Neomycin binding to BHVs prevent glycosaminoglycan loss after storage and in vitro cyclic fatigue

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Introduction: Approximately 300,000 heart valve replacement surgeries are performed every year worldwide. About 60% of the patients requiring replacement valves receive bioprosthetic heart valves (BHVs). Better extracellular matrix stabilization to prevent degeneration will determine the long-term success and durability of these valves. Commercially available BHV's are fixed with Glutaraldehyde (Glut). Glut is known to be a good fixative for the collagenous component of the heart valves. Glycosaminoglycans (GAGs) and elastin are the other two major components of heart valves apart from collagen. Glut does not fix the GAGs and elastin present in the heart valve cusps. It has been shown that GAGs are lost during harvesting, fixation, storage, *in vitro* cyclic fatigue and after *in vivo* animal implantation[1,2]. Clinically explanted BHVs also show GAG depletion[3]. We hypothesized that the loss of GAGs may in part be responsible for degeneration resulting in the ultimate failure of the valves[1]. We have previously demonstrated that incorporating neomycin trisulfate, a hyaluronidase inhibitor, along with carbodiimide fixation chemistry helped to preserve GAGs after in vitro enzymatic degradation and in vivo implantation. The objective of this work was to determine the optimum concentration of neomycin, its effect of preserving the elastin in addition to GAGs. Also, we determined the effect of neomycin fixation on GAG loss during storage of up to 1 year and after in vitro cyclic fatigue of up to 50 million cycles.

Methods: Porcine aortic heart valves were obtained from a local abattoir; aortic cusps were dissected along the commissures and rinsed thoroughly in ice-cold saline. Cusps were chemically crosslinked within 3-4 hours of dissection in different fixation groups (n=6 cusps/group unless otherwise mentioned). Fixation groups and their ID's are follows: **GLUT:** 0.6% glut for 24 hrs followed by 0.2% Glut in HEPES buffer at pH 7.4 for 6 days, **ENG:** 30 mM EDC/ 6 mM NHS (Pierce Biotech, Rockford, IL) for 24 hrs followed by GLUT as above, **NEO:** 1 mM of neomycin trisulfate for 1 hr followed by ENG for 24 hrs and then GLUT as shown in ENG. Fresh tissue was used as control in some studies.
Various concentrations of neomycin were used from 10µM to 1 mM to determine optimum neomycin concentration for GAG preservation. GAGs in the cusps were quantified by hexosamine analysis and released GAGs were quantified by DMMB assay[4, 5]. Stability of GAGs against enzymatic digestion was determined by treating half cusps in 5U/ml hyaluronidase and 0.1 U/ml chondroitinase ABC (Sigma Aldrich Corp, St. Louis, MO) in 100 mM ammonium acetate buffer (37^0C for 24hrs at 650 RPM). Remaining half cusps were placed in ammonium acetate buffer alone as controls. Elastin and collagen stabilities were determined by studying weight loss in the cusps after treatment with collagenase and elastase enzymes. For elastase, cusps were treated with 1.2 ml of elastase (5 U/ml) in 100mM Tris buffer, 1mM $CaCl_2$, 0.02% NaN_3 pH 7.8 and incubated at 37°C for 24 hrs with shaking at 600 rpm. For collagenase, cusps were treated with 1.2 ml of type VII collagenase (75 U/ml) made in 50 mM Tris buffer, 10 mM $CaCl_2$, 0.02% NaN_3, pH 8.0, and incubated at 37°C with orbital shaking at 650 rpm for 48 hrs. For loss of GAGs during storage, cusps were stored in 0.2% GLUT for different time points of 4, 6 and 12 months and the GAG stability determined by hexosamine and DMMB assays.. Whole valves treated with NEO and GLUT were cycled in *in vitro* cyclic fatigue tester for 10 and 50 million cycles and the GAG stability after fatigue was determined.

Results and Discussion: It was found that 1mM of NEO was optimum in preserving GAGs compared to 500 µM, 100 µM and 10 µM. NEO stabilized both collagen and elastin better than GLUT. The percent weight loss was highest in fresh tissue followed by GLUT, ENG and NEO after both collagenase and elastase. NEO was able to prevent enzyme mediated GAG degradation even after 1 year of storage. ENG and GLUT were only partially effective against enzyme mediated GAG degradation as they continued to loose more GAGs as storage time increased. NEO also preserved GAGs after both 10 and 50 million fatigue cycles whereas GLUT continued to loose GAGs after fatigue. The hexosamine data was complemented by the histological assessment using Alcian Blue staining for GAGs. Thus this novel stabilizing strategy for GAGs and elastin may eventually be a stepping stone for increasing the durability of these BHVs.

Conclusions: Current results suggest that NEO preserves collagen and elastin better than ENG and GLUT. NEO preserves GAGs better than other groups even after 1 year of storage and after 50 million cycles of in vitro fatigue. Thus NEO crosslinking may help improve the lifetime and durability of the heart valve prostheses.

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