 (pH 7.8) and incubated at 37 °C for 24 h with shak-

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