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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 (BHV) 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

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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 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-dimethylmethylen 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 H₂O) 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 μ M, 100 μ M and 10 μ M 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 (refer to Table 1) 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 CaCl₂, 0.02% NaN₃ (pH 7.8) and incubated at 37°C for 24 h with shaking at 600 rpm. For collagenase studies, samples were treated with 1.2 ml of type VII collagenase (75 U ml⁻¹) made in 50 mM Tris buffer, 10 mM CaCl₂, 0.02% NaN₃ (pH 8.0), and incubated at 37°C with orbital shaking at 650 rpm for 48 h. The samples were then rinsed, lyophilized and weighed to obtain the final dry weight. The percentage weight loss due to elastin or collagen degradation is calculated from the initial and final dry weights before and after treatments of elastase or collagenase, respectively.

2.7 Immunohistochemistry for elastin

To test the effectiveness of elastin stability, cuspal sections from GLUT and neomycin (NEG) groups (refer to Table 1) with and without elastase treatment were immunostained for elastin. The Vectastain elite ABC kit with rabbit IgG and the diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate kit were used (Vector Laboratories, Burlingame, CA). The sections were incubated overnight at 4 °C in rabbit antielastin primary antibody (1:200 dilution). Elastin for the antibody was pooled from pig, human, dog chicken, rat and cow, and

reacts with all of them. Rat-adsorbed biotinylated secondary anti-rabbit IgG antibody was used to minimize cross-reactivity. Negative staining control was performed with the omission of the primary antibody. The sections were finally counterstained using hematoxylin.

2.8 Desmosine assay to determine elastin content of cusps

Tissues fixed in NEG, ENG, GLUT and FRESH were treated with elastase as described in Sec. 2.6. These tissues were lyophilized and weighed before hydrolysis using 6 N Ultrex hydrochloric acid for 8 h at about 95°C. The samples were dried in nitrogen gas and further reconstituted using 0.01 N HCl. These samples were sent to our collaborator, Dr Barry Starcher, for desmosine analysis. Desmosine, an amino acid unique to elastin, can be used to express elastin content. A radioimmunoassay was performed on the reconstitutes to express desmosine as pmol desmosine per mg protein [20]. Comparison of desmosine content on different groups after elastase treatment can be used to determine the stability of various groups against elastases.

2.9 GAG quantification

Total tissue hexosamine content is measured using hexosamine assay as mentioned previously [15]. Elson and Morgan's modified hexosamine assay was used for this purpose [21]. In brief, lyophilized cusps were hydrolyzed in 2 N HCl for 20 h at 95°C in a vacuum dessicator and further dried under nitrogen gas. The residues were dissolved in 2 ml of 1 M sodium chloride solution and then reacted with 2 ml of 3% acetyl acetone in 1.25 M sodium carbonate. Then 4 ml of 100% ethanol and 2 ml of Ehrlich's reagent (0.18 M *p*-dimethylaminobenzaldehyde in 50% ethanol containing 3 N HCl) were added and solutions were left at room temperature for 45 min. The pinkish-red color product was indicative of the tissue hexosamine quantities and the absorbance was read at 540 nm [21]. A set of D(+)-glucosamine solutions (0–200 µg) were used as a standard curve.

DMMB assay was used as described previously [15] to determine the amount of total GAGs released into the enzyme/buffer liquid. In a 96 well plate, 20 µl of enzyme solution along with 30 µl of PBE buffer (100 mM Na₂HPO₄, 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 into each well and absorbances were read at 525 nm. Chondroitin sulfate (0–1.25 µg) standards were used. As additional controls, the chondroitin sulfate standards were also treated with 20 µl of the enzyme mixture (HAase + CSase).

2.10 Fixation and storage studies

The GAG content of cusps was monitored over time for about 1 year. Thus, cusps treated with GLUT, ENG and NEG (Table 1) were stored for 4 months, 6 months and 12 months in 0.2% GLUT. The effect of the in vitro enzymatic degradation of GAGs was also determined at these time points by hexosamine content and DMMB assay.

2.11 In vitro accelerated cyclic fatigue testing

BHVs were subjected to accelerated cyclic testing to test GAG stability during valve function. Fresh porcine aortic valves were obtained from the local abattoir in saline on ice. Within 3 h after harvesting, the valves were fixed in fixatives (GLUT and NEG only for fatigue testing studies). During fixation, the valve cusps and the valves were stuffed with cotton balls in order for the valves to give a diastolic morphology and also to avoid backflow during fatigue testing. After fixation, the valves were carefully trimmed and sutured onto delrin stents and rings. Utmost care was taken during trimming as any cuts will result in stress propagation and tearing of the valves during testing. The valves were then mounted carefully onto a Dynatek Delta M6 accelerated fatigue tester. 0.2% GLUT is used as the circulating fluid in the tester as per industry

standards. Three valves per group ($n = 9$ cusps) were mounted and these were tested at a rate of ~ 700 cycles min^{-1} . In this protocol, approximately 37 days of testing (~ 38 million cycles) would be equivalent to 1 year of in vivo functioning of the normal cardiac cycle of an adult. The testing includes daily stroboscopic checking of the valves and pressure observations for any discrepancies. The valves were subjected to 10 million or 50 million cycles. Also, in order to determine the effect of storage followed by fatigue, NEG fixed cusps were stored for up to 8 weeks and further subjected to 10 million cycles of accelerated fatigue testing.

2.12 Histological assessment

Histological assessment after accelerated fatigue cycling was performed on paraffin embedded cuspal tissue sections. Alcian blue staining with Brazilliant![®] nuclear fast red (Anatech Ltd, Battle Creek, MI) as a counterstain was used to stain for GAGs. Histological images were captured using a Zeiss Axioskop 2 plus microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) and analyzed with SPOT Advanced software.

2.13 Statistical analysis

All data were expressed as a mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using single factor analysis of variance (ANOVA). Differences between the means were determined using least-significant difference (LSD) with a P-value of 0.05.

3 Results

3.1 Optimization of neomycin concentration

To determine the optimum concentration of tissue-bound neomycin necessary to prevent GAGase-mediated GAG degradation, crosslinking was performed with various concentrations of neomycin ($n = 6$ per group). Neomycin concentrations of 1 mM–100 μM exhibited similar GAG stability (Fig. 1A). At a lower concentration (10 μM) of neomycin, GAGs were lost after enzyme digestion, indicating inadequate GAG stability. DMMB assay showed that at 1 mM and 500 μM neomycin concentrations, no GAGs were released in enzyme solutions, suggesting complete GAG stability (Fig. 1B). 1 mM of neomycin was chosen as the concentration for use in this study as we wanted to completely prevent GAG loss.

3.2 Deoxystreptamine as a control for neomycin

In an attempt to establish that neomycin prevents GAG loss from cusps due to its hyaluronidase inhibitor property, DOS was chosen as control. This has a similar structure to neomycin and can link to the tissue during chemical crosslinking process, but lacks the hyaluronidase inhibitor property. Fig. 1C shows that DOS group was only partially effective in GAG stabilization in cusps against enzymatic degradation ($n = 6$ per group, $P < 0.05$). DMMB assay on enzyme solutions also showed a similar trend and complemented the hexosamine results (Fig. 1D).

3.3 Collagen and elastin stability studies

Per cent weight loss of cusps after treatment with collagenase showed very little loss of weight in all groups except fresh tissue. The NEG group lost least weight followed by ENG (Fig. 2). There was a significant difference among all four groups ($n = 6$ per group, $P < 0.05$). Since GLUT stabilizes collagen and all fixation groups were terminally fixed with GLUT, we observed less than 10% weight loss for all the fixation groups. FRESH, on the other hand, lost about 65% of collagen as it is not chemically crosslinked. Per cent weight loss of the cusps was also determined after subjecting cusps to elastase.

Per cent weight loss was least for the NEG group, followed by ENG ($n = 6$ per group, $P < 0.05$). GLUT and FRESH groups lost significant weight after elastase (Fig. 2), suggesting that

GLUT does not stabilize the elastin present in the cusps. Neomycin-bound cusps lost less weight after elastase treatment than any other group, so this chemistry was also effective to certain extent in elastin stabilization.

3.4 Immunohistochemistry for elastin

Immunohistochemical (IHC) staining showed the presence of elastic fibers in GLUT and NEG groups (Fig. 3A and C, respectively). After elastase enzyme treatment, cusps in the NEG group retained elastic fibers as seen in IHC stain (Fig. 3D). However, there was considerable thinning of fibers in the NEG group, suggesting some degradation of elastin. GLUT cusps, on the other hand, lost elastin and were sparingly stained for elastic fibers (Fig. 3B). Negative controls with the omission of primary antibody also did not show any staining (data not shown). Thus, IHC data further confirmed the weight loss data, showing better elastin stabilization in the NEG group as compared to the GLUT group.

3.4 Desmosine assay to determine elastin content of cusps

The desmosine content of cusps from different groups suggested that there was significant elastin loss in all groups after elastase treatment ($P < 0.05$) (Fig. 4). Although, NEG showed significantly improved stability than GLUT in weight loss studies and IHC after elastase treatment, there was no significant difference in their desmosine content ($P < 0.05$). This data suggests that elastin could still be vulnerable to degradation in fixed prostheses. GAG crosslinking needs to be supplemented with other treatments for further elastin stabilization. FRESH tissue desmosine content after elastase was significantly lower than other fixed groups after elastase ($P < 0.05$).

3.4 Fixation and storage studies

GAGs have been previously found to be lost from BHVs after storage [11]. This becomes an important issue as valves may be stored for up to 3 years prior to implantation. We investigated GAG stability in BHVs that were stored for up to 12 months.

The NEG group was found to be effective against GAG-degrading enzymes for up to 12 months of storage, whereas the ENG and GLUT groups showed progressive loss of GAGs after enzymatic degradation as storage time increased (Fig. 5A–C). GLUT-fixed tissue lost about 40.5% of GAGs after 1 year of storage in GLUT solution, while NEG-stabilized tissue lost only about 2.25% of GAGs. DMMB assay was performed on the enzyme liquid used for digestion of cusps to determine the amount of GAGs released into the enzyme solution. Better crosslinking is depicted by lower amounts of GAGs in the enzyme solution. DMMB assay results on enzyme solutions after 4, 6 and 12 months storage showed progressive loss of GAGs from the GLUT group with significant amount of GAGs lost at each time point ($n = 6$ per group, $P < 0.05$). Significantly lower amounts of GAGs were released from cusps in the NEG group as compared to the GLUT group (Fig. 5D) ($n = 6$ per group, $P < 0.05$). These results suggest that NEG crosslinking was significantly effective in preventing GAG loss for up to 1 year of storage.

3.5 In vitro accelerated cyclic fatigue testing

In vitro cyclic fatigue experiments were conducted to assess whether neomycin crosslinking is effective in GAG stabilization after cyclic fatigue testing for up to 50 million cycles ($n = 9$ per group). After 10 million cycles, cusps from GLUT group lost 9.5% GAGs, while NEG-fixed tissue lost only 6.22% GAGs (Fig. 6A). There was no significant difference between uncycled and 10 million cycled samples in NEG group ($P < 0.05$), suggesting complete GAG stability. Furthermore, the GLUT group continued the trend of losing more GAGs at 50 million cycles (Fig. 6B). The NEG group, on the other hand, experienced only a very slight decrease

in GAG content from 10 million to 50 million cycles, which was insignificant ($P > 0.05$). After 50 million cycles, cusps from the GLUT group lost 42% GAGs while NEG-fixed tissue lost only 7.12% GAGs.

Since GAG loss occurs even during storage in GLUT solution, cusps fixed with NEG and stored up to 2 months in 0.2% GLUT were fatigue tested for 10 million cycles to evaluate the effect of storage coupled with fatigue on NEG cusps only. There was no significant difference in GAG content between the uncycled control and the cycled samples after storage in the NEG group (Fig. 6C).

3.6 Histological assessment

Histological staining was performed on samples in an effort to qualitatively visualize the presence of GAGs after fatigue. The presence of blue stain and its relative intensity in alcian blue staining is related to GAG content. Cusps in the NEG group showed greater intensity of blue stain in the spongiosa layer than cusps in the GLUT group, suggesting that the former retains more GAGs than the GLUT group after 50 million fatigue cycles (Fig. 7). Moreover, cusps in the GLUT group showed voids in the spongiosa layer after fatigue.

4. Discussion

Native heart valves experience complex mechanical forces as they open and close during systole and diastole [3,12], and it is the structure of the valves' highly specialized extracellular matrix that enables them to withstand these stresses, including the collagen in their fibrosa layer, the framework of which provides mechanical strength to the cusps [22,23]. Stresses vary across the valve and the root, probably due to the morphological asymmetry [24]. In the ventricularis layer, elastin imparts elasticity to cusps and contributes to tissue recoil during valve opening and expansion during cuspal coaptation in valve closing. In the cuspal spongiosa layer, GAGs provide a "sliding gap" between the ventricularis and fibrosa. The hydrating nature of GAGs helps to absorb the shear and compressive stresses acting on the valve cusps [3,25,26]. Geometric and structural changes in the valves may affect the valves' biomechanical function when implanted [27]. GLUT, the commercially used fixative for BHVs, is an excellent fixative for the collagenous component of the valves, but it does not effectively stabilize GAGs and elastin, the other two major valve components. We hypothesized that valve long-term durability could be improved by preserving all ECM components in BHVs.

We wanted to choose the concentration of neomycin that inhibited GAG-degrading enzymes so as not to make the valves any stiffer. Both 1 mM and 500 μ M neomycin concentrations were found to stabilize GAGs against GAG-degrading enzymes. When enzyme solutions were assayed with DMMB, 1 mM and 500 μ M neomycin-fixed cusps did not show any released GAGs (compared to 100 and 10 μ M concentrations). We chose 1 mM neomycin concentration, in order to have little excess neomycin during crosslinking.

Because carboxyl groups of GAGs are the active sites for enzyme-mediated degradation [28, 29], we investigated whether the binding of neomycin to the carboxyl groups of GAGs could be the cause of its effectiveness in preventing GAG loss. We used DOS control as a substitute for neomycin. Since the structure of DOS is similar to that of neomycin, yet DOS does not inhibit GAGases, we compared GAG stability. DOS-linking only partially prevented GAG loss. These results suggest that the inhibitory action of neomycin on GAG loss is due to a combination of binding to the carboxyl groups of GAGs and inhibiting GAGase activity within the cusps.

Collagen is the major extracellular component in BHVs. GLUT, which has been used for four decades as a fixative of BHVs, stabilizes collagen against collagenase-mediated degradation.

Thus, a successful crosslinking strategy is one that does not hinder the ability of GLUT to stabilize collagen. Our results showed that neomycin binding prior to GLUT crosslinking improved the resistance of BHV cusp collagen to collagenase as compared to GLUT crosslinking alone. Thus, collagen crosslinking by GLUT was further enhanced by neomycin linking.

In native cusps, elastic fibers in the ventricularis layer, arranged radially, are believed to contribute to recoil of cusps, minimizing surface area during systole (when the valve opens), and to stretch during diastolic back-pressure to form large coaptation areas [30]. The role of elastin in BHVs is not clear, and no studies have focused on elastin stabilization in BHVs. It is believed that elastin forms a matrix and surrounds the collagen fiber bundles that help to return the collagen fibers to their undeformed state, maintaining the resting geometry [31,32]. In the same studies, damage to elastin fibers was found to distend the cusps, resulting in reduced extensibility and increased stiffness. Elastases were also found to remove proteoglycans nonspecifically [33]. We found that neomycin linking increased cusp resistance to elastases in weight loss studies. Immunohistochemistry for elastin after elastase digestion showed intact, albeit thinner, elastic fibers in the NEG group and completely degraded elastic fibers in GLUT-crosslinked cusps. However, desmosine content showed no significant differences among three groups after elastase treatment, suggesting that none of the crosslinking was completely effective in preventing elastase-mediated elastin degradation. Further strategies and studies are warranted to demonstrate the importance of elastin stability in cusps.

BHVs are approved by the FDA for a 3 year storage life; they are routinely stored for a few months to a year before implantation. In the present studies, the effectiveness of GAG stabilization after storage was determined at three time points: 4, 6 and 12 months. NEG-treated cusps inhibited enzymatic degradation of GAGs, for up to 1 year of storage; GLUT-treated cusps progressively lost GAGs during storage, which suggests that GAGs are leaching into the storage solution due to incomplete GAG fixation. Additional studies are needed to confirm whether GLUT-fixed BHVs that are stored for extended period will degenerate earlier after implantation than neomycin-linked BHVs.

Neomycin binding prevented GAG loss from cusps for up to 50 million cycles, while significant GAGs were lost from GLUT-crosslinked BHVs. Because of the risks associated with both surgery and in vivo valve failure, the FDA Replacement Heart Valves Draft Guidance, V. 5.0., requires samples from every batch of BHVs to be cycled in vitro for 200 million cycles and to undergo performance analysis before implantation [34,35]. GLUT-fixed BHVs, first approved for human use four decades ago, perform adequately for a minimum of 200 million cycles in in vitro cyclic fatigue experiments. Clearly, GAG loss during first 50 million cycles does not affect biomechanical performance. In terms of years of functional life, a period of 200 million cycles corresponds to only 5 years. Many BHVs fail due to degeneration after 10–15 years of implantation. We hypothesize that GAG loss during early implantation and function can make tissue vulnerable to subsequent degeneration later after implantation, and that stabilizing GAGs by neomycin binding may increase the functional life of valves. This hypothesis is not tested and further research is necessary.

5. Conclusions and future work

GLUT-stabilized BHVs showed degradation of GAGs and elastin components in BHV cusps. BHVs with bound neomycin showed complete preservation of GAGs after 1 year of storage and after 50 million fatigue cycles. The binding of neomycin also led to less degradation of collagen and elastin components. Such improved extracellular matrix stabilization may further enhance the functional life of BHVs. Future biomechanical, in vitro cyclic fatigue and animal

studies will help determine whether more effective preservation of GAGs and elastin prevents long-term degeneration of BHVs.

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Figure 1.

(A) Hexosamine content of cusps fixed with GLUT and various concentrations of neomycin (NEG). GLUT and 10 μ M neomycin concentration showed significant GAG loss ($P < 0.05$). (B) DMMB assay showing the amount of total GAGs released into the enzyme for the concentration study. Significant amounts of GAGs were released from cusps fixed in 100 and 10 μ M GLUT ($n = 6$). (C) Hexosamine content for 2-deoxystreptamine (DOS) study. DOS was only partially effective in inhibiting the degradation of GAGs ($n = 6$), while NEG group showed no GAG loss. (D) DMMB assay for DOS study showing amount of total GAGs released into the enzyme liquid from different groups ($n = 6$). DMMB data also showed more GAG release in DOS group compared to the NEG group. UD, undetected amounts. *Significant difference ($P < 0.05$).

Figure 2.

Per cent weight loss after treatment with elastase and collagenase. Cusps in the NEG group lost least weight followed by ENG, GLUT and FRESH after collagenase treatment (open bar). There was a significant difference between all groups ($n = 6$, $P < 0.05$). Cusps in the NEG and ENG groups lost significantly less weight compared to GLUT and FRESH ($n = 6$ per group) after elastase treatment (shaded bar).

Figure 3.

Immunohistochemical staining for elastin fibers (A) GLUT, (B) GLUT after elastase, (C) NEG and (D) NEG after elastase. The presence of elastin is indicated by brown staining for the elastin fibers. The arrows indicate the presence of elastin fibers and their location. Cusps in GLUT after elastase treatment did not show elastin fibers, suggesting complete degradation (B). In the NEG group elastin staining after elastases showed the presence of elastin fibers (D) but they were thinner than controls without elastase (C).

Figure 4.

Desmosine assay for determination of elastin content of cusps. All groups showed significantly lower desmosine content after elastases treatment, suggesting elastin degradation. Fresh tissues showed lowest elastin stability after elastases as expected. *Significant difference ($P < 0.05$).

Figure 5.

Long-term storage studies: hexosamine content of cusps fixed in three groups after storage for (A) 4 months, (B) 6 months and (C) 12 months. Cusps in the GLUT group lost more GAGs after different storage times ($n = 6$, $P < 0.05$) while no significant GAG loss was seen in the NEG group. (D) DMMB assay for GAGs released in enzyme solutions showed more GAG loss in GLUT than any other group at each time point ($n = 6$, $P < 0.05$). *Significant difference ($P < 0.05$).

Figure 6.

Accelerated cyclic fatigue testing studies: hexosamine content of cusps. (A) GLUT and NEG cusps after 10 million fatigue cycles. (B) GLUT and NEG cusps after 50 million fatigue cycles. After 50 million fatigue cycles, there was a significant difference in GAG content between the static and cycled GLUT groups ($n = 9$, $P < 0.05$), whereas no significant difference was observed in the NEG group. (C) NEG: groups stored for 2 weeks and 2 months followed by 10 million fatigue cycles. Data suggest storage followed by cyclic fatigue does not affect GAG stability in NEG-fixed cusps. *Significant difference ($P < 0.05$).

Figure 7.

Alcian blue staining for GAGs on cusps after 50 million fatigue cycles: (A) GLUT and (B) NEG. Blue indicates the presence of GAGs. The NEG group showed greater intensity of blue staining compared to GLUT, suggesting NEG crosslinking retained more GAGs even after 50 million fatigue cycles. Cusps in the GLUT group showed void areas in the spongiosa layer, indicating the loss of matrix elements.

Table 1
Crosslinking conditions used for stabilizing porcine aortic valve leaflets

Acronym	Fixatives	Duration of Fixation			
		1 hr	1 day	5 days	1 day
NEG	1 mM Neomycin	█	↓		
	30 mM EDC / 6 mM NHS		█	↓	
	0.6 % GLUT in HEPES			█	↓
	0.2 % GLUT in HEPES				█
ENG	30 mM EDC / 6 mM NHS	→	█	↓	
	0.6 % GLUT in HEPES			█	↓
	0.2 % GLUT in HEPES				█
GLUT	0.6 % GLUT in HEPES	→	█	█	↓
	0.2 % GLUT in HEPES				█
FRESH	→	No fixation			