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Glycosaminoglycan-targeted fixation for improved bioprosthetic heart valve stabilization ☆

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Abstract

Numerous crosslinking chemistries and methodologies have been investigated as alternative fixatives to glutaraldehyde (GLUT) for the stabilization of bioprosthetic heart valves (BHVs). Particular attention has been paid to valve leaflet collagen and elastin stability following fixation. However, the stability of glycosaminoglycans (GAGs), the primary component of the spongiosa layer of the BHV, has been largely overlooked despite recent evidence provided by our group illustrating their structural and functional importance. In the present study we investigate the ability of two different crosslinking chemistries: sodium metaperiodate ($NaIO_4$) followed by GLUT (PG) and 1-Ethyl-3-(3 dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) followed by GLUT (ENG) to stabilize GAGs within BHV leaflets and compare resulting leaflet characteristics with that of GLUT-treated tissue. Incubation of fixed leaflets in GAG-degrading enzymes illustrated in vitro resistance of GAGs towards degradation in PG and ENG treated tissue while GLUT fixation alone was not effective in preventing GAG loss from BHV leaflets. Following subdermal implantation, significant amounts of GAGs were retained in leaflets in the ENG group in comparison to GLUT-treated tissue, although GAG loss was evident in all groups. Utilizing GAG-targeted fixation did not alter calcification potential of the leaflets while collagen stability was maintained at levels similar to that observed in conventional GLUT-treated tissue.

Keywords: Glutaraldehyde; Periodate; Carbodiimide; Hexosamine; Subdermal implant

1. Introduction

In 2001, nearly 300,000 patients worldwide underwent valve-replacement surgery to manage valvular heart disease [1]. Dysfunctional valves are replaced by either mechanical valves fabricated from pyrolytic carbon or chemically crosslinked biological tissue such as porcine aortic valves or bovine pericardium often called bioprosthetic heart valves (BHVs).

Glutaraldehyde (GLUT), an aliphatic dialdehyde, has been routinely used for fixation of BHVs. It has the ability to react with the free amine groups in tissue components, specifically collagen, to create tissue-stabilizing crosslinks. Although GLUT crosslinking provides tissue stability against biological breakdown, minimal immunogenicity, and sterility, it is generally recognized that this fixative contributes to loss of cell viability, leaflet calcification and structural dysfunction due to increased tissue stiffness. Approximately 20–30% of GLUT-fixed BHVs become dysfunctional within 10 years and more than 50% fail due to degeneration within 12 years post-operatively [2]. Glycosaminoglycans (GAGs), an integral component of native leaflets, are critical to valvular biomechanics. GAGs lack the amine functionality necessary for GLUT crosslinking to occur. Unlike collagen, these extracellular matrix components are not stabilized within the GLUT-fixed porcine valve leaflet. As a result GAGs are unremittingly lost from BHVs during in vitro fatigue experiments, storage, as well as when implanted in vivo [3–5]. Alternative fixation techniques using carbodiimides [1], epoxides [6], acyl azides [7], dye-mediated fixation [8], ultraviolet irradiation [9] and sodium periodate [10] have

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been used to over come the inadequacies associated with GLUT. Only periodate crosslinking has been investigated specifically for GAG-targeted fixation.

We hypothesize that loss of GAGs from valve leaflets may contribute to the accelerated degeneration of GLUT-fixed BHVs. Moreover, fixation of these GAGs within the spongiosa layer of the valve leaflet may help to retain leaflet water content while improving valve biomechanics and the long-term durability of the implant.

The primary aim of the present studies was to compare the ability of sodium metaperiodate ($NaIO_4$) and 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) to target GAG molecules and enhance their stabilization within the porcine aortic valve leaflet both in vitro and in vivo.

2. Materials and methods

GLUT (50% stock), hyaluronidase (from bovine testes, type IV-s, 3000–15,000 U/mg), chondroitinase ABC (from Proteus Vulgaris, lyophilized powder, 50–250 U/mg), D(+)-glucosamine-HCL, collagenase type VII from *Clostridium histolyticum* were all purchased from Sigma-Aldrich Corporation (St. Louis, MO). *P*-dimethylaminobenzaldehyde was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) HCL and *N*-hydroxysuccinimide (NHS) and bicinchoninic assay (BCA) protein kits were purchased from Pierce Biotech (Rockford, IL). Sodium periodate (meta) was purchased from Fisher Scientific (Fairlawn, NJ).

2.1. Aortic valve leaflet collection and fixation

Porcine hearts were collected at the time of slaughter from a local abattoir. Aortic roots were immediately dissected and the three leaflets were separated by cutting between the leaflet commissures, leaving each leaflet attached to its corresponding sinus and basal insertion. This procedure was followed in order to minimize unnecessary GAG loss via leaching through leaflet cut surfaces. Excised leaflet–sinus constructs were rinsed, transported to the laboratory on ice in cold saline, and fixed within 3 h of harvest.

GLUT-fixed aortic valve leaflets were prepared by fixing leaflet–sinus constructs using 0.6% GLUT in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline solution at pH 7.4 at ambient temperature. After 24 h, the solution was changed for an identically buffered 0.2% GLUT solution in which the leaflets were stored for a minimum of 6 days at ambient temperature.

Carbodiimide fixation of fresh aortic valve leaflets was performed in a 30 mM EDC/6 mM NHS solution buffered with 50 mM 4-morpholinoethanesulfonic (MES) acid hydrate at a pH of 5.5 for 24 h at ambient temperature. Following fixation leaflets were thoroughly rinsed in a 50 mM HEPES buffered (pH 7.4) saline solution and subsequently crosslinked with 0.6% GLUT for 1 day followed by storage in 0.2% GLUT for 5 days as described for the GLUT group. This group was designated as ENG.

Periodate fixation of leaflet tissue was performed using a 3.25 mM sodium periodate in a 100 mM MES buffer at a pH of 5.0 at 4 °C for a period of 2 h in the absence of light with gentle shaking. Unreacted periodate was quenched in a 10% aqueous glycerol solution for 15 min with rinses in acidic water. The quenching step was followed by an acidic GLUT fixation (0.6% GLUT in 100 mM MES buffer solution at a pH of 5.0) for 22 h in order to further stabilize the leaflet extracellular matrix. After acidic GLUT fixation, the standard GLUT procedure as described above was used to fix and store the valve tissue. This group is designated as PG. Schematics of ENG and PG crosslinking chemistries are shown in Fig. 1.

2.2. Resistance to GAG degrading enzymes

Aortic valve leaflets were removed from their respective storage conditions and dissected from the attached sinus. Leaflets were rinsed thoroughly in 100 mM ammonium acetate buffer (pH 7.4) and cut symmetrically in half in the radial direction. Half leaflets were incubated in 1.2 ml of 5 U/ml hyaluronidase and 0.1 U/ml chondroitinase ABC buffered in the aforementioned ammonium acetate buffer for 24 h at 37 °C under vigorous shaking at 650 RPM. GAGs have been shown to be completely removed from fresh leaflets under these conditions [4]. Undigested controls, consisted of corresponding half leaflets, were placed in 1.2 ml of the ammonium acetate buffer only. Following incubation in enzyme, samples were rinsed thoroughly in three changes of 1.2 ml of distilled water while vigorously shaking for 5 min each time. For studies requiring whole leaflet samples, enzymatic GAG removal was performed using 1.2 ml buffered solutions of 10 U/ml hyaluronidase and 0.2 U/ml chondroitinase ABC.

2.3. GAG quantification by hexosamine analysis

Total tissue hexosamines were quantified as previously published [4]. Briefly, lyophilized half leaflets ($n = 6$ per group) were acid hydrolyzed, reacted with a 3% acetylacetone in 1.25 M sodium carbonate solution, 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 subsequently added. The samples were incubated for 45 min in order to develop a color product indicative of hexosamine quantities.

A baseline hexosamine value was determined from enzyme-treated fresh leaflets. Total hexosamine content represents the sum of non-GAG and GAG-associated hexosamines. From our experiments it was found

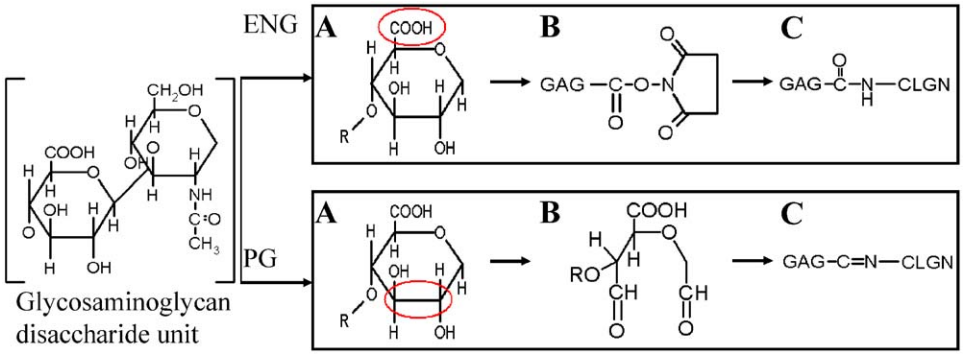

Fig. 1. Proposed reaction schematic for GAG-targeted fixation. Included is the structure of the repeating disaccharide unit: (A) fixative reaction site on GAG (oval), (B) resulting reactive intermediate, (C) resulting crosslink with collagen (CLGN).