Published in final edited form as:

Biomaterials. 2006 March ; 27(8): 1507–1518.

Stability and function of glycosaminoglycans in porcine bioprosthetic heart valves

Joshua J. Lovekamp^a, Dan T. Simionescu^a, Jeremy J. Mercuri^a, Brett Zubiate^b, Michael S. Sacks^b, and Narendra R. Vyavahare^{a,*}

^a Cardiovascular Implant Research Laboratory, Department of Bioengineering, Clemson University, 501 Rhodes Engineering Research Center, Clemson, SC 29634, USA

^b Engineered Tissue Mechanics Laboratory, Department of Bioengineering, University of Pittsburgh, 100 Technology Drive, Suite 200, Pittsburgh, PA 15219, USA

Abstract

Glycosaminoglycans (GAGs) are important structural and functional components in native aortic heart valves and in glutaraldehyde (Glut)-fixed bioprosthetic heart valves (BHVs). However, very little is known about the fate of GAGs within the extracellular matrix of BHVs and their contribution to BHV longevity. BHVs used in heart valve replacement surgery have limited durability due to mechanical failure and pathologic calcification. In the present study we bring evidence for the dramatic loss of GAGs from within the BHV cusp structure during storage in saline and both short- and long-term Glut fixation. In order to gain insight into role of GAGs, we compared properties of fresh and Glut-fixed porcine heart valve cusps before and after complete GAG removal. GAG removal resulted in significant morphological and functional tissue alterations, including decreases in cuspal thickness, reduction of water content and diminution of rehydration capacity. By virtue of this diminished hydration, loss of GAGs also greatly increased the “with-curvature” flexural rigidity of cuspal tissue. However, removal of GAGs did not alter calcification potential of BHV cups when implanted in the rat subdermal model. Controlling the extent of pre-implantation GAG degradation in BHVs and development of improved GAG crosslinking techniques are expected to improve the mechanical durability of future cardiovascular bioprostheses.

Keywords

Aortic valve; Glycosaminoglycans; Glutaraldehyde; Bioprosthesis

1. Introduction

It is estimated that more than 75,000 bioprosthetic heart valves (BHVs) are implanted annually worldwide [1]. These valves offer the advantage of being non-thrombogenic and, therefore, do not require the chronic anticoagulation therapy necessary with mechanical valve implantation. However, their durability is limited when compared to their mechanical counterparts, often failing within 10–15 years of implantation due to calcification and/or structural fatigue [2,3]. As a result, the use of BHVs is typically restricted to those patients who are greater than 60 years of age and to those in which anticoagulation therapy is counter indicated. The remainder of patients, who receive mechanical valves, are also likely to encounter complications because of their valve replacement, primarily related to the requisite anticoagulation therapy [1,2]. Therefore, the development of more durable BHVs capable of being implanted into a younger patient population would be advantageous.

*Corresponding author. Tel.: +1 864 656 5558; fax: +1 864 656 4466. E-mail address: narenv@clemson.edu (N.R. Vyavahare)..

Current BHVs are constructed either from porcine aortic valve tissue or bovine pericardium. In order to reduce the immunologic ramifications of xenograft implantation, tissues are crosslinked with glutaraldehyde (Glut). This crosslinking also serves to improve resistance to enzymatic degradation in vivo and improve durability [4–6]. Tissue crosslinks are formed by means of a number of complex reactions through which covalent bonds are created between the primary amine groups of proteins and the reactive aldehyde functionalities of Glut. The result is a tightly crosslinked matrix of proteins, the majority of which is collagen [7–9]. However, cuspal extracellular matrix components lacking free amine functionalities, such as elastin and glycosaminoglycans (GAGs), are not effectively stabilized [10–13]. In the case of GAGs, this deficiency has previously been documented quantitatively as a reduction in the GAG content of explanted clinical BHVs [14,15] and qualitatively using histology and ultrastructural analysis following Glut fixation [10,16].

GAGs constitute a large fraction of the extracellular matrix of porcine aortic valve cusps, particularly within the central cuspal layer, the spongiosa. GAGs are long, hydrophilic, anionic, unbranched, polymeric molecules consisting of repeating disaccharides. There exists at least five GAG species, the largest of which is hyaluronic acid, which is also the only one that does not contain a sulfate group and is not covalently bound to a protein. The remainder of the tissue GAGs (chondroitin and dermatan sulfates) are typically bound to small link proteins, which are in turn bound to a core protein, forming a proteoglycan [17]. These molecules, because of the high concentration of negative charges and their inherent hydrophilicity are capable of absorbing a large amount of water within the tissue matrix. As a result, they are important components of the extracellular matrix of native heart valve cusps, especially with regards to mechanical behavior [4,10,16]. It has been speculated that their ability to hydrate the spongiosa layer serves to decrease the shear stresses associated with cuspal flexure during valve function [18–20]. In addition, the ability of this hydrated layer to absorb compressive forces may reduce buckling during flexion, which has been attributed to the mechanical failure of BHV cusps [21–25]. Furthermore, it has previously been speculated that the presence of negatively charged GAG molecules within the extracellular matrix of cuspal tissue may reduce calcification by chelating calcium ions, thereby preventing hydroxyapatite nucleation [26,27].

Taken together, these observations suggest that the loss of GAGs may be an important factor in the structural and/or calcific failure of BHVs. However, the exact contribution of GAGs to BHV longevity remains elusive. In the present study we present quantitative and histologic evidence for the progressive loss of GAGs from within the BHV cusp structure during valve fixation and storage in Glut. Furthermore we show that GAGs are essential components of the cuspal extracellular matrix that maintain tissue hydration and consequently influence the mechanical properties of BHVs. As a result, the vulnerability of cuspal GAGs within BHVs to degradation and loss is likely to ultimately affect the long-term durability of these devices following implantation.

2. Materials and methods

2.1. Materials

Glutaraldehyde (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-glucurono-6,3-lactone, carbazole, collagenase Type VII from *Clostridium histolyticum* and D (+)-glucosamine-HCl were all purchased from Sigma-Aldrich Corporation (St. Louis, MO). *p*-dimethylaminobenzaldehyde was purchased from EMD Chemicals Inc. (Gibbstown, NJ).

2.2. Collection and fixation of porcine aortic valves

Porcine hearts were collected at the time of slaughter from a local abattoir, Aortic valves were immediately excised and the three cusps separated by cutting between the cuspal commissures, leaving the cusps attached to the aortic sinus at the basal insertion in order to minimize GAG loss through the cut edge. The cusps were then rinsed and transported to the laboratory in ice cold, unbuffered saline.

The instability of GAGs in fresh cuspal tissue was investigated by storing freshly obtained valve cusps in unbuffered saline for 24 h at 4 °C. Glut-fixed porcine aortic valve cusps were prepared by the incubation of freshly obtained valve cusps (within 3–5 h of collection), intact with its corresponding aortic sinus, at room temperature in 0.6% Glut in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered saline at pH 7.4 for 24 h. Subsequently, this solution was exchanged for a HEPES buffered 0.2% Glut solution, in which cusps were stored for 6 days at 22 °C. Where noted, longer term storage (from 14 days to 5 years) was also conducted during which time valves remained in buffered 0.2% Glut until the indicated analyses were performed. In the case of samples prepared for mechanical testing, cusps and sinuses were lightly stuffed with sterile cotton presoaked in Glut in order to preserve the natural diastolic leaflet shape (closed valve position) during the 7 days of Glut fixation and storage.

2.3. GAG removal by specific enzymes

Aortic valve cusps were dissected from the attached sinus, cut in half in radial direction, and rinsed well (3 × 5 min) in 100 mM ammonium acetate buffer, pH 7.0. Subsequently, cuspal GAGs were selectively removed by incubating half cusps in 1.2 ml of the same buffer containing 5 U/ml high-purity hyaluronidase and 0.1 U/ml high-purity chondroitinase ABC, or, where noted, a dilution thereof. As an undigested control, the opposing half of each cusp was incubated in buffer alone. For studies requiring whole cusp samples, enzymatic GAG removal was performed using 1.2 ml of buffered 10 U/ml hyaluronidase and 0.2 U/ml chondroitinase ABC. In all cases, incubation was performed with vigorous shaking (650 rpm) at 37 °C for 24 h. Following enzyme incubation, samples prepared for stiffness testing were stored in 0.2% HEPES buffered Glut (pH 7.4) prior to analysis for no more than 7 additional days.

2.4. GAG quantification by uronic acid analysis

The GAG content of fresh and Glut-fixed porcine aortic valve cusps ($n = 6$ per group) was determined by GAG extraction followed by uronic acid analysis, as described previously in detail [26], with the exception of some modifications noted below.

- a. *GAG extraction:* Half cusps were frozen in liquid nitrogen and crushed using a bio-pulverizer (Biospec Products Inc., Bartlesville, OK). This ground tissue was then lyophilized and the dry weight recorded for normalization. GAG extraction was performed by tissue digestion for 24 h in sodium hydroxide at 4 °C. Subsequently, proteins were precipitated with trichloroacetic acid for an additional 24 h at 4 °C, after which time the samples were centrifuged (12,000g, 12 min) and the supernatant dialyzed against several changes of water for 24 h (3500 kD MWCO). The GAGs from the resulting solution were then precipitated with cetylpyridinium chloride for 24 h at 37 °C in glass centrifuge tubes. The precipitated GAGs were collected by centrifugation (2000g, 10 min), dissolved in sodium acetate, and reprecipitated with absolute ethanol for 48 h at –20 °C. These precipitates were then collected by centrifugation (2000g, 10 min), the supernatants poured off, and the resulting GAG pellets dried under nitrogen gas.

- b. *Uronic acid analysis*: The dried GAG pellets were dissolved in 1 ml of benzoic acid saturated water, to which 5 ml of borax-sulfuric acid reagent was added with constant cooling in a dry ice/acetone bath. These samples were then heated for 10 min in a boiling water bath and allowed to return to room temperature. Subsequently, 0.2 ml of carbazole color reagent was added and the samples were placed back into the boiling water bath for an additional 15 min. After again cooling to room temperature, the optical absorbance at 530 nm was measured using a microplate spectrophotometer (μ Quant, Biotek Instruments Inc., Winooski, VT). Quantification was made possible by performing analogous measurements of D-glucurono-6,3-lactone standards (0–150 μ g/ml) dissolved in benzoic acid saturated water. The same procedure was followed for the standards with the exception that the carbazole reagent was added immediately after the addition of the borax-sulfuric acid reagent and the samples were heated in the boiling water bath for 25 min without interruption.

2.5. GAG quantification by hexosamine analysis

Total tissue hexosamines were quantified as previously published [28] with minor modifications. To summarize, half cusps ($n = 6$ per group) were first hydrolyzed for 20 h in 2 N hydrochloric acid (HCl) in a vacuum desiccator at 95 °C, after which time the solution was dried under nitrogen gas in a hot water bath. Subsequently, tissue hydrolysates and standards of D(+)-glucosamine (0–200 μ g) were dissolved in 2 ml of 1 M sodium chloride, which was followed by the addition of 2 ml of 3% acetylacetone in 1.25 M sodium carbonate and 1 h of incubation at 96 °C. After allowing to cool back to room temperature, 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 added sequentially to each sample. The resulting color product was then allowed to develop for 45 min at 22 °C after which time the optical absorbance at 540 nm was recorded and the hexosamine content of unknowns determined by comparison with the standard curve.

2.6. Collagen stability studies

The thermal denaturation temperatures (T_d) and resistance to collagenase, indicators of collagen crosslinking stability, were measured in samples obtained from Glut-fixed cusps collected before and after treatment with hyaluronidase and chondroitinase, as described before [13]. T_d was measured in cusp samples ($n = 3$) using a differential scanning calorimeter (DSC) (Model DSC 7, Perkin-Elmer, Boston, MA) by heating tissue samples at a rate of 10 °C/min from 20 to 110 °C and T_d determined as the temperature measured at the endothermic peak. For resistance to collagenase, cusp samples ($n = 6$), were rinsed, lyophilized to record dry weight and incubated in 150 U/ml collagenase buffered in 50 mM Tris, 10 mM calcium chloride, pH 8.0 at 37 °C for 48 h with shaking. After rinsing, cusps were lyophilized again to obtain dry weight after collagenase, and the percent of digested tissue was calculated.

2.7. Measurement of water content

The water content of both fresh and Glut-fixed half cusps with or without GAGs was determined by comparing the dry tissue weight with that of the same sample when wet. Wet weights were obtained by gently drying the exterior surfaces of the cuspal tissue with tissue paper (Kimwipes EX-L Delicate Task Wipers, Kimberly Clark Inc, Roswell, GA) without applying any pressure, prior to weighing. The initial water content was determined by comparing the wet weight of the tissue prior to lyophilization with its dry weight after lyophilization ($n = 13$). The rehydrated water content was determined by soaking dried cuspal tissue in 1 ml of deionized water for 24 h at 4 °C, after which time the wet weight was obtained and compared with previously measured dry weight ($n = 13$).

2.8. Measurement of cuspal thickness

The thickness of whole porcine aortic valve cusps (fresh and Glut-fixed) was measured before and after 24 h of incubation at 37 °C in either enzyme (10 U/ml hyaluronidase and 0.2 U chondroitinase ABC in 0.1 M ammonium acetate buffer pH 7) or buffer solution. Thickness was measured using an analog dial indicator (Peacock model 1331, Ozaki Manufacturing Co LTD, Japan) with an accuracy of 0.01 mm and a sliding shaft that exerted a compressive force of 30 g over a circular area 5 mm in diameter. The sample thickness was taken from three different points in the center of each cusp sample. These values were then averaged to yield a single thickness value per specimen. A percentage decrease in thickness was obtained by comparing the thickness of a sample before and after enzyme or buffer incubation.

2.9. Bending stiffness measurements

The contribution of cuspal GAGs to the mechanical properties of Glut-fixed porcine aortic valve cusps was investigated by measuring the flexural rigidity of cuspal tissue during bending in the “with curvature” direction with and without having undergone enzymatic GAG depletion ($n = 15$ and 17 , respectively). Specimens were prepared by cutting a 3 mm wide strip in the circumferential direction from the belly region of the cusp (Fig. 1A), and gluing three small graphite markers with a cyanoacrylate adhesive (Permabond 268, Permabond LLC, Somerset, NJ) along its long edge. Next, two metal hollow posts cut from 19 gauge syringe needles were glued to either end of the strip for attachment to the mechanical testing apparatus (Fig. 1B). Testing was conducted with the sample submerged in phosphate buffered saline, pH 7.4, at 22 °C.

The device used for flexural measurements is presented in detail elsewhere [29]. Briefly, the testing apparatus consisted of a Plexiglas tank to which one end of the test specimen was attached via a rigid bar. The other end of the specimen was attached to a flexible bending bar (316V stainless steel, 0.011 in diameter) that was connected to a stationary mount (Fig. 1C). The test was conducted by sliding the tank toward the bending bar by means of a computer controlled electric motor, thus shortening the distance between the two specimen attachment points, applying a compressive force to the naturally curved tissue. The effect of this was a gradual increase in tissue curvature and a simultaneous deflection of the bending bar (Fig. 1C). This event was captured via a computer controlled CCD camera, which tracked the motion of the three graphite markers on the tissue as well as the two attachment points on either end of the sample. The force applied to the end of the sample was calculated by virtue of knowing both the displacement of the flexible bending bar, which was equivalent to the translation of the corresponding attachment point, and the force–displacement ratio for the particular bar being used. This force data was coupled with a curve-fitting program, allowing for the calculation of both the bending moment applied to the tissue and the corresponding change in curvature.

The resulting moment versus change in curvature data was plotted and the data fit to the exponential function, $M = a(1 - e^{-b\Delta k})$, where M is the applied moment, Δk is the change in sample curvature, and a and b are constants. The derivative of this equation was then taken to yield the flexural rigidity (EI) of the sample over the range of curvatures tested, $M' = EI = abe^{-b\Delta k}$.

2.10. Subdermal implantation and calcium analysis

Glut-fixed half cusps ($n = 6$ per group) were incubated in either enzyme or buffer solution as described above and rinsed in three changes of sterile saline over a period of 24 h prior to implantation. Male juvenile Sprague-Dawley rats (40–50 g, Harlan Laboratories, Indianapolis, IN) were placed under general anesthesia by inhalation of isoflurane (2–4%) and a small dorsal incision was made, through which subdermal pockets were created. Tissue samples were

inserted (one per pocket) and incisions closed with surgical staples. Animals were sacrificed by CO₂ asphyxiation after 7 and 21 days and the samples were retrieved. All animals received humane care in compliance with protocols approved by the Clemson University Animal Research Committee as formulated by the NIH (Publication No. 86-23, revised 1985). Calcium analysis of explanted samples was performed as previously described [30]. Briefly, explanted samples were lyophilized and their dry weights recorded for normalization. The dry samples were hydrolyzed in 6 N HCl in a boiling water bath for 8–10 h. The calcium content of each sample was obtained by atomic absorption spectrophotometry (Perkin-Elmer 3030 Atomic Absorption Spectrophotometer, Norwalk, CT).

2.11. Histology

Multiple representative tissue samples were fixed in alcoholic acid formalin, embedded in paraffin wax, and sectioned (5 μ m) for light microscopic examination. Histologic evaluation of cuspal GAG content after storage in Glut or after enzyme digestion was performed by Alcian Blue staining with a Brazilliant!® nuclear fast red counter stain (Anatech Ltd., Battle Creek, MI). Digital photographs were taken from central region of cusp samples (approximately midway between cusp base and tip). Samples explanted from subdermal studies were evaluated histologically for calcification using Alizarin Red with a Fast Light Green counterstain.

2.12. Statistical analysis

Results are expressed as mean $\pm$ standard error of the mean (SEM). Results obtained by uronic acid analysis before and after storage in saline or Glut were compared by single-factor ANOVA analysis. In all other cases, statistical significance was determined by two-sided student's paired *t*-test. Differences were considered statistically significant when $p < 0.05$.

3. Results

3.1. Progressive loss of cuspal GAGs

Quantitative measurements consistently showed a progressive loss of GAGs from cuspal tissue after 7-day fixation in 0.6% Glut and with increasing storage time in 0.2% Glut (Fig. 2). This is illustrated by the fact that valve cusps stored under static conditions in 0.2% Glut for 6 months lost greater than 50% of their original GAG content and valves stored for 5 years experienced an 80% decrease in cuspal GAG content. Quantitative results were confirmed by histological analysis, which showed greatly reduced Alcian Blue staining in cusps after long-term Glut storage (Figs. 3C and D) as compared to fresh tissue (Fig. 3A).

3.2. Enzymatic removal of cuspal GAGs

The procedure for enzymatic GAG removal was optimized by measuring the total hexosamine content of cuspal tissue that had been incubated in various concentrations of hyaluronidase and chondroitinase ABC (Fig. 4). We have previously used this approach for the analysis of GAG composition in cuspal tissues using fluorophore assisted carbohydrate electrophoresis [11]. Fresh tissue appeared to be easily depleted of its GAG content with even the lowest enzyme concentrations, while Glut-fixed cusps required slightly higher enzyme concentrations for successful GAG removal (Fig. 4). The complete removal of cuspal GAGs from half cusps incubated in 5 U/ml hyaluronidase and 0.10 U/ml chondroitinase ABC was confirmed by measuring the uronic acid content, which revealed a negligible amount of GAGs remaining in the digested fresh and Glut-fixed samples as compared to tissues incubated in buffer alone (Fig. 5). GAG removal was also confirmed by histological analysis of fresh (Fig. 3E) and Glut-fixed (Fig. 3F) cusps. Thus, this enzyme concentration was chosen for all further studies.

In order to assure that GAG removal did not alter the chemistry of collagen crosslinking, the thermal denaturation temperatures (T_d) and resistance to collagenase were measured in fresh and Glut-fixed cusps, with and without treatment with hyaluronidase and chondroitinase. Enzymatic GAG removal from fresh cusps did not significantly change T_d (68.65 ± 0.57 °C for untreated cusps vs. 67.71 ± 0.51 °C for enzyme treated cusps, $p = 0.28$). Likewise, T_d of control Glut-fixed cusps (90.19 ± 0.57 °C) did not significantly differ from Glut-fixed cusps analyzed after GAG removal (89.16 ± 0.48 °C, $p = 0.24$). In addition, enzymatic GAG removal from Glut-fixed cusps did not significantly alter resistance to collagenase ($92.7 \pm 0.67\%$ tissue remaining for untreated cusps vs. $92.56 \pm 0.62\%$ for hyaluronidase/chondroitinase treated cusps, $p = 0.88$). These results indicate that the enzymatic removal of GAGs from cuspal tissue does not affect collagen stability or crosslinking.

3.3. Effects of GAG removal on cuspal water content

The amount of water within cuspal tissue before lyophilization and following subsequent rehydration was measured in order to ascertain the effect of cuspal GAGs on tissue hydration (Fig. 6). Enzymatic removal of GAGs from fresh tissue had a profound effect on the water content, decreasing it significantly ($p < 0.001$). This effect was compounded when examining the ability of the tissue to be rehydrated following lyophilization. Fresh tissue that did not undergo enzymatic GAG removal was able to rehydrate completely ($p = 0.26$), while that which underwent enzyme digestion lost much of its hydrating capacity ($p < 0.001$). Cuspal tissue did not undergo any significant change in hydration resulting from Glut fixation ($p = 0.18$), although its ability to undergo rehydration was impaired ($p < 0.001$) compared to fresh tissue. As was the case for fresh tissue, the removal of GAGs from Glut-fixed cuspal tissue was found to significantly decrease water content ($p = 0.002$).

3.4. Effects of GAG removal on cusp thickness

The impact of GAG removal on the thickness of both fresh and Glut-fixed cuspal tissue was investigated as a method of understanding the importance of GAGs to cuspal morphology. Fresh cusps incubated in buffer alone experienced no significant change in thickness ($0.39 \pm 2.28\%$ decrease). By comparison, cusps that were incubated in GAG-degrading enzymes underwent an average decrease in thickness of $18.17 \pm 2.38\%$ ($p < 0.01$). Glut-fixed tissues were found to behave similarly, remaining unaffected by incubation in buffer ($-0.45 \pm 0.68\%$) and experiencing a decrease in thickness following enzyme incubation. However, this effect appeared to be dampened by fixation, with these samples experiencing an average decrease in thickness of just $3.22 \pm 1.29\%$ ($p < 0.05$).

3.5. Effects of GAG removal on cuspal stiffness

Mechanical testing revealed a non-linear relationship between the applied moment (M) and the resulting change in specimen curvature of cusps (Δk) (Fig. 7A). For this reason, it was also necessary to plot the resultant flexural rigidity (EI) as a function of change in specimen curvature (Fig. 7B). The data obtained demonstrated that the removal of cuspal GAGs resulted in a 60% increase ($p = 0.007$) in flexural rigidity at the relaxed position ($\Delta k = 0$). As the curvature of the tissue increased from the relaxed state the stiffness of both the control and digested tissues decreased, as did the difference between them, losing significance ($p < 0.05$) between Δk of 0.10 and 0.15 mm⁻¹.

3.6. Effects of GAG removal on cuspal calcification

The juvenile rat subdermal model was used to investigate the calcification of Glut-fixed porcine aortic valve cuspal tissue with and without GAGs. GAG content in samples before implantation was 122.59 ± 3.09 µg uronic acid/10 mg dry tissue for buffer-treated Glut-fixed control cusps and 1.92 ± 1.63 µg uronic acid/10 mg dry tissue in enzyme-treated cusps. Implanted cusps

exhibited a progressive increase in tissue calcium content, typical of pathologic calcification in this model (Fig. 8A). However, no statistically significant difference in calcium levels was associated with the removal of GAG molecules from the cuspal matrix at either 7 or 21 days ($p = 0.23$ and 0.70 , respectively). Histological analysis using Alizarin Red staining confirmed quantitative results showing extensive areas of calcification within the cuspal structure (Figs. 8B and C). Careful analysis of marginal, less calcified areas showed that morphology of the round calcium deposits was not different between the two tissues (Figs. 8B and C).

4. Discussion

GAGs play important roles throughout the body in tissue mechanics, tissue hydration and as participants in the regulation of extracellular calcium homeostasis. Mechanically, their large molecular weight, anionic nature, and hydrophilicity make them excellent lubricants and shock absorbers. Their ability to perform these functions has been extensively investigated with particular attention to the synovial joints such as the knee [31,32]. In addition, their proposed function in the regulation of new bone formation by virtue of their ability to chelate positively charged calcium ions has also been widely studied [33–35]. However, despite their relative abundance within the matrix of cardiac valves and BHVs, the potential for similar mechanical and biochemical functions within these tissues has only recently been proposed, with very little evidence thus far [10,11,14,16,36].

4.1. Stability of GAGs during BHV preparation and storage

In the present study we bring evidence for progressive loss of GAGs from within the BHV cusp structure during fixation and storage in Glut. Furthermore we demonstrate that GAGs are essential components of the cuspal matrix that maintain tissue hydration and consequently influence the mechanical properties of BHVs.

Major degenerative changes occur in the extracellular matrix ultrastructure of porcine cusp during preparation for use as BHVs [10,37,38]. To the best of our knowledge the BHV preparation procedure described here mimics those in use at typical commercial manufacturing facilities. Since procedures may differ among different companies, extrapolation of these results to commercially available BHVs should be done with caution.

Our quantitative assays for GAG content in porcine aortic valve cusps clearly establish that GAGs are lost in vitro during BHV preparation, fixation and storage. The data presented here extend the findings of previous quantitative and qualitative studies that have described the instability of GAGs within the extracellular matrix of porcine BHVs. [10,11,14–16].

Routinely, valves are harvested at slaughterhouses, transported to heart valve manufacturing facilities on ice, cleaned and dissected in cold saline and fixed in Glut. In fresh aortic valves, GAGs occupy the majority of the interfibrillar space and closely associate with collagen fibers. However, following saline storage and/or Glut fixation of BHVs, GAGs both in the interfibrillar space and associated with collagen fibers are almost entirely absent as assessed previously by electron microscopy [10]. Our current results show that a significant loss of GAGs (about 25%) occurs after only 24 h of storage in ice-cold saline. In addition to the clear potential for GAG loss during fresh tissue storage and transport, our results also indicate that the loss of cuspal GAGs was not prevented by Glut fixation of fresh tissue within 3–5 h of collection. However, it did appear that those GAGs most susceptible to degradation were lost within the first 24 h of fixation, with no additional significant reduction during fixation and storage in Glut for up to 14 days. By contrast, prolonged storage for 6 months or more in 0.2% Glut was accompanied by the loss of greater than 50% of cuspal GAGs.

The relatively rapid loss of GAGs from the extracellular matrix of porcine aortic valve cusps during incubation in the static environments of fixation and storage suggests active mechanisms responsible for this degradation. One such mechanism is possibly that of enzymatic degradation, which we have implicated previously by demonstrating the presence of active GAG-degrading enzymes within BHV tissues following Glut fixation [11]. Synergistic with this is simple passive diffusion of these highly water soluble molecules with time into storage and fixation solutions, which has been confirmed by us through the examination of the uronic acid content of long-term Glut storage solutions (data not shown).

This study goes further to examine the possible effect that GAG degeneration and loss might have on the morphology and mechanical behavior of implanted porcine valves. We have previously shown that the main GAGs in porcine aortic cusps are hyaluronic acid, chondroitin sulfate and dermatan sulfate [11]. Furthermore, we have demonstrated that despite Glut fixation, these GAGs are vulnerable to enzymatic degradation [11]. In present studies, we determined optimal conditions required for the complete enzymatic removal of GAGs from fresh and Glut-fixed cusps in order to evaluate the properties of GAG-depleted tissues, comparing their properties with those of undigested, control tissues.

Cuspal thickness measurements before and after enzyme digestion revealed an almost 20% decrease in the thickness of fresh tissue due to GAG removal. This is expected considering the large volume that GAG molecules occupy within the extracellular matrix when fully hydrated [39]. GAG removal was also shown to significantly decrease the thickness of Glut-fixed cusps, although to a much lesser extent. This is speculated to be due to the increased stiffness of matrix collagen fibers following Glut fixation, which are capable of supporting the GAG-depleted spongiosa layer, maintaining much of the tissue's thickness. Despite this effect, these results are suggestive of the fact that GAGs are important in maintaining the optimal three-dimensional spongiosa structure.

Freshly collected porcine valves contain about 90% water. It is theorized that the intrinsic GAGs within the cuspal matrix serve to maintain this elevated hydration status naturally. This is supported by our results in that fresh, GAG-rich cuspal tissue was shown to be quite resilient, returning fully to its original hydration state after lyophilization and rehydration. However, fresh cuspal tissue that was subjected to enzymatic GAG removal not only lost a significant amount of its water content upon digestion, but its ability to rehydrate was also drastically impaired. Similarly, the water content of Glut-fixed cuspal tissue was also decreased by GAG removal, although by much less than that of fresh tissue. This is possibly related to the fact that Glut fixation itself had an effect on the hydration properties of the cuspal tissue, impairing its ability to be rehydrated after lyophilization. This is theorized to be the result of the reaction of Glut with hydrophilic amine groups in proteins, including collagen, reducing the tissue's hydrating capacity. Taken together, the data collected demonstrate that the loss of GAGs during fixation may significantly affect the hydration properties of BHVs, thereby reducing the cushioning properties of the spongiosa, which may in turn, directly influence cuspal mechanics and durability [40].

4.2. Biomechanical effects of GAG removal

A look into the basic effect of GAG removal on cuspal mechanics was obtained by examining the change in with-curvature flexural rigidity. When GAG-depleted cusps were flexed in the natural with-curvature direction, there was an increase in flexural rigidity at the resting position of approximately 60%, as compared to controls. This trend was maintained as the change in curvature increased. This is in agreement with the hypothesized function of the hydrated spongiosa, which is typically presumed to be responsible for reducing the stresses associated with cuspal flexure [18–20]. Accordingly, Talman et al. [20] have previously shown that tissue dehydration using organic solvents results in increased shear stiffness, highlighting the

importance of hydration status on BHV biomechanics. This is because, theoretically, when cusps are flexed in the natural with-curvature direction, the function of the spongiosa is primarily to allow shearing between the two major cuspal layers (the fibrosa and ventricularis). Without this capability, it is speculated that shearing is resisted by the remaining fibrillar extracellular matrix components, thereby increasing the rigidity. In addition, this increase in rigidity might be exacerbated by additional crosslinking reactions between collagen fibers within the ventricularis and fibrosa during glutaraldehyde storage following GAG removal. Such an increase in cuspal rigidity is speculated to precipitate the fatigue events seen both in vitro and in vivo that often ultimately result in BHV failure, particularly cuspal delamination [16,41,42]. Our results also indicate that the GAG depletion in the spongiosa is also likely to increase the vulnerability of the flexing valve cusp to buckling, a phenomenon that has been directly linked to an increase in the potential for the mechanical failure of BHVs [22,23].

4.3. Effects of GAG removal on calcification

The role of GAGs in BHV calcification has previously been studied indirectly, mostly by correlations made between their loss and valve mineralization. These studies include one in which we showed that the calcification of Glut fixed cusps in the rat subdermal model was accompanied by a marked decrease in GAG content and a concomitant increase in GAG-degrading enzyme activities [11]. Lower quantities of GAGs have also been reported in calcified natural valves [43], as well as in clinically failed, calcified BHVs [15]. Using the rat subdermal model it has also previously been demonstrated that the extraction of GAGs before Glut fixation stimulated the calcification of bovine pericardium [44] and conversely, the covalent immobilization of hyaluronic acid mitigated pericardial calcification [45]. Taken together, these results are indicative of the possibility that GAG degeneration and calcium deposition in BHVs are interconnected and that GAGs may act as intrinsic inhibitors of cuspal calcification in porcine BHVs.

By implanting porcine aortic valve cuspal tissue with and without its native GAG molecules we were able to test this theory. The results we obtained demonstrated that, contrary to those obtained for bovine pericardium, no significant difference in the degree of cuspal calcification of control Glut-fixed cusps and GAG-depleted Glut-fixed cusps was found. This suggests that native GAGs play only a minor role, if any, in the calcification of Glut-fixed porcine BHV cuspal tissue in the rat subdermal model. This is theorized to be due to the fact that the majority of cuspal GAGs are concentrated within the central spongiosa layer of valve cusps rather than near the tissue's surface as is the case with surface-grafted hyaluronic acid. The central location of the GAGs within the spongiosa is, by this rationale, unlikely to be able to significantly impact the influx of calcium ions and inhibit calcification.

Furthermore, it is widely accepted that the major substrates of calcification in bioprosthetic cusps are devitalized cells and Glut-fixed collagen [41]. In the study presented here, our histologic observations reveal that cell and collagen-mediated calcification was not altered by GAG removal. This suggests that any change in the potential for calcification due to enzymatic GAG removal would be the direct result of their loss from the tissue. By contrast, one previous study conducted by this group indicated that calcification was inhibited by GAG cross-linking using periodate fixation [26]. However, the process of periodate fixation might also significantly alter the collagenous component of the cuspal tissue in addition to cuspal GAGs. For this reason, it is possible that the reduction in calcification noted for tissues undergoing GAG fixation was actually the result of a reduction in nucleation sites due to collagen modification rather than the preservation of cuspal GAGs, as originally proposed.

The importance of GAGs within the structure of heart valve tissue has been demonstrated indirectly, by correlations made between their loss and valve failure [14,46,47]. Accordingly, we used a controlled system of enzymatic degradation to completely remove GAGs and studied

properties of GAG-deprived cusps as compared to untreated controls. Undoubtedly, more studies are needed to directly demonstrate the role of GAGs in heart valves.

The use of animal models for studies of BHV calcification and anti-calcification strategies is also a matter of controversy [48]. We showed in the present paper that GAGs play an insignificant role in BHV calcification in the juvenile rat subdermal model; however, extrapolation of these results to other animal models, or to clinical implants should be done with caution.

5. Conclusions

Several important aspects concerning the stability and function of GAGs in BHVs have been identified in the present study. First, GAGs are progressively lost from within the structure of porcine aortic cusps during routine BHV preparation steps. This unaided loss is translated into significant morphological and functional tissue alterations, including decrease in cuspal thickness, reduction of water content and diminution of rehydration capacity. By virtue of these effects, the loss of GAGs also greatly influences the bending properties of cuspal tissue and thus most likely also impacts the mechanical durability of BHVs. Lastly, native cuspal GAGs apparently play a minor role in the calcification of Glut-fixed BHV tissues when implanted in the rat subdermal model. This is thought to be due to their concentration within the center of the cuspal tissue rather than near the tissue's periphery, where they might serve a greater role in limiting the influx of calcium.

The detailed examination of the effect of GAG loss on porcine BHV cuspal mechanics and durability are currently underway in our laboratory. In addition, more fundamental studies are also needed to fully understand the role of GAGs in the physiology and pathology of both native and BHVs. In this regard, more experiments are also required to better understand the kinetics and mechanisms of GAG degradation both during BHV preparation and following implantation in vivo. As for the prevention of these degenerative events, controlling the extent of pre-implantation GAG loss from BHVs and the development of improved GAG crosslinking techniques remain a major focus of our group with the expectations that these efforts will improve the durability of cardiovascular bioprostheses.

Acknowledgements

This work was supported by NIH Grant # HL61652 to NRV. MSS would also like to acknowledge the support of NIH Grant # HL63026 and an Established Investigator Award from AHA.

References

1. Schoen, FJ.; Padera, RF. Cardiac surgical pathology. In: Cohn, LH.; Edmunds, LH., Jr, editors. Cardiac surgery in the adult. 2. New York: McGraw-Hill; 2003. p. 119-85.
2. Hammermeister K, Sethi GK, Henderson WG, Grover FL, Oprian C, Rahimtoola SH. Outcomes 15 years after valve replacement with a mechanical versus a bioprosthetic valve: final report of the Veterans Affairs randomized trial. *J Am Coll Cardiol* 2000;36(4):1152-8. [PubMed: 11028464]
3. Sako EY. Newer concepts in the surgical treatment of valvular heart disease. *Curr Cardiol Rep* 2004;6(2):100-5. [PubMed: 14759352]
4. Schoen FJ, Levy RJ. Tissue heart valves: current challenges and future research perspectives. *J Biomed Mater Res* 1999;47(4):439-65. [PubMed: 10497280]
5. Human P, Zilla P. Inflammatory and immune processes: the neglected villain of bioprosthetic degeneration? *J Long Term Eff Med Implants* 2001;11(3-4):199-220. [PubMed: 11921664]
6. Human P, Zilla P. Characterization of the immune response to valve bioprostheses and its role in primary tissue failure. *Ann Thorac Surg* 2001;71(5 Suppl):S385-8. [PubMed: 11388230]

7. Woodroof EA. Use of glutaraldehyde and formaldehyde to process tissue heart valves. *J Bioeng* 1978;2(1-2):1-9. [PubMed: 98522]
8. Cheung DT, Perelman N, Ko EC, Nimni ME. Mechanism of crosslinking of proteins by glutaraldehyde III. Reaction with collagen in tissues. *Connect Tissue Res* 1985;13(2):109-15. [PubMed: 3157539]
9. Jayakrishnan A, Jameela SR. Glutaraldehyde as a fixative in bioprostheses and drug delivery matrices. *Biomaterials* 1996;17(5):471-84. [PubMed: 8991478]
10. Simionescu DT, Lovekamp JJ, Vyavahare NR. Degeneration of bioprosthetic heart valve cusp and wall tissues is initiated during tissue preparation: an ultrastructural study. *J Heart Valve Dis* 2003;12(2):226-34. [PubMed: 12701796]
11. Simionescu DT, Lovekamp JJ, Vyavahare NR. Glycosaminoglycan-degrading enzymes in porcine aortic heart valves: implications for bioprosthetic heart valve degeneration. *J Heart Valve Dis* 2003;12(2):217-25. [PubMed: 12701795]
12. Simionescu DT, Lovekamp JJ, Vyavahare NR. Extracellular matrix degrading enzymes are active in porcine stentless aortic bioprosthetic heart valves. *J Biomed Mater Res* 2003;66A(4):755-63.
13. Isenburg JC, Simionescu DT, Vyavahare NR. Tannic acid treatment enhances biostability and reduces calcification of glutaraldehyde fixed aortic wall. *Biomaterials* 2005;26(11):1237-45. [PubMed: 15475053]
14. Grande-Allen KJ, Mako WJ, Calabro A, Shi Y, Ratliff NB, Vesely I. Loss of chondroitin 6-sulfate and hyaluronan from failed porcine bioprosthetic valves. *J Biomed Mater Res* 2003;65A(2):251-9.
15. Mako WJ, Calabro A, Ratliff NB, Vesely I. Loss of glycosaminoglycans (GAGs) from implanted bioprosthetic heart valves. *Circulation* 1997;I-155:863.
16. Vyavahare N, Ogle M, Schoen FJ, Zand R, Gloeckner DC, Sacks M, et al. Mechanisms of bioprosthetic heart valve failure: fatigue causes collagen denaturation and glycosaminoglycan loss. *J Biomed Mater Res* 1999;46(1):44-50. [PubMed: 10357134]
17. Fedarko NS. Isolation and purification of proteoglycans. *Experientia* 1993;49(5):369-83. [PubMed: 8500593]
18. Thubrikar M, Eppink RT. A method for analysis of bending and shearing deformations in biological tissue. *J Biomech* 1982;15(7):529-35. [PubMed: 7130208]
19. Thubrikar MJ, Skinner JR, Eppink RT, Nolan SP. Stress analysis of porcine bioprosthetic heart valves in vivo. *J Biomed Mater Res* 1982;16(6):811-26. [PubMed: 7174710]
20. Talman EA, Boughner DR. Effect of altered hydration on the internal shear properties of porcine aortic valve cusps. *Ann Thorac Surg* 2001;71(5 Suppl):S375-8. [PubMed: 11388228]
21. Sacks MS. The biomechanical effects of fatigue on the porcine bioprosthetic heart valve. *J Long Term Eff Med Implants* 2001;11(3-4):231-47. [PubMed: 11921666]
22. Sacks MS, Gloeckner DC, Vyavahare N, Levy RJ. Loss of flexural rigidity in bioprosthetic heart valves with fatigue: new findings and the relation to collagen damage. *Journal of Biomechanical Engineering*. in press
23. Vesely I, Boughner D, Song T. Tissue buckling as a mechanism of bioprosthetic valve failure. *Ann Thorac Surg* 1988;46(3):302-8. [PubMed: 3137903]
24. Vesely I, Mako WJ. Comparison of the compressive buckling of porcine aortic valve cusps and bovine pericardium. *J Heart Valve Dis* 1998;7(1):34-9. [PubMed: 9502137]
25. Smith DB, Sacks MS, Pattany PM, Schroeder R. Fatigue-induced changes in bioprosthetic heart valve three-dimensional geometry and the relation to tissue damage. *J Heart Valve Dis* 1999;8(1):25-33. [PubMed: 10096478]
26. Lovekamp J, Vyavahare N. Periodate-mediated glycosaminoglycan stabilization in bioprosthetic heart valves. *J Biomed Mater Res* 2001;56(4):478-86. [PubMed: 11400125]
27. Hunter GK. An ion-exchange mechanism of cartilage calcification. *Connect Tissue Res* 1987;16(2):111-20. [PubMed: 3032508]
28. Blix G. The determination of hexosamines according to Elson and Morgan. *Acta Chem Scan* 1948;2:467-73.
29. Lam TV, Sacks MS. A novel device for determination of the transmural strains in soft biological tissues under flexure. *Journal of Biomechanical Engineering*. in press

30. Vyavahare N, Hirsch D, Lerner E, Baskin JZ, Schoen FJ, Bianco R, et al. Prevention of bioprosthetic heart valve calcification by ethanol preincubation. Efficacy and mechanisms [see comments]. *Circulation* 1997;95(2):479–88. [PubMed: 9008467]
31. Quinn TM, Dierickx P, Grodzinsky AJ. Glycosaminoglycan network geometry may contribute to anisotropic hydraulic permeability in cartilage under compression. *J Biomech* 2001;34(11):1483–90. [PubMed: 11672723]
32. Laurent TC, Laurent UB, Fraser JR. The structure and function of hyaluronan: an overview. *Immunol Cell Biol* 1996;74(2):A1–7. [PubMed: 8724014]
33. Hunter GK. Role of proteoglycan in the provisional calcification of cartilage. A review and reinterpretation *Clin Orthop* 1991;262:256–80.
34. Poole AR, Pidoux I, Rosenberg L. Role of proteoglycans in endochondral ossification: immunofluorescent localization of link protein and proteoglycan monomer in bovine fetal epiphyseal growth plate. *J Cell Biol* 1982;92(2):249–60. [PubMed: 7037793]
35. Engfeldt B, Hjerpe A. Glycosaminoglycans and proteoglycans of human bone tissue at different stages of mineralization. *Acta Pathol Microbiol Scand A* 1976;84(1):95–106. [PubMed: 130054]
36. Grande-Allen KJ, Griffin BP, Calabro A, Ratliff NB, Cosgrove DM III, Vesely I. Myxomatous mitral valve chordae. II: selective elevation of glycosaminoglycan content. *J Heart Valve Dis* 2001;10(3):325–32. [PubMed: 11380095]discussion 332–3
37. Ferrans VJ, Tomita Y, Hilbert SL, Jones M, Roberts WC. Pathology of bioprosthetic cardiac valves. *Hum Pathol* 1987;18(6):586–95. [PubMed: 3596579]
38. Warnes CA, Scott ML, Silver GM, Smith CW, Ferrans VJ, Roberts WC. Comparison of late degenerative changes in porcine bioprostheses in the mitral and aortic valve position in the same patient. *Am J Cardiol* 1983;51(6):965–8. [PubMed: 6829473]
39. Neame, PJ.; Barry, FP. The link proteins. In: Jolles, P., editor. *Proteoglycans*. Basel, Switzerland: Birkhauser Verlag; 1994.
40. Talman EA, Boughner DR. Glutaraldehyde fixation alters the internal shear properties of porcine aortic heart valve tissue. *Ann Thorac Surg* 1995;60(2 Suppl):S369–73. [PubMed: 7646190]
41. Schoen FJ, Hirsch D, Bianco RW, Levy RJ. Onset and progression of calcification in porcine aortic bioprosthetic valves implanted as orthotopic mitral valve replacements in juvenile sheep. *J Thorac Cardiovasc Surg* 1994;108(5):880–7. [PubMed: 7967670]
42. Ishihara T, Ferrans VJ, Boyce SW, Jones M, Roberts WC. Structure and classification of cuspal tears and perforations in porcine bioprosthetic cardiac valves implanted in patients. *Am J Cardiol* 1981;48(4):665–78. [PubMed: 7282549]
43. Baig MM, Daicoff GR, Ayoub EM. Comparative studies of the acid mucopolysaccharide composition of rheumatic and normal heart valves in man. *Circ Res* 1978;42(2):271–5. [PubMed: 145919]
44. Jorge-Herrero E, Fernandez P, Gutierrez M, Castillo-Olivares JL. Study of the calcification of bovine pericardium: analysis of the implication of lipids and proteoglycans. *Biomaterials* 1991;12(7):683–9. [PubMed: 1742413]
45. Ohri R, Hahn SK, Hoffman AS, Stayton PS, Giachelli CM. Hyaluronic acid grafting mitigates calcification of glutaraldehyde-fixed bovine pericardium. *J Biomed Mater Res A* 2004;70A(2):328–34. [PubMed: 15227678]
46. Torii S, Bashey RI, Nakao K. Acid mucopolysaccharide composition of human-heart valve. *Biochim Biophys Acta* 1965;101(3):285–91. [PubMed: 4222030]
47. Murata K. Acidic glycosaminoglycans in human heart valves. *J Mol Cell Cardiol* 1981;13(3):281–92. [PubMed: 6790717]
48. Simionescu DT. Prevention of calcification in bioprosthetic heart valves: challenges and perspectives. *Expert Opin Biol Ther* 2004;4(12):1971–85. [PubMed: 15571459]

Fig. 1.

Experimental setup for bending stiffness evaluation of Glut-fixed porcine aortic valve cusps. A circumferential strip of tissue was cut from the belly region of a cusp (A), graphite markers were attached to the cut area of the tissue (dark spots, B) and the cusp strip was glued to two cylindrical steel posts (black cylinders, B). Finally the tissue strip was mounted on the testing apparatus and subjected to compressive end loading (C). See more experimental details in the text.

Fig. 2.

Uronic acid content of cusp tissues showing progressive loss of GAGs during typical BHV processing steps. GAG values obtained for tissues fixed in Glut for 24 h and longer are significantly lower than those of fresh tissue ($p < 0.05$). GAG values after 6 months and 5 years of storage in Glut were statistically lower than those stored for less than 6 months ($p < 0.05$).

Fig. 3. Histological analysis of valvular GAGs using Alcian blue staining. Sections shown are taken from the center of fresh porcine aortic valve cusps (A) and after Glut fixation and storage for 7 days (B), 1 year (C), and 5 years (D). Note the almost complete disappearance of Alcian blue-stainable tissue components after prolonged storage in Glut. Also shown are fresh (E) and 7 day Glut-fixed (F) cusps following treatment with the optimized hyaluronidase/chondroitinase enzyme mixture. Collagen and cells stain red, GAGs stain blue. Original magnification, 100 $\times$.

Fig. 4.

Optimization of the enzymatic removal of GAGs from fresh and 7 day Glut-fixed cuspal tissue was performed by exposing tissues to various mixtures of hyaluronidase (HAase) and chondroitinase ABC (CSase) followed by GAG quantification using total hexosamine analysis. Note the different kinetics of GAG removal for Glut-fixed tissue as compared to fresh tissue.

Fig. 5. Uronic acid analysis demonstrated the complete removal of GAGs from both fresh and 7-day Glut-fixed cuspal tissues using the optimized enzyme protocol.

Fig. 6. Effects of GAG removal on tissue water content. The removal of GAGs from fresh tissue resulted in a decreased cuspal water content as well as a decreased capacity for rehydration. Seven day Glut-fixed cuspal tissue, which itself was less capable of being rehydrated, also demonstrated a decrease in water content as a consequence of GAG removal ($p = 0.002$).

Fig. 7. Flexural rigidity analysis. (A) Representative moment versus change in curvature (M vs. Δk) plots (gray dots) and exponential fits (dark lines) for Glut-fixed cusps with and without GAGs. (B) Total enzymatic removal of GAGs from Glut-fixed cuspal tissue ($n = 15$) resulted in a statistically significant ($p < 0.05$) increase in flexural rigidity (EI) for with-curvature bending for the range of curvatures (Δk) from resting (0 mm^{-1}) to 0.01 mm^{-1} .

Fig. 8. Rat subdermal calcification studies showed that complete GAG removal from 7 day Glut-fixed cusps did not alter the extent of subdermal calcium accumulation at 7 or 21 days of implantation (A). Alizarin red staining of histological sections from Glut-fixed cusps explanted after 21 days showed no differences between the calcification patterns of GAG-free tissues (C) as compared with untreated 7 day Glut-fixed controls (B). Calcified deposits stain red, collagen light green. Original magnification, 100 $\times$.