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Neomycin fixation followed by ethanol pretreatment leads to reduced buckling and inhibition of calcification in bioprosthetic valves

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Abstract

Glutaraldehyde crosslinked bioprosthetic heart valves (BHVs) have two modalities of failure: degeneration (cuspal tear due to matrix failure) and calcification. They can occur independently as well as one can lead to the other causing co-existence. Calcific failure has been extensively studied before and several anticalcification treatments have been developed; however, little research is directed to understand mechanisms of valvular degeneration. One of the shortcomings of glutaraldehyde fixation is its inability to stabilize all extracellular matrix components in the tissue. Previous studies from our lab have demonstrated that neomycin could be used as a fixative to stabilize glycosaminoglycans (GAGs) present in the valve to improve matrix properties. But neomycin fixation did not prevent cuspal calcification.

In the present study, we wanted to enhance the anti-calcification potential of neomycin fixed valves by pre-treating with ethanol or removing the free aldehydes by sodium borohydride treatment. Ethanol treatment has been previously used and found to have excellent anti-calcification properties for valve cusps. Results demonstrated in this study suggest that neomycin followed by ethanol treatment effectively preserves GAGs both in vitro as well as in vivo after subdermal implantation in rats. In vivo calcification was inhibited in neomycin fixed cusps pretreated with ethanol compared to glutaraldehyde (GLUT) control. Sodium borohydride treatment by itself did not inhibit calcification nor stabilized GAGs against enzymatic degradation. Neomycin fixation followed by ethanol treatment of BHVs could prevent both modalities of failure, thereby increasing the effective durability and lifetime of these bioprostheses several fold.

1. Introduction

Glutaraldehyde crosslinked porcine aortic valve bioprosthesis (BHV) failure occurs either due to calcification or degeneration. Calcification process of valve cusps can make tissue brittle and prone to tears. Several decades of research on the mechanisms of calcification has led to new generation of valves that have anti-calcification treatments to prevent calcific failure. However, many valves also fail due to matrix failure independent of calcification leading to cuspal tears. We hypothesize that such failure is due to improper fixation of all extracellular matrix components by glutaraldehyde. We have developed neomycin based crosslinking (NEO) to stabilize glycosaminoglycans in the BHVs^{1,2}. Such stabilization could reduce valvular degenerative failure. In those studies we showed that despite effective GAG preservation, neomycin based fixation did not completely prevent cusp calcification after subdermal implantation in juvenile rats¹. Many approaches to prevent calcification

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were based on chemical treatment or removal of calcifiable compounds or by binding a calcium chelator^{3,4}. Diphosphonates such as ethane hydroxybisphosphate (EHBP) inhibits calcification by poisoning the growth of calcium crystals^{3,4}. Trivalent ions such as iron and aluminum prevent calcification as these cations complexes with phosphates and prevent calcium phosphate formation and nucleation^{3,4}. Amino oleic acid (AOA) binds to bioprosthetic tissue through amine linkage and prevents calcium flux through the cusps⁵⁻⁷. Surfactants and detergents such as sodium dodecyl sulfate (SDS) extract the phospholipids associated with calcification⁸. Ethanol preincubation especially greater than 80% is known to extract almost all the phospholipids and cholesterol from the cusps and affects the cusps interactions with water and lipids⁴. Also ethanol treatment was found to cause permanent alteration to the collagen conformation and also it enhances cuspal resistance to collagenase, thus preventing collagen associated calcification^{4,8}. Ethanol based anti-calcification pretreatment is currently clinically used for BHVs as a Linx[®] technology by St. Jude Medical Inc.

It has been long known that the free aldehyde groups present in the bioprosthetic tissue after glutaraldehyde fixation are also partially responsible for the bioprostheses calcification⁸⁻¹⁰. Sodium borohydride had been used as a reducing agent or Schiff bond quenching agent to reduce and eliminate the free aldehyde groups of tissues processed with glutaraldehyde¹¹⁻¹⁴. It has been previously shown by Connelly *et al* that, sodium borohydride reduction followed by ethanol pretreatment of the valves was very effective in inhibiting cuspal calcification¹¹.

The goal of current study was to evaluate whether we can implement ethanol pretreatment and/or sodium borohydride neutralization in combination with NEO stabilization to prevent calcification of BHVs as well as to prevent GAG loss. Such treatments might result in the prolonged durability for bioprosthetic heart valves.

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). Glutaraldehyde (50 wt% in H₂O) was obtained from Polysciences, Inc. (Warrington, PA), elastase from porcine pancreas (135 units/mg) 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, calcium standard and HEPES were purchased from Fisher Scientific (Fair Lawn, NJ), MES hydrate was obtained from Acros Organics, (Somerville, NJ).

2.2. Tissue preparation

Porcine aortic valve cusps were obtained at the time of slaughter from a local abattoir (Snow Creek Meat Processing, Seneca, SC). The valves were transported to the laboratory in saline on ice. The aortic root was cut along the cuspal commissures and the cusps were left attached to the base of the aortic sinuses. The valves were rinsed in buffered saline for three rinses of 10 minutes each on an orbit shaker. The aortic cusps were chemically crosslinked within 3–4 hours of harvesting to minimize the amount of GAGs loss during collection and transportation. These cusps were fixed in NEO or GLUT fixation. Valve cusps fixed with

NEO and GLUT were treated with ethanol and / or sodium borohydride to evaluate their anticalcification effects. The fixation and treatment conditions for different groups used for this study are shown in Table 1. Number of samples used were n=6 per group per study unless otherwise mentioned.

2.3. Stability of cusps against in vitro GAG degrading enzyme

The cusps fixed in different fixatives as mentioned above were exposed to in vitro enzymatic digestion of GAGs to determine GAG stability. Briefly, cusps cut from respective sinuses were rinsed thoroughly in 100 mM ammonium acetate buffer. The cusps were cut into two halves along the radial direction. One half of the cusp was placed in 5 U/ml hyaluronidase and 0.1 U/ml chondroitinase mixture in 100 mM ammonium acetate buffer and the remaining half was placed in 100 mM ammonium acetate alone as buffer control. The samples were placed at 37°C and shaken vigorously at 650 rpm for 24 hrs (Lab-Line Orbit Environ-Shaker, Melrose Park, IL).

The cusp samples after digestion were lyophilized and weighed. The dried samples were analyzed for cuspal tissue hexosamine content. The GAGs released into the buffer / enzyme were quantified using dimethylmethylene blue (DMMB) assay. The tissue hexosamine content or the total GAGs measured were finally normalized to tissue dry weights.

2.4 GAG quantification

Total tissue hexosamine content was measured using hexosamine assay as mentioned previously¹. Elson and Morgan's modified hexosamine assay was used for this purpose¹⁵. In brief, lyophilized cusps were hydrolyzed in 2N 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 1M sodium chloride solution and then reacted with 2 ml of 3% acetyl acetone in 1.25M sodium carbonate. Then 4 ml of 100% ethanol and 2ml of Ehrlich's reagent (0.18M p-dimethylaminobenzaldehyde in 50% ethanol containing 3N 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 540nm¹⁵. A set of D(+)-glucosamine solutions (0 – 200 µg) were used as a standard curve.

Dimethylmethylene blue (DMMB) assay was used as described previously¹ to determine the amount of sulfated 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).

2.5. Stability against collagenase and elastases mediated tissue degradation

Collagen and elastin are the two major extracellular matrix components in the valvular cusps apart from GAGs. For a valve to function and mimic the native valve, these ECM components should be preserved properly as well. Therefore, cuspal tissue weight loss against elastase or collagenase was determined. Briefly cusps were dried, lyophilized and weighed to measure the initial dry weights. The cusps were further rehydrated and treated with porcine pancreatic elastase or type VII collagenase as mentioned previously^{16–18}. Briefly, the cusps were treated with 1.2 ml of elastase (5 U/ml) in 100mM Tris buffer, 1mM CaCl₂, 0.02% NaN₃ (pH 7.8) and incubated at 37°C for 24 hrs with shaking at 600 rpm. For collagenase studies, cusps were treated with 1.2 ml of type VII collagenase (75 U/ml) 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 hrs. The enzyme digested samples were again dried,

lyophilized and the final dry weights measured. The percentage weight loss due to elastin or collagen degradation was calculated from the initial and final dry weights before and after treatments of elastase or collagenase respectively.

2.6. Measurement of thermal stability of collagen crosslinks

Thermal stability of the collagen crosslinks were determined using differential scanning calorimetry (DSC) (Model DSC 7, Perkin-Elmer, Boston, MA). The denaturation temperature (T_d) denotes the temperature at which the collagen triple helix is denatured and is a direct indicative of the collagen crosslink stability^{19–21}. Samples were heated in hermetically sealed pans from 20 to 110°C at a rate of 10°C/min. T_d , the temperature at the endothermic peak was determined.

2.7. Subdermal implantation studies

The cusps were treated in different groups as mentioned in Table 1 (n=10 per group). Cusps were rinsed in sterile saline prior to implantation to remove residual glutaraldehyde or ethanol. Male juvenile Sprague-Dawley rats (35–40g, 21 day old, Harlan Laboratories, Indianapolis, IN) were anesthetized by inhalation of 3% isoflurane gas and two subdermal pockets were created in the dorsal side. Cusps were implanted into the pocket (one cusp per pocket, 2 cusps per rat) and the incision was closed using surgical staples. Cusps and the capsule tissue surrounding the cusps were explanted after three weeks (21 days). All animals received humane care in compliance with protocols approved by Clemson University Animal Research Committee as formulated by NIH (Publication No. 86-23, revised 1996). Cuspal explants were further used for hexosamine, calcium, phosphorous and histological analysis. Cuspal explants for GAG quantification were immediately frozen on dry ice and lyophilized.

2.8. Calcium and phosphorous analysis

In order to determine the extent of calcification, a portion of the sample from the explants was used for calcium and phosphorous quantification. Calcium analysis was performed using atomic absorption spectroscopy^{1,18,20} and phosphorous quantification was done using a molybdate complexation assay.

For phosphorous analysis, samples are usually diluted twice that of which was used for calcium analysis in deionized water to make a final volume of 1 ml. 1 ml of reagent C (2.5% ammonium molybdate with 6N sulfuric acid and 10% L-ascorbic acid) was added and reacted at 37 °C for 2 hours. Samples were cooled for 15 minutes and 250 µl aliquots from each samples was placed in a 96 well plate. Sample absorbance was measured at 820 nm and phosphorous content determined from the standards used. The total phosphorous content was finally normalized to the tissue dry weight.

2.9. Histological analysis

For histological evaluation using paraffin sections, samples were placed in 10% alcoholic acid formalin at room temperature for 24h prior to infiltration and embedding. Following fixation, samples were dehydrated, infiltrated with paraffin wax in a tissue processor. Radial cuspal sections were embedded into paraffin blocks after processing the tissue. 5 µm sections were cut using a microtome and scooped onto microscope slides. These sections were used for various histological analyses.

Cuspal morphology was analyzed by hematoxylin and eosin staining. Dahl's alizarin red stain for calcium was used with light green counter stain to visualize the presence of calcium deposits after implantation. Light green is an anionic dye that stains components like cytoplasm and collagen so that the red calcium stain can be seen.

2.10. Specimen preparation for buckling depth studies

For bending studies to measure the tissue buckling, cusps fixed in NEO and GLUT followed by ethanol treatment alone (i.e., GLUT + EtOH and NEO + EtOH) were bent against the native curvature. Cusps were excised from the aortic root and 5 mm circumferential strips were obtained from the belly region of the cuspal tissue. These strips were bent to desired curvatures in the belly region of cusps (Figure 1A). Stainless steel pins were pierced through either ends of the strips and the ends were separated to a desired radius of curvature. They were further held in place by using cork stoppers at either ends of the pin for 24 hours in 0.2 % Glut solution. These samples were sectioned with bent configuration for histological observations to determine tissue buckling.

The radius of curvature was varied by changing the length of the tissue to satisfy the following relationship:

$$s=r*\theta_{\text{radians}}$$

whereby s denotes the arc length of the curvature, r represents the radius of curvature, and θ_{radians} is the radian angle of the arc.

2.11. Tissue buckling quantification

Zeiss Axioskop 2 plus (Carl Zeiss MicroImaging, Inc., Thornwood, NY) along with SPOT Advanced software were used to quantify the extent of buckling. The actual curvature of the bending, tissue thickness, and depth of buckling were measured using measuring and drafting functions such as circular and linear dimension features of the SPOT Advanced software. A circle was fitted visually to the semi-circular arc of the tissue to determine the radius of curvature. The tissue thickness was measured by averaging the local thickness of the tissue away from the sites of tissue buckling. Depth of tissue buckling was quantified by measuring the distance between the deepest point of buckling and the inner boundary of the tissue thickness (Figure 1B). The fractional depth of buckling represents the ratio of buckling depth to the local tissue thickness. The curvature was multiplied by the local thickness of tissue in order to normalize the variation in tissue thickness between samples. Thus, the degree of buckling depth was affected by both curvature and tissue thickness.

The extent of buckling increased as the radius of curvature decreased or as the curvature of bending increased. This was demonstrated by plotting fractional depth of buckling versus the product of tissue thickness and curvature of bending as described previously by Vesely, I. *et al*22·23.

2.12. 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. Stability against GAG degrading enzymes

In order to determine stability of the cusps against GAG degrading enzymes, cusps treated with sodium borohydride and / or ethanol were analyzed using hexosamine and DMMB assays with and without GAG digestion, as previously reported^{1·20}.

After in vitro GAG digestion, all the NEO groups retained GAGs as there was no significant difference between GAG digested and undigested samples ($p<0.05$) (Figure 2A). There was significant loss of GAGs after GAG digestion in all the GLUT groups ($p<0.05$) (Figure 2A).

Also, NEO+EtOH and NEO+NaBH₄+EtOH retained more GAGs, before and after digestion, compared to other groups ($p<0.05$). Though NEO+NaBH₄ resisted GAG degradation, it retained significantly lower amount of GAGs compared to NEO+EtOH and NEO+NaBH₄+EtOH. DMMB assay results shown in Figure 2B also correlated with the hexosamine results, showing lower amount of sulfated GAGs released into enzymes / buffer in NEO groups compared to their corresponding GLUT groups. DMMB assay measures only sulfated GAGs. It can be seen in GLUT + NaBH₄ groups that sulfated GAGs were leached even without GAGase.

3.2. Stability against collagenase and elastase

Cusps were either treated with elastase or collagenase to study the collagen and elastin stability. After elastase digestion, there was a significant lower weight loss in NEO groups as compared to GLUT ($p<0.05$) group (Figure 3A). This suggested that NEO treated cusps had superior stability against elastase in addition to GAG degrading enzymes. After collagenase digestion, groups that were treated with both NaBH₄ and ethanol lost least weight suggesting better collagen stability (Figure 3B). However all groups showed significant collagen stability very similar to GLUT crosslinked cusps reported earlier (10.2% weight loss)²⁴.

3.3. Measurement of thermal stability of collagen crosslinks

Thermal denaturation temperature (T_d) was measured by DSC for cusps in each fixation group to determine the denaturation temperature, a measure of the stability of the collagen crosslinks. Table 2 shows that the denaturation temperature of cusps fixed in different fixatives were in the acceptable range ($> 90^\circ\text{C}$) and there was no significant difference between groups suggesting no adverse effects on collagen stability.

3.4. Subdermal Implantation Studies

The purpose of the subdermal implantation studies were two fold: (i) determination of GAG retention following implantation on cusps that underwent anticalcification and neutralization treatment and (ii) determination of calcification of cusps fixed in different groups after anticalcification and neutralization treatment. Hexosamine data obtained from the explanted cusps suggested that NEO+EtOH group retained most GAGs after in vivo implantation (Figure 4A, $p<0.05$). NEO+NaBH₄+EtOH and NEO+NaBH₄, though resisting in vitro GAG digestion as shown in Figure 2, did not resist in vivo GAG loss compared to the unimplanted control (Figure 4A). GLUT fixed groups without neomycin also lost significant amounts of GAGs after in vivo implantation ($p<0.05$). Histological staining for GAGs (alcian blue) also corroborated the hexosamine data showing higher GAG staining in NEO+EtOH group (data not shown).

Calcium data obtained for cusps after explants confirmed that there is a significant reduction in calcification in case of ethanol treated groups. Sodium borohydride treatment by itself did not inhibit calcification as can be seen by higher amount of calcium content in NEO+NaBH₄ and GLUT+NaBH₄ groups ($p<0.05$) (Figure 4B). Calcification was significantly prevented in NEO+EtOH cusps as compared to all other groups ($p<0.05$). GLUT+EtOH though calcified less did not inhibit in vivo GAG digestion. Thus, only NEO+EtOH group showed inhibition of calcification as well as GAG retention in vivo. When phosphorus contents were measured, all NaBH₄ treated groups showed a very high calcium: phosphorous molar ratio (Figure 4B) clearly suggesting that calcification in these groups was not due to the deposition of poorly crystalline hydroxyapatite, which is generally seen in GLUT crosslinked cusps (Ca:P molar ratio of 1.67).

3.5. Histological Analysis

Dahl's alizarin red staining for calcium deposits were performed on histological sections of cusps obtained after in vivo implantation (Figure 5). As expected all ethanol treated groups showed absence of red deposits confirming that it is an effective anticalcification treatment. Samples treated with NaBH₄ alone showed lots of calcium deposits suggesting that NaBH₄ treatment alone does not inhibit calcification. The histological data correlated well with quantitative calcium data.

3.6. Depth of buckling analysis

Cusps fixed in NEO and GLUT and further incubated in ethanol for anticalcification treatment were analyzed for the depth of buckling by bending the cusps against the native curvature. Cuspal tissue fixed with NEO + EtOH experienced least degree of buckling ($p < 0.05$) when compared to their GLUT + EtOH counterparts bent to similar configurations (Figure 7). GAG digestion of GLUT + EtOH cusps resulted in a significant increase in the depth of buckling ($p < 0.05$) due to GAG loss. This suggested a greater propensity of the GLUT + EtOH group to buckle locally. On the other hand, there was no significant change in the depth of buckling of NEO + EtOH after GAG digestion ($p < 0.05$). This was expected as GAGs were resistant to GAGase digestion in NEO + EtOH group.

6.4. Discussion

Deterioration is a major cause of bioprosthetic heart valves (BHVs) dysfunction which is synergistically caused by two major factors (i) calcification and (ii) non-calcific degradation of the matrix elements^{3,25,26}. Despite, more than four decades of work in the field of BHVs, an ideal BHV free from degeneration and calcification is yet to be achieved. Calcification occurs in the valves due to multi-factorial reasons. Age, mitral or aortic implant position, prosthesis design (stented or non-stented), structure (porcine or pericardium, aortic wall portion or cusp), glutaraldehyde crosslinking, and presence of cellular debris are some of the factors²⁷ responsible for cusp calcification. Several anti-calcification treatments primarily focused on neutralization of aldehydes and phospholipid removal are used on current BHVs. These anti-calcification treatments only have been clinically used for last 5–7 years so long-term data of their effectiveness is yet not available. Clinical experiences also show that cuspal matrix degeneration and tearing can also occur independent of calcification²⁸. There are no treatments available to prevent tissue degeneration. We have shown earlier that glutaraldehyde crosslinking is unable to stabilize all components of extracellular matrix of tissues, especially elastin and glycosaminoglycans (GAGs)^{1,2,19,20,29–31}. We believe that loss of these components during valve function would lead to valvular matrix deterioration and lead to valve failure. Deterioration of the matrix elements such as GAGs are prevented by using a neomycin as a fixative, which might prevent the degenerative mode of BHV failure^{1,2}. However GAG stabilization alone could not prevent cuspal calcification. Therefore in present studies we looked to combine ethanol based anticalcification with neomycin based GAG fixation. We also looked at whether additional neutralization of aldehyde groups using sodium borohydride would be helpful. We chose these two treatments as extensive published studies have demonstrated the effectiveness of ethanol pretreatment and sodium borohydride in preventing calcification^{1,32–36}. The goal was to eventually prevent both degeneration and calcification of BHVs. This would ideally improve the durability of the valves and extend the functional life of bioprosthesis.

Previously we have shown that neomycin followed by GLUT fixation prevent GAG loss during storage, in vitro cyclic fatigue, and after in vivo implantation^{1,2}. We wanted to make sure that additional ethanol and NaBH₄ treatments would not make GAGs vulnerable to

loss. Especially ethanol is used as dehydrating agent and for removal of phospholipids and could extract GAGs from the tissue. We used in vitro exposure to high concentrations of chondroitinase and hyaluronidase as an accelerated method to test GAG stability after these treatments. It was encouraging to see that NEO fixed valve resisted GAG degradation due to GAGases even after treatments with ethanol of NaBH₄. Neomycin fixation was essential for GAG stability as all groups without neomycin lost GAGs after enzymatic degradation ($p < 0.05$). Thus, anticalcification treatments such as ethanol and NaBH₄ alone were unable to prevent GAG loss from the cusps.

Collagen and elastin are the other two major ECM components present in the valve. Any crosslinking and anti-calcification treatments should maintain the stability of these components for the valves to be durable. Again we used in vitro exposure to higher concentrations to elastases and collagenase as an accelerated method to test their stability. Previously we have shown that additional step of neomycin fixation prior to standard glutaraldehyde fixation did not alter the collagen and elastin stability. The present studies clearly show that even after ethanol and NaBH₄ treatments after crosslinking, collagen and elastin components were resistant to degradation in all groups. In fact, all neomycin groups showed increased stability against elastases. This data clearly shows that neomycin based GAG stabilization also improves elastin stability in the cusps. Previously it has been shown that loss of elastin from BHV cusps can lead to increase in stiffness and decrease in radial extensibility thus altering mechanical behavior of cusps³⁷. Thus improving elastin stability could be important in preventing mechanical failure. Neither ethanol treatment nor neutralization using sodium borohydride had a detrimental effect on the collagen crosslink stability as can be seen by DSC studies.

The next step was to study in vivo stability of GAGs and calcification of cusps. We used rat subdermal implantation model as it has been extensively used as a primary model to study calcification of BHVs^{1,19,20}. We have also shown previously that GAG degrading enzymes are active in subdermal tissue and GAGs are degraded from GLUT crosslinked cusps in this model while neomycin fixation prior to GLUT prevents GAG degradation¹. Although all NEO fixed group retained GAGs after GAG digestion in vitro, only NEO+EtOH retained all GAGs after 21 days of subdermal implantation while all other groups showed loss of GAGs ($p < 0.05$). Importantly, the same NEO+EtOH had least amount of calcification compared to other groups ($p < 0.05$). Sodium borohydride treatment by itself did not inhibit calcification. All NaBH₄ treated groups showed higher molar ratios of calcium deposition with significantly lower phosphorus levels. This high ratio suggests that calcification did not show poorly crystalline hydroxyapatite generally seen in BHV calcification in these groups. It has been suggested that reduction of free aldehydes by sodium borohydride in glutaraldehyde crosslinked tissues reduces calcification³⁸ but we did not see any difference in the present study. Many of the previous studies included other neutralizing agents such as glutamic acid or l-lysine in addition to NaBH₄ that may have affected calcification³⁹. Two other studies with NaBH₄ reduction of glutaraldehyde did not prevent calcification similar to what we observe in our studies^{40,41}. Neither ethanol treatment or NaBH₄ treatment or their combination prevented calcification and GAG loss. Only when neomycin fixation was combined with ethanol treatment (NEO + EtOH) we found tissue devoid of calcification and resistant to in vivo GAG loss.

Depth of buckling studies were performed on only the NEO + EtOH and GLUT + EtOH samples as our in vivo implantation results suggested that ethanol treatment results in improved resistance to calcification and increased GAG stability against enzymes in NEO fixed groups. We chose reverse bending for measuring buckling depth for the following reason. In native valves, the annulus expands when the valve opens. Thus valve only bend in one direction. In bioprosthetic valves, since they are crosslinked (thus increasing stiffness

several folds) and stented, the expansion of annulus is restricted resulting in irregular folding of cusps⁴². This results in reverse bending curvatures, characterized by the fibrosa on the outside of the bend^{22,43}. This unusual folding can lead to local stresses and failure of valve tissue. In the fractional depth of buckling studies NEO + EtOH groups showed significantly lower buckling depth as compared to GLUT + EtOH group. Moreover when Glut + EtOH group was exposed to GAG degrading enzymes, the buckling depth increased further due to loss of GAGs. While exposure to GAGase to NEO + EtOH group did not increase buckling depth, most probably due to retention of native GAGs. The buckling depths for NEO + EtOH group were very similar to native uncrosslinked cusps². This is a significant finding suggesting that such valve would cause less local structural damage during bending thus improve durability of valve tissue.

In conclusion, the results presented here suggest that neomycin fixation followed by ethanol incubation could be used to prevent both modes of valvular failure namely calcification and degeneration. Such modified crosslinking would eventually lead to increased durability of BHVs.

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Figure 1.

(A) Specimen bending preparation for tissue buckling studies. (B) Buckling depth quantification: Buckling depth was quantified by measuring the deepest point of the buckling to the inner boundary of the tissue. Arc length is measured by fitting a circle to the outer boundary of the tissue.

Figure 2.

In Vitro GAG stability against GAG degrading enzymes: (A) Cuspal tissue hexosamine content showed that NEO groups resisted enzyme mediated GAG degradation while there was significant GAG removal in case of GLUT groups after anti-calcification treatments ($p < 0.05$, $n = 6$). (B) DMMB assay for sulfated GAGs released in solution complemented the hexosamine data. When compared to their NEO counterparts, GLUT groups leached more GAGs. In particular GLUT combined with NaBH₄ crosslinking lead to increased sulfated GAG loss without GAGase treatment. * denotes significant difference.

Figure 3.

Percent weight loss studies: (A) Percent weight loss after in vitro elastase digestion and (B) Percent weight loss after in vitro collagenase digestion. Both A and B suggested that, except for ethanol treatment there was significant difference between the corresponding NEO and GLUT groups ($p < 0.05$, $n = 6$). * denotes significant difference.

Figure 4.

In Vivo implantation study: (A) Hexosamine content of cusps fixed in different groups following 21 days implantation in rats. Unimplanted cusps were used as control for comparison to determine GAG loss due to implantation. NEO+EtOH alone retained most GAGs after implantation, whereas all other groups lost GAGs following implantation ($p < 0.05$, $n = 10$). (B) Calcium and phosphorus content of cusps from different groups following 21 day subdermal implantation showed that NEO fixation followed by ethanol treatment inhibited calcification. It decreased calcification by more than 2 fold compared to GLUT followed by ethanol treatment ($p < 0.05$, $n = 10$). NaBH₄ treatments by itself did not inhibit calcification. The numbers on top of the bar denotes the Ca:P ratio for each group.

Figure 5.

Alizarin red staining with light green counter staining for calcium deposits in cusps after 21 days implantation in male Sprague Dawley rats. Red staining indicates presence of calcium deposits. (A) NEO+EtOH, (B) GLUT + EtOH, (C) NEO + NaBH₄, (D) GLUT + NaBH₄, (E) NEO+NaBH₄+EtOH and (F) GLUT+NaBH₄+EtOH. Ethanol treatment inhibits calcification while cusps treated with NaBH₄ alone show calcification.

Figure 6.

Depth of buckling analysis: (A) GLUT+EtOH buckling depth analysis and (B) NEO+EtOH buckling depth analysis. Graphs show the fractional depth of buckling plotted against the product of thickness and curvature. NEO+EtOH group had significantly lower fractional depth of buckling compared to GLUT+EtOH both before and after GAG digestion ($p < 0.05$, $n = 6$).

Table 1

Group identification for fixation conditions and treatments

Group ID	Acronyms	Treatment Conditions
NEO	Neomycin fixation of cusps	1 mM neomycin trisulfate in 50 mM MES hydrate (pH 5.5) for 1 hr followed by 30 mM EDC and 6 mM NHS in MES hydrate (pH 5.5) for 24 hrs followed by 0.6% glutaraldehyde (in 50 mM HEPES buffered saline, pH 7.4) for 24 hrs followed by 0.2% glutaraldehyde in 50 mM HEPES buffered saline (pH 7.4) for 5 days
GLUT	Glutaraldehyde fixation of cusps	0.6% glutaraldehyde (in 50 mM HEPES buffered saline, pH 7.4) for 24 hrs followed by 0.2% glutaraldehyde in 50 mM HEPES buffered saline (pH 7.4) for 6 days
NEO+EtOH	Neomycin fixation followed by ethanol	NEO treatment followed by 80% ethanol in HEPES (pH 7.4) for 24 hours
GLUT+EtOH	Glutaraldehyde fixation followed by ethanol	GLUT treatment followed by 80% ethanol in HEPES (pH 7.4) for 24 hours
NEO+NaBH ₄	Neomycin fixation followed by NaBH ₄	NEO treatment followed by NaBH ₄ treatment (0.08 M) in carbonate buffer (0.1 M NaHCO ₃ , 0.1 M Na ₂ CO ₃ , pH 10.3) for 24 hours
GLUT+NaBH ₄	Glutaraldehyde fixation followed by NaBH ₄	GLUT treatment followed by NaBH ₄ treatment (0.08 M) in carbonate buffer (0.1 M NaHCO ₃ , 0.1 M Na ₂ CO ₃ , pH 10.3) for 24 hours
NEO+NaBH ₄ +EtOH	Neomycin fixation followed by NaBH ₄ and ethanol	NEO treatment followed by NaBH ₄ treatment (0.08 M) in carbonate buffer (0.1 M NaHCO ₃ , 0.1 M Na ₂ CO ₃ , pH 10.3) for 24 hours. This is followed by 80% ethanol treatment in HEPES (pH 7.4) for 24 hours
GLUT+NaBH ₄ +EtOH	Glutaraldehyde fixation followed by NaBH ₄ and ethanol	GLUT treatment followed by NaBH ₄ treatment (0.08 M) in carbonate buffer (0.1 M NaHCO ₃ , 0.1 M Na ₂ CO ₃ , pH 10.3) for 24 hours. This is followed by 80% ethanol treatment in HEPES (pH 7.4) for 24 hours

Table 2Denaturation temperature T_d of cusps measured using DSC

Treatment	$T_d \pm \text{SEM}$ ($^{\circ}\text{C}$)
NEO + EtOH	92.75 ± 0.15
GLUT + EtOH	93.31 ± 0.40
NEO + NaBH_4	90.63 ± 1.25
GLUT + NaBH_4	95.31 ± 1.06
NEO + NaBH_4 + EtOH	92.08 ± 0.53
GLUT + NaBH_4 + ETOH	93.97 ± 0.4