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Structural and Biomechanical Changes to Dentin Extracellular Matrix Following Chemical Removal of Proteoglycans

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Abstract

Proteoglycans are biomacromolecules with significant biomineralization and structural roles in the dentin extracellular matrix. This study comprehensively assessed the mechanical properties and morphology of the dentin extracellular matrix following chemical removal of proteoglycans to elucidate the structural roles of proteoglycans in dentin. Dentin extracellular matrix was prepared from extracted teeth after complete tissue demineralization. Chemical removal of proteoglycans was carried-out using guanidine hydrochloride (GuHCl) for up to 10 days. The removal of proteoglycans was determined by dimethylmethylene blue colorimetric assay and histological staining analyses using transmission electron microscopy and optical microscopy. The modulus of elasticity of dentin matrix was determined by a 3-point bending test method. Partial removal of proteoglycans induced significant modifications to the dentin matrix, particularly in the type I collagen. Removal of proteoglycans significantly decreased the modulus of elasticity of dentin extracellular matrix ($p < 0.0001$). In conclusion, the subtle disruption of proteoglycans induce pronounced changes to the collagen network packing and the bulk modulus of elasticity of dentin matrix.

Keywords

proteoglycans; collagen; dentin; modulus of elasticity; histology

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Conflict of Interest

The authors state no conflict of interest with the content of the manuscript.

Introduction

Dentin constitutes the bulk of the tooth and shares many compositional and mechanical similarities to bone. Dentin is a vital semi-permeable barrier between enamel and pulp with organizational and mechanical features that prevent crack propagation from the overlying highly mineralized enamel [1, 2], and provide biological shielding to the vascularized pulp tissue. The extracellular matrix of dentin includes collagen and non-collagenous components [3], such as phosphoproteins and proteoglycans. The dentin extracellular matrix provides essential toughening mechanism and serves as a scaffold for the organized mineral phase deposition [4].

Proteoglycans are major biomacromolecules of mineralized and non-mineralized connective tissues [5]. Found in low quantities in the mature dentin, proteoglycans regulate the biophysical properties of the dentin as well as its three-dimensional spatial structure. These molecules fill space, bind and organize water molecules, and repel negatively charged molecules. In load bearing tissues, proteoglycans contribute to the osmotic pressure by providing sites of electrostatic interaction that assure higher hydrophilicity to the tissue [6–14]. The high hydrophilicity is driven by one or more glycosaminoglycans (GAGs) covalently attached to specific core proteins [15]. In dentin, chondroitin-4-sulfate (C4S)/chondroitin-6-sulfate (C6S)-rich decorin and biglycan are the most predominant GAGs and proteoglycans; and lumican, versican [6] and fibromodulin [5, 16] have also been immunolocalized in the tissue.

Laboratorial methods to extract GAGs-proteoglycans from dentin include enzymatic digestion by trypsin (TRY) [17] or chondroitinase-ABC (C-ABC) [18] and the chemical extraction with guanidine hydrochloride (GuHCl) [19–21]. Such methods are utilized experimentally to elucidate the roles of proteoglycans in the tissue structure and function. GuHCl is particularly interesting due to the continuous removal of proteoglycans.

As the prominent role of proteoglycans in the function and aging of organs and tissues take prime interest with a worldwide aging population, this study will take initial steps to further elucidate the role of these biomacromolecules on the physicochemical properties of dentin. The aim of this study was to assess experimentally the time related effects of the depletion of proteoglycans on the modulus of elasticity and morphology of the dentin matrix. The hypothesis tested is that complete depletion of proteoglycans from dentin will decrease the bulk tissue mechanical behavior and matrix organization.

MATERIAL AND METHODS

Biomechanical properties of the dentin matrix

Freshly extracted sound human molars were selected following approval by an Institution Review Board (University of Illinois at Chicago, protocol #2011–0312). Mid-coronal dentin disks were sectioned into 1.7 width × 6.0 length × 0.5 mm thickness specimens with a slow speed diamond wafering blade (Buehler-Series 15LC Diamond). Specimens were demineralized in 10% phosphoric acid solution (LabChem Inc., Pittsburgh, PA, USA) for 5 h and thoroughly rinsed with distilled water for 10 min [22]. Dry mass of demineralized

specimens was estimated after 24 h desiccated in a vacuum desiccator containing anhydrous calcium sulfate. Following re-hydration, the specimens were randomly divided into two groups (n = 20): proteoglycans extraction with 4 M GuHCl (Sigma-Aldrich, Co, St Louis, USA) in 50 mM sodium acetate and 1mM iodoacetic acid buffer pH 7.0 at 4 °C; and no proteoglycans extraction (control) in buffer without GuHCl. Incubation solutions were freshly prepared and changed daily for 10 days. The apparent modulus of elasticity (E) of the specimens determined at baseline and daily during the 10 days incubation. The modulus of elasticity of the dentin specimens were assessed in a three-point bending flexural test with a 1-N load cell mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) at crosshead speed of 0.5mm/min [23, 24]. The data were analyzed by Levene's and Shapiro-Wilk tests, followed by two-way and one-way ANOVA with Fisher LSD post hoc test ($\alpha=0.05$).

Quantification of proteoglycans removal from dentin

Supernatants collected throughout the 10-day incubation period were used to quantify removal of proteoglycans by detection of GAGs [20]. Briefly, a 100 μ L aliquot of supernatant from each day of extraction was incubated with 1 mL of 1,9-dimethylmethylene blue assay (DMMB) solution under vigorous agitation for 30 min at room temperature (RT) to promote DMMB complexation with proteoglycans. Then, PG-DMMB complex was separated by centrifugation (12.000 xg for 10 min), supernatant was discarded and pellet was incubated with 500 μ L of decomplexation solution under agitation for 30 min. A standard curve was generated using chondroitin sulfate sodium salt from shark with concentrations from 0.5 to 64 μ g/mL (Sigma-Aldrich). Absorbance was measured at 656 nm in a spectrophotometer (Spectramax Plus, Molecular devices, Sunnyvale, CA). Proteoglycans concentration was expressed in μ g/ml/mg of dry dentin after subtracting the GuHCl solution background. The data were subjected to Levene's test and Two-way ANOVA/One-way ANOVA and Games Howell post hoc test ($\alpha = 0.05$).

Quantification of collagen degradation

Using the same supernatant (n=5), collagen solubilization was determined by standard hydroxyproline assay [25], with minor modifications. A 500 μ L aliquot of supernatant was lyophilized, re-suspended in 10 μ L of distilled water and incubated with 40 μ L 2N NaOH at 120°C for 60min. After, 450 μ L of 0.056 M chloramine T buffer was mixed in vortex and incubated for 25min at RT. A 500 μ L of 1M Erlich's reagent was mixed in vortex and incubated for 40min at 65°C. Standard curve was generated using 0.5, 1, 2, 3, 4 and 5 μ g/mL of hydroxyproline (Sigma). Absorbance was measured at 550 nm in a spectrophotometer (Spectramax Plus, Molecular devices, Sunnyvale, CA) and hydroxyproline concentration was expressed in μ g/ml. Statistical analysis was performed by ANOVA/Tukey HSD with ($\alpha=0.05$).

Histological localization of proteoglycans in the dentin matrix – optical and transmission electron microscopy

The distribution and presence of proteoglycans in the dentin matrix was quantified using alcian blue (AB) staining under optical microscopy. Paraffin-embedded demineralized dentin obtained from freshly extracted molars (as described above) (n = 3) were cut into 4- μ m-thick

sections (RM 2135, Leica Microsystems GmbH, Wetzlar, Germany). Deparaffinized sections were incubated in GuHCl as described above for 4, 6 days and 10 days. Proteoglycans staining was performed by incubation with 1% AB (Sigma-Aldrich, St. Louis, MO) in 3% acetic acid (pH 2.5) for 30 min followed by 1% Nuclear Fast Red (Sigma-Aldrich) for 5 min [26, 27]. Digital images of stained section were captured using a light microscope (Axioskop 40 optical microscope, Carl Zeiss Microscopy, Thornwood, NY, USA) at 40x magnification. Proteoglycans in mid dentin were quantified from a set of images from each tooth using ImageJ software (version 1.48v) as previously described [28]. Images were converted to grayscale and bright and dark regions were inverted. Threshold tool settings were determined in negative control images and duplicated for every following image and the analyze particles command was used to quantify the counts of staining. Statistical analysis was performed by one-way ANOVA after conversion to log10 ($\alpha=0.05$).

Proteoglycans removal by GuHCl was also visualized using cupromeronic blue staining (CB) of GAG chains [29] under transmission electron microscopy (TEM). Demineralized middle crown dentin blocks ($1.5 \times 1.5 \times 0.5$ mm) were incubated with GuHCl as described above for 4 and 6 days ($n = 5$). Then, dentin blocks were immersed in 0.05% CB solution for 3h at 37°C for glycosaminoglycans chain staining [23]. Dentin sections of 70 nm were mounted in grids and observed under TEM (JEOL JEM-1220, JEOL Ltd., Tokyo, Japan).

Morphological analysis of the dentin matrix

Picrosirius red staining assessed the collagen fibrils network packing (interfibrillar spacing) under light microscopy [30–32]. Ten sections from dentin matrix (from same dentin blocks described above) were deparaffinized, progressively rehydrated and incubated in GuHCl up to 6 days. Then, sections were treated with 0.2% phosphomolybdic acid aqueous for 3min, after briefly rinse in DW and treated 0.1% Sirius red in Picric acid (Sigma-Aldrich) for 90min at RT [33–35] followed by 5 min of nuclear-fast red. Sections were washed twice in 0.01N hydrochloric acid for 1 min, dehydrated, cleaned and mounted in Permount. Digital images of stained section were captured at 40x magnification in an optical microscope (Axioskop 40 optical microscope) and quantitative assessment using Image J software as described for AB staining. The data was statistically analyzed by one-tailed t test ($\alpha = 0.05$).

RESULTS

Biomechanical properties of the dentin matrix

The bulk apparent modulus of elasticity (E) of dentin matrices are shown in Figure 1. Statistical analysis by Shapiro-Wilk test revealed normal data distribution and Levene's test showed no equality of variances ($p<0.0001$). Two-way ANOVA revealed significant difference between control and GuHCl incubation ($p<0.0001$) and no difference between incubation times ($p=0.898$). One-way ANOVA/Fisher LSD revealed significant differences in E values for both control and GuHCl groups ($p = 0.0329$ and $p < 0.001$, respectively). GuHCl incubation significantly decreased the E of dentin matrices after 1-day treatment when compared to baseline ($p < 0.05$). No statistically significant differences were observed with additional GuHCl exposure time ($p > 0.05$). Control group was statistically higher than GuHCl groups, except for baseline ($p < 0.05$).

Quantification of proteoglycan and solubilized collagen in incubation media

Estimated proteoglycan removal by GuHCl are depicted in Table 1. Levene's test of equality of variance assumption was rejected ($p < 0.001$). There were statistically significant differences between GuHCl and control, days of incubation and interaction between these two factors ($p < 0.001$). The higher proteoglycans removal occurred in the first, second and third days of GuHCl incubation, with statistical difference among these three days ($p < 0.05$). After the fourth day of incubation, there were negligible proteoglycans removal, with no statistically significant difference from 4 to 10 days ($p > 0.05$). The control group showed no proteoglycans removal throughout the incubation periods.

GuHCl extraction protocol did not degrade collagen, as observed by absence of statistical differences in the hydroxyproline content of the incubation media as compared to control ($p > 0.05$, Table 2).

Detection of proteoglycans in the dentin matrix

Both optical and TEM images confirmed the presence of proteoglycans after 10 days of incubation with GuHCl (Figure 2 and 3). Intense AB staining is observed in both control and GuHCl-treated dentin after 4, 6 and 10 days of incubation, especially surrounding the dentin tubules (Figure 2). A slight decrease in proteoglycans quantification in GuHCl-treated dentin is observed when compared to control (Figure 2), however there is no significant difference among incubation protocols and control ($p = 0.1893$). Representative TEM images reveal the presence of proteoglycans in the control group, by the visualization of GAGs (dark filaments, Figure 3) surrounding collagen fibrils and dentin tubules. Although less predominant, GAGs are still visualized in GuHCl treated dentin after 10-day incubation, confirming the incomplete removal of proteoglycans by GuHCl.

Morphological analysis of type I dentin collagen

The effects of chemical removal of proteoglycans by GuHCl on collagen fibrils arrangements is shown in Figure 4. Control group exhibited an intense red staining in contrast to GuHCl group. This was confirmed by a statistically significant decrease in the picrosirius red staining count was observed on GuHCl group after 6 days incubation when compared to control ($p = 0.044$). The increase in type I collagen interfibrillar spaces (decreases packing) points to the pivotal role proteoglycans have in sustaining the architecture of the dentin organic matrix.

DISCUSSION

The findings demonstrate that partial removal of proteoglycans in dentin induce pronounced decrease in the bulk mechanical properties, thus the tested hypothesis was rejected. The results are in agreement with previous studies reporting significant decrease in the modulus of elasticity of dentin matrix after the enzymatic removal of proteoglycans [1, 8, 36, 37]. In fact, such effect was observed after the first day of incubation with GuHCl (Figure 1) and indicates a disruption in the protein-protein interaction between proteoglycans and collagen fibrils. In collagen-rich tissues such as dentin, proteoglycans contribute to the fibrillar organization by binding of collagen fibril [37], stabilizing collagen molecules and sustaining

a scaffold of microfibrillar organization [38]. Within the fibrillar level, collagen is stabilized by intra- and inter-molecular cross-links. The disruption of the hierarchical organization of the collagen fibril network by PGs, is most likely the reason for decrease in the modulus of elasticity. This degree of disruption is supported by reduced fibrillar collagen packing (Figure 4) and the lack of collagen solubilization (Table 2), facilitating collagen-collagen sliding. In addition, proteoglycans contribute to the hydrostatic and osmotic pressure of dentin and regulation of biophysical properties of load bearing tissues by filling spaces and organizing water molecules [39]. The free movement of water within the dentin matrix may also contribute for reducing resistant to collagen fibrils sliding, resulting in decreases modulus of elasticity.

Despite the decrease in the apparent modulus of elasticity, GuHCl did not completely deplete proteoglycans from the dentin matrix (Figures 2 and 3, Table 1). However, the partial removal of proteoglycans induced significant modifications to the type I collagen architecture in the dentin matrix (Figure 4). In partially depleted-proteoglycans dentin matrices, a sparse collagen network is observed after GuHCl-treatment, which is likely due to the lack of microfibrillar stabilization and fibrillar space regulation by proteoglycans [40]. Considerable disorganization on collagen microfibrillar bundles in tendon has been previously reported after GuHCl digestion [21] and such modifications are primarily driven by the removal of proteoglycans. Similar results are found here for dentin. It is important to note that GuHCl might have removed other non-collagenous proteins from demineralized dentin matrices [41], with unknown structural role in the dentin matrix.

Structural changes in collagen microfibrillar architecture may have hindered the ability of GuHCl to continue removal of proteoglycans from the dentin matrix, as seen by staining of type I collagen in dentin (Figure 4). A rapid change in the collagen fibril band pattern with short period treatment with GuHCl was shown to increase the number and decrease the width of collagen bands; especially with high GuHCl concentrations as used in the present study [21]. Also, the fibrillar organization of collagen in the inner layers of dentin possibly remained intact over treatment periods, so the proteoglycans extraction rate could be different from the outermost region and from the inner collagen, as the inner layers come into less contact with the extraction solution [41].

There could be a preferential GuHCl extraction of proteoglycans, leaving certain proteoglycans attached to the dentin matrix, which explains the presence of GAGs in TEM and histological evaluations (Figures 2, 3 and 4). Dentin is rich in chondroitin, keratan and dermatan sulfated GAGs [19, 42] that may have been selectively removed from the dentin matrix. Non-sulfated GAGs, not detectable by DMMB assay, could also exhibit a slow release rate by GuHCl. The hypothesis that sulfated GAGs cannot be fully removed by GuHCl requires further systematic investigation.

Proteoglycans are remarkable proteins at regulating bulk tissue physical properties and minor modifications to their content can dramatically diminish the biomechanics of the tissue. It is evident that GuHCl significantly reduced the mechanical properties of the dentin matrix despite the partial removal of proteoglycans. Two outcomes can be drawn from the findings: (1) disturbance of the ratio of proteoglycans-collagen may have a detrimental effect

in the modulus of elasticity of the dentin matrix, and (2) a loss of function of the remaining proteoglycans induced by GuHCl may have permanently disrupted the collagen-proteoglycans-collagen stress-bearing system in the dense dentin matrix leading to reduced mechanical properties.

In conclusion, the subtle disruption of the proteoglycans-collagen complex by guanidine hydrochloride induce structural changes to the type I collagen network resulting in significant decrease in the modulus of elasticity of dentin matrix. Guanidine hydrochloride is widely used to remove proteoglycans complex from collagen-based tissues but does not completely remove these proteins from the dentin organic matrix, even after long-term exposure.

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Fig. 1.

Results of the apparent modulus of elasticity (E) of dentin matrix incubated in GuHCl for 10 days. Symbol (δ) indicate where statistically significant differences were found between control and GuHCl groups at each incubation day ($p < 0.05$). Asterisk (*) indicates statistical differences between baseline measurements and incubation time for both groups ($p < 0.05$). There were no differences in E among incubation times for control and GuHCl groups ($p > 0.05$). Control group: incubation media; GuHCL: incubation with guanidine hydrochloride; D: incubation days.

Fig. 2.

Representative light microscopy images of dentin section stained with Alcian blue (AB) to evaluate proteoglycans removal by GuHCl. Black arrows points to dentinal tubules. A, untreated group (control); B, 4 days of GuHCl incubation; C, 6 days of GuHCl incubation; D, 10 days of GuHCl incubation. Graph shows quantification of AB-staining in light microscopy images of AB-stained dentin sections after proteoglycans removal by GuHCl incubation for 4, 6 and 10 days. There were no statistical differences among groups ($p > 0.05$). D: Days. Scale bar = 50 µm.

Fig. 3. Representative transmission electron microscopy images of demineralized dentin matrix. A, control group specimens display dentin matrix morphology with abundance of proteoglycans (dark filaments), more prominent surrounding the dentin tubules (T); B and C, dentin matrices treated with GuHCl solution for 4 and 6 days respectively show similar dark filaments (proteoglycans) distribution as the control samples. Scale bar = 100 nm.

Fig. 4.

Light microscopy representative images of demineralized dentin sections stained with picrosirius red. A-C, control group, without GuHCl; D-F, 6 days of GuHCl incubation, exhibiting loss on interfibrillar packing (increase interfibrillar spacing). Graph shows quantification of picrosirius red staining of collagen observed in light microscopy images of dentin sections after GuHCl or control incubation for 6 days (n= 10). (*) depicts statistical differences between groups ($p > 0.05$). Scale bar = 100 μ m.

Table 1.

Quantification of proteoglycans in incubation media using DMMB assay for 10 days.

Groups	Proteoglycans of incubation media ($\mu\text{g/ml/mg}$ dentin)									
	1D	2D	3D	4D	5D	6D	7D	8D	9D	10D
Control	0	0	0	0	0	0	0	0	0	0
	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)	(0)
GuHCl	7.31b	10.92a	4.49c	0.86d	0f	0.80de	0.27ef	1.94d	0.61ef	0.13f
	(1.89)	(2.55)	(1.45)	(1.00)	(0.01)	(0.60)	(1.16)	(1.51)	(0.72)	(0.22)

Different letters indicate statistically significant difference among incubation time in the GuHCl group ($p < 0.05$). The highest rate of proteoglycan was detected in the first 3 days. There were no detectable amounts of proteoglycan in supernatant of the control group media at any incubation time. Control: incubation media; GuHCl: incubation media with guanidine hydrochloride; D: incubation days.

Table 2.
Collagen solubilization detection by hydroxyproline (HYP) in incubation media for 10 days.

Groups	Hydroxyproline ($\mu\text{g/ml/mg dentin}$) in incubation media									
	Mean ($\pm$ Standard Deviation)									
	1D	2D	3D	4D	5D	6D	7D	8D	9D	10D
Control	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0
GuHCl	0.49 (0.7)	0.17 (0.28)	0.08 (0.2)	0.17 (0.29)	0.07 (0.19)	0 (0)	0 (0)	0 (0)	0.06 (0.18)	0 (0)

Control: incubation media; GuHCl: incubation media with guanidine hydrochloride; D: incubation days. There are no statistically significant difference among groups ($p>0.05$)