Neomycin Enhances Glutaraldehyde Cross-linking and Glycosaminoglycan Stability in Bioprosthetic Heart Valves

Vincent M. Friebe, Amy E. Munnelly, Brandon T. Mikulis, Michael S. Sacks, Naren R. Vyavahare
Department of Bioengineering, Clemson University, Clemson, SC 29634

Introduction: Glutaraldehyde (Glut) cross-linked porcine aortic heart valves are used in over 125,000 heart valve replacement surgeries every year. While this type of bioprosthetic heart valve (BHV) has significantly improved the quality and length of life of many, it is encumbered with a limited durability that results in degenerative structural failure within 12-15 years post-operatively. Glut is known as an excellent fixative of the essential load-bearing collagen fibers in these tissues. However, valvular glycosaminoglycans (GAGs) are not stabilized, owing to their lack of amine moieties that are necessary for Glut cross-linking[1]. Previous work has shown that GAGs function in maintaining appropriate morphology, hydration, and biomechanical behavior in heart valves[1,2]. However, these components are subsequently lost throughout fixation, storage, *in vitro* cyclic fatigue and *in vivo* implantation[1]. Furthermore, clinically failed BHVs exhibit GAG loss upwards of 90% after 5 years[3], and have been similar after only 1 year in explanted sheep models[4]. Thus, we hypothesize that loss of GAGs may be partially responsible for the structural degeneration and failure of BHVs. This study examines the ability of neomycin to enhance glutaraldehyde cross-linking (NG) and stabilize cuspal GAGs against, without GAG targeted carbodiimide chemistry. We evaluate the resistance of NG fixed leaflets against *in vitro* enzymatic, fatigue and storage induced GAG loss. Furthermore, we indirectly investigate whether neomycin enhances Glut cross-linking by evaluating enzymatic resistance of collagen and elastin fibers, and examine biaxial biomechanics of NG treated leaflets versus GLUT.

Methods: Porcine aortic heart valves were obtained from a local abattoir, rinsed thoroughly, and transported back to the lab in ice-cold saline. Within 4 hours of excision, valves were cross-linked in the following methodologies:

GLUT: 0.6% glut for 24 hrs followed by 0.2% Glut in HEPES buffer at pH 7.4 for 6 days

NG: 1 mM of neomycin trisulfate (50 mM MES buffer) for 1hr followed by standard GLUT as shown. (n=6 for all groups). To analyze GAGase resistance, leaflets were excised at cuspal commisures, rinsed, and cut symmetrically in half. One half underwent GAGase incubation (5U/ml hyaluronidase and .1 U/ml chondroitinase ABC in 100 mM ammonium acetate buffer). The other half (controls) was placed in buffer mentioned, and both groups underwent incubation at 37 °C for 24 hrs under vigorous shaking. GAG content within cuspal tissues was assessed using hexosamine analysis. GAGs leached into surrounding buffer were quantified using DMMB assay. Leaflets were also qualitatively assessed for GAGs via alcian blue histological staining and transmission electron microscopy (TEM). Fatigued NG and GLUT treated valves underwent 50 million cycles of accelerated cyclic fatigue (corresponding to 1.5 years in the average adult) and were assessed for GAG content using aforementioned methods. Furthermore, analysis of GAG content of these groups in storage was assessed over a 1 year time period. Collagen and elastin stabilities were assessed by comparing weight loss after incubation in collagenase and elastase.

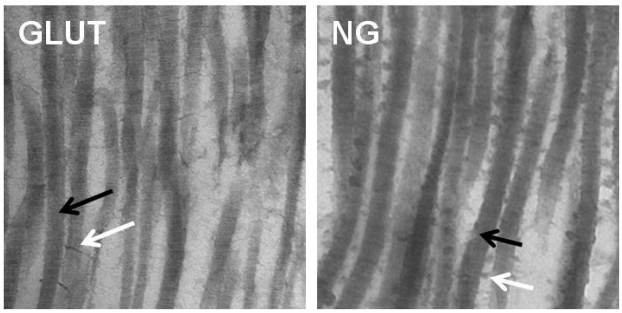

Figure 1.TEM; GLUT and NG leaflets; collagen fibers and associated GAGs (black and white arrows respectively).

Results: NG treatment was found to be an effective inhibitor of GAGases in BHVs, while GLUT experienced large amounts of GAG loss as assessed via hexosamine analysis (6% and 55% GAG loss, respectively). During storage, NG leaflets retained a substantial amount of original the GAG content (72%), comparative to GLUT valves (55%). However, GAG loss in NG valves in storage, while comparatively attenuated, is still substantial and suggests NG is not able to fully stabilize GAGs. During fatigue cycling, GLUT fatigued valves lost ~15% of GAGs, while NG GAG losses were negligible. All studies are further complemented by analysis of leached GAGs using DMB analysis and histological staining for GAGs. NG treated leaflets subjected to collagenase and elastase incubation revealed only 3% and 12% weight losses, respectively, while GLUT treated leaflets experienced weight losses of 17% and 26%. Ultra-structural analysis of NG treated leaflets revealed prominent GAG stabilization, while GLUT leaflets were substantially diminished (**Figure 1**). Biaxial tensile characterization revealed similar areal compliance of NG when compared to standard GLUT controls. The only significance comparison was examined by an increased low-tension radial stiffness in NG valves, a by-product likely accrued from enhanced collagen inter-fiber cross-linking.

Conclusions: Current results suggest that neomycin binding to collagen inhibits GAG degradation within BHVs. Furthermore; neomycin enhanced the Glut cross-linking densities, improving collagen and elastin stabilities as well. Collectively, NG cross-linking may improve the durability and long-term success of BHVs.

Acknowledgement: NIH grant #(HL070969).

References:

1. Lovekamp, J. *Biomaterials*. 2006; **27** (8): 1507-1518
2. Shah, S.R. *Biomaterials*. 2008; **29** (11): 1645-1653
3. Grande-Allen, K.J., *Biomed Mater Res A*. 2003; **65** (2)
4. Vavahare, N. *Biomed Mater Res*. 1999; 46 (1) 44-50