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Role of proteoglycans on the biochemical and biomechanical properties of dentin organic matrix

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Abstract

Objective—Proteoglycans (PGs) are multifunctional biomacromolecules of the extracellular matrix of collagen-based tissues. In teeth, besides a pivotal regulatory role on dentin biomineralization, PGs provide mechanical support to the mineralized tissue and compressive strength to the biosystem. This study assessed enzymatic protocols for selective PGs removal from demineralized dentin to determine the roles of these biomacromolecules in the bulk mechanical properties and biostability of type I collagen.

Methods—Selective removal of glycosaminoglycans chains (GAGs) and PGs from demineralized dentin was carried out by enzymatic digestion protocols using chondroitinase ABC (c-ABC) and trypsin (Try). A comprehensive study design included assessment of dentin matrix mass loss, biodegradability of the PGs/GAGs-depleted dentin matrix, ultimate tensile strength (UTS) and energy to fracture tests. Quantitative data was statistically analyzed by two-way and one-way ANOVA followed by the appropriate post hoc tests ($\alpha = 0.05$).

Results—Transmission electron microscopy images show effective GAGs removal by c-ABC and Try and both enzymatic methods released statistically similar amounts of GAGs from the demineralized dentin. Try digestion resulted in about 25% dentin matrix mass loss and increased susceptibility to collagenolytic digestion when compared to c-ABC ($p = 0.0224$) and control ($p =$

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Disclosure

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0.0901). Moreover, PGs digestion by Try decreased the tensile strengths of dentin. Statistically lower energy to fracture was observed in c-ABC-treated dentin matrix.

Conclusions—GAGs plays a pivotal role on tissue mechanics and anisotropy, while the core protein of PGs have a protective role on matrix biostability.

Keywords

dentin; proteoglycans; glycosaminoglycans; ultimate tensile strength; biodegradation

Introduction

Dentin is a mineralized tissue composed of 70 wt. % hydroxyapatite crystals and 20 wt. % organic extracellular matrix (ECM). The latter is mainly represented by type I collagen and small amounts of non-collagenous components (Goldberg, Kulkarni, Young, & Boskey, 2011; Linde & Goldberg, 1993). Dentin forms the bulk of tooth and thus is the main dental tissue affected in pathological conditions such as caries disease and mal-formations. The determination of functional roles of the extracellular matrix to the biomechanics and biostability of dentin could lead to the development of innovative biomimetic based preventive and restorative therapies. The functional roles of the mineral content and collagenous network has been largely investigated (Kinney, Habelitz, Marshall, & Marshall, 2003); however, only recently the contributions of non-collagenous components such as proteoglycans (PGs) on tissue's physical properties has gained attention.

PGs are glycosylated biomacromolecules formed by a core protein with one or more covalently attached glycosaminoglycan chains (GAGs), present in many collagen-based tissue including dentin, cartilage, skin and bone. PGs represent a small fraction of the dentin ECM while sustaining pivotal roles in the formation and arrangement of this tissue. GAGs are linear disaccharide chains that contain acidic sugar residues and/or sulfate groups that are negatively charged and can attract cations and/or water molecules (Prydz, 2015). Unlike cartilage, dentin holds mainly members of the small leucine rich proteoglycans (SLRPs), including chondroitin sulfate (CS)-rich decorin and biglycan, and keratin-sulphate (KS)-rich fibromodulin and lumican (Embery, Hall, Waddington, Septier, & Goldberg, 2001). A transition in the PGs profile from the pre-dentin to dentin and different GAG chains length confirm the existence of pools of PGs and different molecule structures attributed to their specific roles in dentin formation and mineralization (Goldberg et al., 2003; Waddington, Hall, Embery, & Lloyd, 2003).

PGs attract water molecules into the interfibrillar spaces of the dentin matrix regulating a hydraulic mechanical support system to the type I collagen network. The intricate interactions between GAG chains among PG and between PGs and collagen (Bertassoni, Orgel, Antipova, & Swain, 2012; Bertassoni & Swain, 2014) suggest that PGs interact with type I collagen by protein core binding to four or more collagen microfibrils via hydrogen bonds assuming a helical configuration. The GAG chain forms interfibrillar bridges by connecting adjacent collagen fibrils at specific intervals when wrapping around them in an anti-parallel position (Bertassoni et al., 2012; Bertassoni & Swain, 2014; Orgel, Eid, Antipova, Bella, & Scott, 2009). Those observations were confirmed in few studies on

dentin (Breschi et al., 2003; Ruggeri et al., 2007), but scarce reports are available on the specific roles of PGs and GAGs in the mechanical behavior of mineralized tissues such as dentin. More recently, their site contribution to the mechanical properties of dentin has been nano-characterized in a creep deformation study after selective removal of PGs or GAGs (Bertassoni, Kury, Rathsam, Little, & Swain, 2015).

Correlations between an imbalance of PGs synthesis or concentration with tissue strength and biodegradability are reported in cartilage and bone (Leyh et al., 2014; Martel-Pelletier, Kwan Tat, & Pelletier, 2010), while it is unclear if PGs have any role in this aspect in dentin. Moreover, there is a lack of knowledge in the specific mechanisms involved in the participation of these biomacromolecules on the bulk tissue mechanical behavior and overall ECM biostability. The present study aimed to selectively remove PGs components to determine their roles on the biomechanical properties and biochemical stability of demineralized dentin matrices. Different enzymatic methods to remove GAGs and the core protein of PGs were used to determine the efficacy, specificity and effects on the ECM of dentin.

Materials and Methods

PGs/GAGs removal quantification

Mid-coronal dentin was cut in a low-speed diamond saw (Isomet, Buehler, Lake Bluff, IL, USA) under water irrigation to obtain 45 dentin specimens (1.5 mm × 1.5 mm × 0.5 mm) from extracted intact human molars (IRB # 2011-0312). All specimens were demineralized in 10% phosphoric acid (Ricca Chemical Company, Arlington, TX, USA) for 5 hours under agitation (Castellan, Pereira, Grande, & Bedran-Russo, 2010). Specimens were then rinsed in distilled water (DW) and dried in desiccator for 24 hours. The specimens were weighed on an analytical balance (XS105DU, Mettler Toledo Inc., Columbus, OH, USA), rehydrated in distilled water for 1 hour and selective removal of PGs/GAGs was carried out as follow (n= 15): incubation in 0.67 U/ml chondroitinase ABC (c-ABC) from *Proteus vulgaris* (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.1M Tris-Acetate pH 7.8 for 48 hours at 37 °C under agitation or incubation in 1 mg/ml trypsin (Try) from bovine pancreas TPCK treated (Sigma-Aldrich) diluted in 0.2 M ammonium bicarbonate at the same conditions. Control group was incubated in DW. Solutions were replaced after 24 hours of incubation. Then, specimens were washed in DW for 24 hours in the same conditions and placed in desiccator to obtain the dry weight after PGs/GAGs removal. To determine the remaining amount of PGs/GAGs in dentin, specimens were re-hydrated in distilled water and incubated overnight with 50 µg/ml proteinase K (Thermo Scientific, Waltham, MA, USA) diluted in 100 mM K₂HPO₄ pH 8.0 at 56 °C (Barbosa et al., 2003).

The supernatant was collected and sulfated GAGs removal was quantified spectrophotometrically (Barbosa et al., 2003). Briefly, 100 µl of each solution was mixed with 1,9-dimethylmethylene blue (DMMB) (Sigma-Aldrich) solution to promote GAG complexation with DMMB. The complex was separated from the soluble material and solubilized with decomplexation solution. GAGs concentration was determined using a standard curve with different concentrations of chondroitin sulfate sodium salt from shark (Sigma-Aldrich) from 0.5 to 64 µg/ml. Absorbance was read at 656 nm in a

spectrophotometer (Spectramax Plus, Molecular devices, Sunnyvale, CA, USA). Background subtraction was done using absorbance readings of the buffers used to dilute c-ABC and Try enzymes. GAGs concentration was normalized by the dry weight of dentin specimens before GAGs removal and expressed in $\mu\text{g}/\text{ml}/\text{mg}$ of dentin. Statistical analysis was done using one-way ANOVA and Scheffe post-hoc test ($\alpha = 0.05$).

Biodegradability following PGs/GAGs removal

Dentin specimens were prepared as described above. After GAGs removal, dentin was washed and incubated with 100 $\mu\text{g}/\text{ml}$ of collagenase from *Clostridium histolyticum* (Sigma-Aldrich) in 0.2 M ammonium bicarbonate buffer (pH 7.9) for 24 hours at 37°C under agitation (Bedran-Russo, Castellan, Shinohara, Hassan, & Antunes, 2011) ($n = 10$). Collagenase solution was replaced after 1, 2, 4, 8 and 24 hours, and every day until complete degradation of specimens (up to 7 days of incubation). Collagen solubilization was estimated by hydroxyproline (HYP) release at each time point using a method previously described (Reddy & Enwemeka, 1996) with minor modifications. In brief, lyophilized collagenase solution was re-suspended in 10 μl of DW and 40 μl of 2 M sodium hydroxide and hydrolyzed by incubation at 120 °C for 1 hour. Then, solutions were incubated with chloramine T reagent for 25 min at room temperature followed by 1M Erlich's reagent for 40 min at 65°C. Standard curve was done using 2 to 25 $\mu\text{g}/\text{ml}$ of trans-4-hydroxy-L-proline (Sigma-Aldrich). Absorbance was read at 550 nm in a spectrophotometer (Spectramax Plus). HYP release was calculated according to dry weight of the specimens and expressed in $\mu\text{g}/\text{ml}/\text{mg}$ dentin. Total HYP release was statistically analyzed by one-way ANOVA and Scheffe post-hoc test ($\alpha = 0.05$).

PGs/GAGs removal evaluation by transmission electron microscopy

Freshly extracted molars were incubated with 10 % buffered neutral formalin at 4 °C for 24 hours. Demineralized dentin specimens were prepared as described above, stored in 10% buffered neutral formalin for 3 more days at 4 °C and PGs/GAGs removal was performed following the same protocols described above ($n = 5$). Then, dentin specimens were fixed with 25 mM sodium acetate pH 5.8 containing 2.5% glutaraldehyde for 30 min, washed with sodium acetate solution without glutaraldehyde for 30 min, stained with 0.05% cupromeronic blue (CB) (Sigma-Aldrich) in 25 mM sodium acetate containing 0.1 M MgCl_2 and 2.5% glutaraldehyde for 3 hours at 37 °C (Bedran-Russo, Pereira, Duarte, Okuyama, & Yamauchi, 2008). Specimens were stained with 0.034 M sodium tungstate for 30 min and dehydrated in ascending concentrations of 200 proof ethanol (25%, 50%, 75%, 90%, 95% and 100%). Then, specimens were embedded in epoxy resin and sections of 70 nm were obtained and observed under transmission electron microscopy (TEM) (JEOL JEM-1220, JEOL Ltd., Tokyo, Japan).

Ultimate tensile strength and energy to fracture of PGs/GAGs-depleted dentin

Twenty sound molars had their roots removed and the crowns were sectioned in the occlusal-cervical direction into approximately 0.5 mm thick sections. The sections were further trimmed to obtain hour-glass shape slabs with 0.5 ± 0.1 mm at the mid-coronal dentin according to the tubule orientation [parallel (PL) and perpendicular (PP)] (Bedran-de-Castro, Pereira, & Thompson, 2004) (Figure 1). Specimens were demineralized in 10% phosphoric

acid for 5 hours (Castellan et al., 2010) and further divided (n=15) according to PGs/GAGs removal strategies described above [c-ABC, Try, and no removal (DW)]. The edge of the specimens were glued with a cyanoacrylate adhesive to a Ciucchi's jig mounted on a universal testing machine (EZ Graph, Shimadzu, Kyoto, Japan) and tested in tensile at a crosshead speed of 1 mm/min. All specimens were kept hydrated in DW prior and during the tensile test. Means and standard deviations were calculated and expressed in MPa. Data were statistically analyzed by two-way ANOVA and Tukey's post hoc test ($\alpha = 0.05$).

The energy to fracture of dentin specimens was determined from the stress-strain curves obtained in the tensile test. At the moment of fracture, the load and displacement raw data were plotted as a stress-strain curve and the energy was calculated as the area under the curve using the trapezoid rule in the software MatLab (MathWorks Inc., Natick, MA, US) (Murphy, Arzi, Hu, & Athanasiou, 2013). Data were statistically analyzed by two-way and one-way ANOVA and Games-Howell post hoc tests ($\alpha = 0.05$).

Results

Quantitative removal of PGs/GAGs is shown in Table 1. C-ABC and Try digestion removed GAGs from the demineralized dentin, with no significant difference after 2 days of incubation ($p = 0.276$). No measurable GAGs release was detected in the control group (DW). Curiously, no GAGs were quantified in dentin (remaining GAGs) for Try, while total GAGs removal (enzymatic removal + dentin/proteinase K digestion) were similar for c-ABC and control groups ($p = 0.225$). Moreover, significant reduction in the dry weight was observed after treatment with Try, while similar results were observed between control and c-ABC ($p = 0.735$) (Table 1).

HYP quantification results are shown in Figure 2. HYP release is observed after 4 h of incubation with collagenase. Similar cumulative HYP is observed for control and c-ABC from 4 h to 7 days, while Try shows significantly faster HYP release, with high HYP release between 8 h and 2 days. Complete degradation of Try-treated dentin was observed after 3 days of incubation, while specimens treated with c-ABC and DW completely degraded only after 1-week incubation (Figure 2A). The total content of HYP released during incubation with collagenase was statistically similar for control and c-ABC ($p = 0.090$, Figure 2B), with significant lower total content of HYP for Try-treated dentin matrix ($p < 0.001$ and $p = 0.0224$ when compared to control and c-ABC, respectively).

Complete removal of GAGs was observed in TEM for both enzymatic methods (Figure 3). Control group exhibited GAGs as dark filaments present around the collagen fibrils. In Try- (Figure 3C and 3D) and c-ABC-treated dentin (Figure 3E and 3F), CB staining for GAGs was markedly reduced or absent.

The removal of PGs/GAGs showed significant effects on the dentin mechanical properties (Figure 4). There were statistically significant interactions between UTS study factors (treatment vs. tubule orientation, $p < 0.031$) and significant differences in the tubule orientation and treatments ($p < 0.001$). The UTS of the dentin matrix is statistically higher for groups tested perpendicular (PP) to the tubule orientation when compared to parallel (PL)

($p < 0.001$), except for Try digestion. Try significantly reduced UTS when compared to control and c-ABC for PP testing condition ($p < 0.002$, Figure 4A). There were no differences in the UTS between c-ABC and control ($p > 0.05$). The energy to fracture was significantly decreased in Try-treated dentin when compared to control in PP condition ($p = 0.011$, Figure 4B). The control group exhibited significantly higher energy to fracture in PP direction when compared to PL ($p = 0.012$). There were no significant differences in the energy to fracture of groups tested PP and PL directions following removal of PGs/GAGs by c-ABC ($p = 0.299$) and Try ($p = 0.343$).

Discussion

This study assessed the contributions of PGs components to the biomechanical and biochemical properties of demineralized dentin matrix. Selective removal of these biomacromolecules components was performed by enzymatic methods commonly reported in the scientific literature. Our results indicate that PGs and their GAGs have an important role in bulk tissue mechanical behavior and may also regulate dentin proteolysis.

C-ABC and Try cleave PGs molecules at different sites. While the former cleaves GAGs such as CS-4, CS-6, dermatan sulfate and also hyaluronate from the protein core (which is preserved) (Bertassoni et al., 2015; Prabhakar et al., 2005), the latter digests the protein core releasing intact GAG chains (Bertassoni et al., 2015; Rapraeger & Bernfield, 1985). TEM images confirm that both enzymatic digestion protocols (c-ABC and Try) effectively removed GAGs surrounding type I collagen (Figure 3). CB is a cationic dye that binds preferentially to highly negatively charge sulfated GAGs. The absence of such protein component from the demineralized dentin observed in TEM indicates removal of GAGs, but it does not provide information on the removal of the core protein. However, results of the DMMB assay show that GAGs were not completely removed by enzymatic methods, with a high residual release after proteinase K digestion for c-ABC.

Disagreement in the DMMB assay and TEM results might be related to the methods restrictions. Detached GAGs (released from core protein by Try for example) may have been removed by TEM processing and were not stained by CB. Although DMMB assay is a widely used spectrophotometric analysis to quantify GAGs in biological tissues (Attia et al., 2014; Barbosa et al., 2003; Farndale, Sayers, & Barrett, 1982; Li et al., 2003), limitations to this protocol include low accuracy for low concentrations of GAGs (less than 5 $\mu\text{g/ml}$) (Barbosa et al., 2003; Farndale et al., 1982) and preferential binding to sulfated versus non-sulfated or to different types of PGs/GAGs (Farndale, Buttle, & Barrett, 1986). Moreover, proteinase K dentin digestion might have released GAGs chains already cleaved by the enzymes (especially c-ABC) but trapped in the dentin matrix, increasing GAGs concentration. For Try, this assumption is supported by different patterns of PGs/GAGs digestion without complete removal reported in cartilage (DiSilvestro & Suh, 2002). Digested PGs may remain within the matrix due to a faster rate of Try penetration in the tissue than the rate of PGs removal along its path. Based on that, a high release of remaining GAGs in DMMB assay of Try-treated specimens from dentin digestion would be expected, but no GAGs were quantified. Try was shown to modify ECM architecture and removes

non-collagenous proteins (Bertassoni & Swain, 2016), which could have impaired binding of DMMB to GAG chains, resulting in very low results in GAGs quantification.

Try increased the susceptibility of type I collagen to collagenase digestion resulting in faster ECM degradation (Figure 2) and higher weight loss (Table 1). These results suggest that the core protein might have an additional protective role in collagen fibrils degradability. The specific content of GAGs in dentin organic matrix is unknown due to variations in extraction protocols, but minor amounts of 5 to 7% of non-collagenous proteins were suggested (Bertassoni et al., 2015; Leaver, Triffitt, & Holbrook, 1975), accounting for about 1 wt. % of the total organic content. While the difference between control and c-ABC treated dentin weight loss was about 0.7%, Try digestion resulted in a 25% weight loss, supporting the removal of other non-collagenous components of ECM, as previously shown in dentin (Bedran-Russo et al., 2008; Bertassoni & Swain, 2016). Collagen modifications by Try include disaggregation into thinner fibrils and untwisted rope like appearance (Bertassoni et al., 2012; Bertassoni & Swain, 2016). It should also be mentioned that Try might cleave the C-telopeptide ends of collagen as previously suggested (Bertassoni & Swain, 2016), which might have resulted in release of minor amounts of collagen during Try incubation and explains the lower total HYP content in Try-treated dentin after collagenase incubation (Figure 2B). Collagen degradation/digestion mechanisms by Try need to be further explored in dentin, especially at low hierarchical levels. Our results support the fact that Try modifications to the collagen fibrils and ECM architecture will increase tissue's susceptible to further proteolysis. There is a correlation between collagen fibrils diameter and tissue tensile strength (Ottani, Raspanti, & Ruggeri, 2001), which also explains the reduced UTS values for Try-treated dentin.

Indeed, such modifications on collagen were significant to the mechanical properties of PGs/GAGs-depleted dentin. Two important aspects should be addressed here, first, the PGs/GAGs interaction with collagen is characterized by the core protein, mainly decorin, binding to four or more collagen fibrils by multiple hydrogen bonds forming interfibrillar aggregates, while the GAG chains interact with one another bridging fibrils together and expanding the collagen network due to the water molecules attached to them (Bertassoni et al., 2012; Orgel et al., 2009). Such complex scaffold created by PGs/GAGs and collagen fibrils regulate the mechanical behavior of mineralized tissues (Bertassoni et al., 2015) by mechanisms that include hydrostatic pressure, tissue viscoelasticity and intramolecular stretching and sliding of GAG chains connecting fibrils that reverses deformation of the interfibrillar aggregates (Bertassoni & Swain, 2014). Besides removal of PGs and GAGs, modifications on collagen promoted by Try may have facilitated molecules and fibrils rupture resulting in decreased UTS in PP orientation. Interestingly, no differences were found for c-ABC-treated dentin and control, which disagrees with nanoscale findings (Bertassoni et al., 2015). Herein at the microscale, we can assume that there is a compensatory mechanism by dentin organic matrix components other than PGs/GAGs, so mechanical properties of c-ABC-treated dentin were similar to control. Second, the energy to fracture of PGs/GAGs depleted was similar when the force was applied PP or PL to tubule orientation, showing that the tissue lost its anisotropic behavior when these biomacromolecules were removed. Peritubular and intertubular structure of dentin together with dentinal tubules orientation dictate different angles of crack growth direction and characterize the tissue anisotropy (Arola & Reprogel,

2006). However, energy to fracture and dentin viscoelastic properties are strongly determined by the collagen fibrils, and not by the dentinal tubules only (Nalla, Kinney, & Ritchie, 2003). Our results indicate that such phenomenon is driven by the interaction of collagen with PGs/GAGs. Considering the PGs/GAGs arrangement in dentin, in the PL condition GAG chains would be organized mainly parallel to the force direction. When tested under tension, bonds between GAG chains are disrupted and they slip as described in the sliding filament theory (Bertassoni & Swain, 2014; Haverkamp, Williams, & Scott, 2005), which will occur until complete disruption of such bonding, followed by separation of GAG chains and collagen fibrils within their orientation plane. On the other hand, in PP condition, PGs will be oriented mainly perpendicular to the force, so breakdown of both PGs/GAGs and collagen fibrils is needed for fracture, contributing for higher UTS (Figure 4, control group). In PGs/GAGs depleted dentin tested PP to tubules, there is no bridging mechanism promoted by GAGs and UTS and energy to fracture are only dependent on remaining unbroken collagen fibrils that act preventing crack propagation parallel to them (Nalla et al., 2003).

In conclusion, PGs/GAGs are important biomacromolecules that regulate dentin mechanical behavior and matrix biostability. More specifically, PGs/GAGs interaction with collagen molecules possibly influence crack growth propagation as part of a bridging mechanism that controls dentin anisotropy, confirming their significant role in the bulk tissue mechanical behavior. Also, PGs core protein may contribute to the resistance of the ECM against enzymatic degradation.

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Highlights

Selective enzymatic removal of main glycosaminoglycan chains from dentin proteoglycans decreased the energy to fracture of dentin matrix; while full enzymatic removal of proteoglycans reduced the strength and altered the anisotropic behavior of the dentin matrix.

Depletion of PGs by trypsin digestion significantly increased the susceptibility to degradation of the dentin matrix; providing clues to the protective role of the core protein in the biodegradation of the abundant type I collagen scaffold.

Proteoglycans are biomacromolecules in the dentin extracellular matrix shown here to have pivotal role in the tooth function by regulating dentin matrix mechanical behavior and biostability.

Figure 1.

Schematic of dentin preparation for ultimate tensile strength evaluation. Dentin specimens were sectioned axially and trimmed into an hour-glass shape according to the dentin tubules orientation [parallel (PL) and perpendicular (PP)]. Specimens were then demineralized, subjected to the digestion protocols for PGs/GAGs removal, and positioned in testing jig with dentin tubules positioned PP or PL to the loading tensile force.

Figure 2.

Means and standard deviations of (A) cumulative Hydroxyproline (HYP) (μg/ml/mg of dentin) release at different time points and (B) total Hydroxyproline release (μg/ml/mg of dentin) by collagenase digestion after PGs/GAGs removal by c-ABC, Try, and Control (DW). Asterisk (*) indicates statistically significant difference ($p < 0.05$).

Figure 3. Representative transmission electron microscopy images of dentin stained for glycosaminoglycans visualization (cupromeronic blue) after PGs/GAGs enzymatic removal methods: Control (DW) (A and B), Try (C and D), and c-ABC (E and F) incubation. Arrows indicate the presence of PGs/GAGs agglomerates. Scale bar corresponds to 200 nm.

Figure 4.

Results of the ultimate tensile strength (MPa) (A) and the energy to fracture (B) of dentin matrix with and without PGs/GAGs removal by c-ABC and Try when loaded perpendicular (PP) (light gray colored bars) or parallel (PL) (dark gray colored bars) to the dentin tubule orientation. Different symbols depict statistically significant ($p < 0.05$) difference among digestion protocols and dentinal tubules orientation.

Table 1

Results [mean (standard deviation)] of glycosaminoglycans ($\mu\text{g/ml/mg}$ dentin) in media and percentage of weight loss of dentin specimens after incubation with different enzymatic solutions and control (DW).

Protocol of PGs/GAGs removal	GAGs removal ($\mu\text{g/ml/mg}$ dentin)			Weight loss (%)
	Incubation media (2 days)	Dentin (proteinase K digestion)	TOTAL	
Control	0 ^B	33.83 (5.28) ^A	33.83 (5.28) ^A	3.75 (1.41) ^A
Trypsin	7.72 (2.30) ^A	0 ^C	7.72 (2.30) ^B	28.66 (3.32) ^B
c-ABC	9.35 (3.77) ^A	27.50 (4.97) ^B	36.85 (5.18) ^A	4.43 (1.80) ^A

Different letters indicate statistically significant differences among protocols of extraction (in each column) ($p < 0.05$).