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ScienceDirect
Biomaterials 29 (2008) 1645–1653
Biomaterials
www.elsevier.com/locate/biomaterials

The effect of glycosaminoglycan stabilization on tissue buckling in bioprosthetic heart valves

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Received 27 September 2007; accepted 9 December 2007
Available online 15 January 2008

Abstract

Bioprosthetic valves are used in thousands of heart valve replacement surgeries. Existing glutaraldehyde-crosslinked bioprosthetic valves fail due to either calcification or degeneration. Glutaraldehyde crosslinking does not stabilize valvular glycosaminoglycans (GAGs). GAGs, predominantly present in the medial spongiosa layer of native heart valve cusps, play an important role in regulating physico-mechanical behavior of the native cuspal tissue during dynamic motion. The primary objective of this study was to identify the role of cuspal GAGs in valve tissue buckling. Glutaraldehyde-crosslinked cusps showed extensive buckling compared to fresh, native cusps. Removal of GAGs by treatment with GAG-degrading enzymes led to a marked increase in buckling behavior in glutaraldehyde-crosslinked cusps. We demonstrate that the retention of valvular GAGs by carbodiimide crosslinking together with chemical attachment of neomycin trisulfate (a hyaluronidase inhibitor), prior to glutaraldehyde crosslinking, reduces the extent of buckling in bioprosthetic heart valves. Furthermore, following exposure to GAG-digestive enzymes, neomycin-trisulfate-bound cusps experienced no alterations in buckling behavior. Such moderate buckling patterns mimicked that of fresh, untreated cusps subjected to similar bending curvatures. Thus, GAG stabilization may subsequently improve the durability of these bioprostheses.

Keywords: Tissue buckling; GAGs; Neomycin; Carbodiimide

1. Introduction

Currently, bioprosthetic heart valves are crosslinked with glutaraldehyde to prevent tissue degradation and to reduce tissue antigenicity. Glutaraldehyde forms stable crosslinks with collagen via a Schiff base reaction of the aldehyde with an amine group of the hydroxylysine/lysine in collagen. However, within a decade of implantation, 20–30% of these bioprostheses will become dysfunctional, and over 50% will fail due to degeneration within 12–15 years post-operatively [1,2].

Glycosaminoglycans, a major constituent of valvular tissue, play an important role in maintaining a hydrated environment necessary for absorbing compressive loads, modulating shear stresses, and resisting tissue buckling in native heart valves. One of the disadvantages of glutaraldehyde crosslinking is its incomplete stabilization of GAGs [3,4], which lack the amine functionalities necessary for fixation by aldehydes. Previous studies have reported a greater depth of buckling in glutaraldehyde-crosslinked aortic valves as compared to fresh uncrosslinked tissue, which was primarily attributed to collagen crosslinking [5,6]. Buckling occurs at sites of sharp bending, producing large stresses that can eventually lead to mechanical fatigue and consequent valvular degeneration. Local structural collapse occurs at these areas of tissue buckling to minimize compressive stresses and subsequent reduction in tissue length.

We have reported the loss of GAGs in glutaraldehyde-crosslinked porcine cusps during fixation, storage, *in vitro* fatigue experimentation, and *in vivo* subdermal implantation due to enzyme-mediated GAG degradation [3,4,7,8]. Additionally,

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doi:10.1016/j.biomaterials.2007.12.009

GAG loss has been observed in failed porcine bioprosthetic heart valves following clinical use [9].

Therefore, to evaluate the potential role of GAGs in cusp buckling in bioprosthetic heart valves, we compared glutaraldehyde crosslinking to 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) based crosslinking chemistries that link GAG carboxyl groups to the amine groups of proteins. Furthermore, neomycin trisulfate, a hyaluronidase inhibitor, was chemically linked to the cusps to prevent enzymatic degradation of GAGs. Previously, such fixation chemistry was found to resist *in vitro* and *in vivo* enzymatic degradation of GAGs [10]. Using these GAG-targeted fixation strategies, we demonstrate that the retention of valvular GAGs reduces the extent of buckling in bioprosthetic heart valves, which may subsequently improve the durability of these bioprostheses.

2. Materials and methods

Porcine aortic heart valves were obtained from a local USDA-approved abattoir, Snow Creek Meat Processing, in Seneca, SC. The following materials were purchased from the noted vendors and used in the present studies: ammonium acetate, neomycin trisulfate salt hydrate, glutaraldehyde (50% stock), hyaluronidase (from bovine testes, type IV-S, 3000–15,000 U/mg), chondroitinase ABC (from *Proteus vulgaris*, lyophilized powder, 50–250 Umg), D(+)-glucosamine-HCl, collagenase Type VII from *Clostridium histolyticum*, and 1,9-dimethylmethylene blue (DMMB) were all purchased from Sigma–Aldrich Corporation (St. Louis, MO); 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) from Pierce Biotech (Rockford, IL); *p*-dimethylaminobenzaldehyde, acetyl acetone, and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Fisher Scientific (Fair Lawn, NJ); and 4-morpholinoethanesulfonic acid hydrate (MES) was obtained from Acros Organics, NJ.

2.1. Tissue harvesting and fixation

Fresh porcine aortic heart valves were thoroughly rinsed in ice-cold saline. Within 3 h of harvesting, intact aortic valves were stuffed with cotton to maintain diastolic morphology and chemically crosslinked in the following three fixation groups.

2.1.1. Group I

Glutaraldehyde (0.6%) in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline solution at pH 7.4 at ambient temperature for 24 h, followed by 0.2% glutaraldehyde in 50 mM HEPES buffered saline solution at pH 7.4 for 6 days at ambient temperature.

2.1.2. Group II

EDC–NHS solution (30 mM EDC/6 mM NHS) was buffered with 50 mM 4-morpholinoethanesulfonic acid hydrate (MES) at a pH of 5.5 for 24 h at ambient temperature. Following the carbodiimide fixation, valves were thoroughly rinsed in a 50-mM HEPES buffered (pH 7.4) saline solution and subsequently crosslinked with 0.6% glutaraldehyde for 24 h, followed by storage in 0.2% glutaraldehyde for the remaining 5 days.

2.1.3. Group III

One hour incubation in 1 mM neomycin trisulfate in MES buffer solution at a pH of 7.4. Next, valves were rinsed with deionized water and subsequently fixed with carbodiimide fixation chemistry as outlined above, in Group II. followed by storage in 0.2% glutaraldehyde.

2.1.4. Group IV

Fresh porcine aortic valves that were not chemically fixed were used as controls to observe buckling in native valve tissue.

2.2. Enzymatic degradation of GAGs

Following the above-mentioned respective tissue fixation and storage procedures, cusps were excised from their subtending aortic walls and thoroughly rinsed in 100 mM ammonium acetate buffer (pH 7.4). Whole cusps were incubated in 1.2 ml of 10 U/ml high purity hyaluronidase and 0.2 U/ml high purity chondroitinase ABC buffered in the aforementioned ammonium acetate buffer for 24 h at 37 °C under vigorous shaking at 650 rpm. Fresh cuspal tissue exposed to these enzymatic conditions has shown to completely deplete the valvular tissues of GAGs [3]. Following incubation in enzyme-buffered solutions, samples were thoroughly rinsed in deionized water.

2.3. GAG quantification by hexosamine analysis

Previously published methods were employed to quantify total hexosamine content in the respective tissue groups [3]. Briefly, lyophilized cusps were acid hydrolyzed using 2 M hydrochloric acid for 20 h at 95 °C in a vacuum desiccator. After thorough drying under nitrogen gas flow in a boiling water bath, tissue hydrolysates were dissolved in 2 ml of 1 M sodium chloride solution and reacted with 2 ml of 3% acetyl acetone in 1.25 M sodium carbonate. Next, these samples were incubated for 1 h at 96 °C. Following the cooling at room temperature, 4 ml of absolute ethanol and 2 ml of Ehrlich's reagent (0.18 M *p*-dimethylaminobenzaldehyde in 50% ethanol containing 3 N HCl) were added to the tube. An incubation period of 45 min at room temperature allowed formation of a color product reflective of the hexosamine quantities present in the cuspal tissue. Using the optical absorbance readings at 540 nm of the tissue hydrolysate and D(+)-glucosamine (0–200 μg) standards, the hexosamine quantities were determined.

2.4. Glycosaminoglycan quantification by dimethylmethylene blue assay

Following enzymatic digestion of GAGs using above-mentioned procedures, GAGs released into the enzyme solutions were quantified by 1,9-dimethylmethylene blue (DMMB) assay using previously described methods [10–13] with minor modifications. In a 96-well plate, 20 μl of the aforementioned enzyme solution, 30 μl of PBE buffer solution (100 mM Na_2HPO_4, 5 mM EDTA, pH 7.5), and 200 μl of DMMB reagent solution (40 mM NaCl, 40 mM glycine, 46 μM DMMB, pH 3.0) were added to each well. Next, optical absorbance readings were read at 525 nm. GAG release in buffer solutions (100 mM ammonium acetate buffer at pH 7.4) was used as controls. Chondroitin sulfate (0–1.25 μg) standards were also treated with 20 μl of the above-mentioned enzyme solution. Likewise, chondroitin sulfate (0–1.25 μg) standards without any exposure to GAG-degrading enzymes were employed to determine GAG release in buffer solution.

2.5. In vitro cyclic fatigue

Following the above-mentioned respective tissue fixation and storage procedures, valves were mounted on Delrin stents for accelerated wear testing. Using a Dynatek M6 machine, 3 stented valves from Group I and Group III were tested at 700 cycles/min and subjected to 10 million cycles in the accelerated fatigue tester. Daily tests included stroboscopic observations and pressure checks. After fatigue testing, cusps were dissected from the aortic wall and the extent of buckling depth was evaluated in cuspal strips (procedure described in the proceeding sections).

2.6. Specimen bending preparation

The cusps were excised from the aortic root, and circumferential strips were obtained from the belly region of the cuspal tissue. These 5 mm wide strips (with varied length to fit the desired curvature, see below) were bent to desired curvatures by bending them against natural curvature to mimic physiological bending in the belly region of the cusps [5,14].

To maintain a bent configuration of the cusps, stainless steel pins were pierced through both ends of the strips; the ends were separated to a desired