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Host-derived Loss of Dentin Matrix Stiffness Associated with Solubilization of Collagen

Marcela R. Carrilho^{1,2}, Franklin R. Tay^{1,3}, Adam M. Donnelly¹, Kelli A. Agee¹, Leo Tjäderhane⁴, Annalisa Mazzoni⁵, Lorenzo Breschi⁶, Stephen Foulger⁷, and David H. Pashley^{1,*}

¹Department of Oral Biology, School of Dentistry, Medical College of Georgia, Augusta, GA, USA

²Department of Restorative Dentistry, Piracicaba School of Dentistry; University of Campinas,

Piracicaba/SP, Brazil ³Department of Endodontics, School of Dentistry, Medical College of

Georgia, Augusta, GA, USA ⁴Institute of Dentistry, University of Oulu and, Oulu University

Hospital, Oulu, Finland ⁵Department of SAU and FAL, University of Bologna, Bologna, Italy

⁶Division of Dental Sciences and Biomaterials, Department of Biomedicine, University of Trieste,

Trieste, Italy and IGM-CNR, Unit of Bologna c/o IOR, Bologna, Italy ⁷Department of Material

Sciences Engineering, Clemson University, Clemson, SC, USA

Abstract

Matrix metalloproteinases (MMPs) bound to dentin matrices are activated during adhesive bonding procedures and are thought to contribute to the progressive degradation of resin-dentin bonds over time. The purpose of this study was to evaluate the changes in mechanical, biochemical and structural properties of demineralized dentin treated with or without chlorhexidine (CHX), a known MMP-inhibitor. After demineralizing dentin beams in EDTA or phosphoric acid (PA), the baseline modulus of elasticity (E) of each beam was measured by 3-point flexure. Specimens were pretreated with water (control) or with 2% CHX (experimental) and then incubated in artificial saliva (AS) at 37°C for 4 weeks. The E of each specimen was remeasured weekly and, the media was analyzed for solubilized dentin collagen at first and fourth week of incubation. Some specimens were processed for electron microscopy (TEM) immediately after demineralization and after 4 weeks of incubation. In EDTA and PA-demineralized specimens, the E of the control specimens fell ($p < 0.05$) after incubation in AS, while there were no changes in E in the CHX-pretreated specimens over time. More collagen was solubilized from PA-demineralized controls ($p < 0.05$) than from EDTA-demineralized matrices after 1 or 4 weeks. Less collagen ($p < 0.05$) was solubilized from CHX-pretreated specimens demineralized in EDTA compared to PA. TEM examination of control beams revealed that prolonged demineralization of dentin in 10% PA (12 h) did not denature the collagen fibrils.

Keywords

dentin collagen; electron microscopy; matrix metalloproteinase; mechanical properties

INTRODUCTION

Dentin, the major mineralized hard tissue in teeth, is a hydrated composite of type I collagen fibrils and non-collagenous proteins embedded with nanocrystals of carbonated apatite. The

*Correspondence: Dr. David H. Pashley, Department of Oral, School of Dentistry, Medical College of Georgia, Augusta, GA, 30912-1129, USA, Tel: 706-721-2033; Fax: 706-721-6252; Email: DPASHLEY@mail.mcg.edu.

compressive mechanical properties of dentin are thought to be due to the packing and density of mineral apatite crystallites^{1,2}. The collagen matrix alone contributes significantly to the tensile strength of dentin^{3,4}. The collagen fibrils are considered the principal tensile stressbearing component of dentin. Thus, the study of the mechanical properties of dentin may generate new information that allows a better understanding of the mechanical behaviour of mineralized/demineralized tissues of the teeth.

A high degree of intermolecular cross-linking and a tight mechanical weave are thought to be responsible to the formidable resistance of dentin collagen to thermal and proteolytic cleavage⁵⁻⁸. Recent studies on the non-collagenous proteins associated with dentin collagen suggest that complexed and active forms of matrix metalloproteinases, identified in both odontoblasts and non-mineralized/mineralized compartments of human dentin⁹⁻¹¹ could exert an important role in the degradation of collagen matrix in numerous pathological processes occurring in oral tissues^{9,12-15}.

For successful adhesion to dentin, it is widely accepted that the formation of a hybrid layer must be achieved via the full impregnation of resin monomers into water-saturated acidetched dentin. The integrity and stability of collagen fibrils are the structural basis for hybrid layer matrices, as well as for their durability¹⁶. However, recent studies have pointed out the limited durability of *in vivo* and *in vitro* hybrid layers. Both mechanical and ultrastructural disruptions of hybrid layers formed in human dentin have been demonstrated in many of these studies¹⁷⁻²². The premature degradation of hybrid layers has been associated to the inability of current adhesives to durably seal the dentin substrate^{23,24}. This degradation process is quite likely to be the consequence of a myriad of factors, including deficient resin monomer infiltration of demineralized dentin, and elution of unpolymerized monomers from polymerized adhesives. This results in zones of exposed collagen fibrils within hybrid layers²⁵ that are prone to be attacked by host-derived proteolytic/hydrolytic enzymes. In fact, evidence of collagenolytic/gelatinolytic activity in dentin demineralized with etch-and-rinse adhesives in the absence of bacteria^{10,26,27} highlights the potential involvement of host-derived proteases in the disruption of incompletely-infiltrated collagen fibrils within hybrid layers^{19,20,22,27}.

Thus, the aim of this work was to evaluate the possible effect of host-derived proteases of the dentin matrix on the mechanical, biochemical and ultrastructural characteristics of dentin matrices demineralized with ethylenediamine tetra-acetic acid (EDTA) or phosphoric acid (PA). As chlorhexidine is a well-known MMP inhibitor²⁸, the null hypothesis tested was that there are no differences in the mechanical properties, collagen solubility and morphological features of dentin matrices demineralized with EDTA or PA when they were pretreated with or without chlorhexidine and then incubated in CaCl₂/ZnCl₂-containing artificial saliva.

MATERIALS AND METHODS

Dentin beam preparation and demineralization

Fifteen extracted non-carious human third molars were collected after the patients' informed consent had been obtained under a protocol reviewed and approved by the Human Assurance Committee of the Medical College of Georgia, USA. They were stored in 0.9% NaCl containing 0.02% sodium azide at 4 °C for less than three months. Enamel, cementum and pulpal soft tissue were completely removed using high-speed diamond burs under copious water cooling. A dentin disk (approximately 0.75 ± 0.04 mm thick) was obtained from the mid-coronal portion of each tooth using a slow speed diamond saw (Isomet, Buehler Ltd, Lake Bluff, IL, USA) under water cooling. Then, three to four dentin beams, measuring approximately 0.9 × 0.75 × 8.5 mm were obtained from each dentin disk as

described previously by Carvalho *et al.* [29]. The dentin beams were demineralized by immersion in either 0.5 mol/L EDTA (EDTA - pH 7.4; Sigma-Aldrich, St. Louis, MO, USA) for 6 days at 25°C 32/33 or 10% liquid phosphoric acid (PA - Sigma-Aldrich) (pH 0.9) for 12 hours at 25°C. Both EDTA- and PA-demineralized dentin beams were thoroughly washed in deionized water under constant stirring at 4 °C for 72 hours. After drying over anhydrous calcium sulfate for 8 hours, the dry mass of each specimen was measured with an analytical microbalance (Model MT5, Mettler Toledo, Hightstown, NJ, USA). All specimens were subsequently rehydrated in 0.9% NaCl containing 0.02% sodium azide for 24 h.

Three-point bending of demineralized dentin beams

The baseline stiffness of both EDTA- and PA-demineralized dentin beams (i.e. 20 specimens/demineralizing solution) was measured by means a three-point bending testing device. Measurement was performed with a 2.5 mm support span, using a universal testing machine (Vitrodyne V1000; John Chatillon & Sons, Greensboro, NC, USA) and a displacement rate of 0.5 mm min⁻¹. The latter was found in a pilot study to be sufficient to induce a maximum strain of 15% along the center of a demineralized dentin beam of similar dimensions. The specimens were tested while immersed in deionized water. The compressive force necessary to induce strain in dentin beams was measured in a 1N load cell (Transducer Techniques, Temecula, CA, USA). The modulus of elasticity of each specimen (E) was calculated as the steepest slope of the linear portion of the load-displacement curve using the following formula:

$$E = \frac{mL^3}{4bh^3}$$

where: m is the steepest slope along the linear portion of the load-displacement curve (N/mm), L is the span length (2.5 mm), b is the width of test specimens (0.9 ± 0.03 mm) and h is the thickness (0.75 ± 0.04 mm). E was expressed in MPa.

Demineralized dentin beams incubation

Half (10 specimens/demineralizing solution) of the EDTA- and PA-demineralized dentin beams were individually transferred to polypropylene screw-capped tubes containing 1 mL of water supplemented with 3 mmol/L NaN₃, while the rest (10 specimens/demineralizing solution) of the EDTA- and PA-demineralized dentin beams were pretreated with 1 mL of 2 mass% (40 mmol/L) chlorhexidine digluconate (CHX; pH 7.2) supplemented with 3 mmol/L NaN₃. After 30 minutes of preincubation (water or CHX) at 37°C, all specimens were blot-dried and then placed in 1 mL of artificial saliva (AS) (50 mmol/L HEPES, 5 mmol/L CaCl₂·2H₂O, 0.001 mmol/L ZnCl₂, 150 mmol/L NaCl, and 3 mmol/L NaN₃; pH 7.2). Individual polypropylene tubes containing the demineralized specimens were incubated in a water-shaker bath at 37 °C up to 4 weeks. After the first week of incubation, the AS storage medium was completely removed and analysed for dentin collagen solubilization. Fresh 1 mL of AS was replaced in the polypropylene screw-capped tubes where specimens were individually stored. After the fourth week of incubation, the storage media was removed and analysed for dentin collagen solubilization. In addition, the E of each demineralized specimen was repeatedly measured after each week of incubation (i.e. after 1, 2, 3 and 4 weeks), as previously described.

Determination of dentin collagen solubilization

A 0.5 mL aliquot of the AS incubation medium was added to an equal volume of 12 N HCl and hydrolyzed in 5 mL capacity Pyrex®-capped test tubes at 120 °C for 18 h. Aliquots of

of the hydrolyzates (25 μ L) were transferred to separate disposable cuvettes and allowed to evaporate to dryness under vacuum over sodium hydroxide/calcium sulfate desiccant for 24 h. Hydroxyproline (HYP) standards were prepared from a 1 mg/mL HYP stock solution in 50% isopropanol. Concentrations of HYP in the standard solution were: 0, 0.01, 0.05, 0.1, 0.15, 0.20, 0.50 or 0.75 mg/mL. Aliquots of these standards were hydrolyzed and allowed to evaporate to dryness for 24 h, similar to unknowns.

Collagenolytic activity was assessed by measuring the HYP content of the hydrolyzed specimens. The rationale for evaluating collagen degradation by means HYP assays relies on the fact that type I collagen contains about 10 mass% HYP, but most of other proteins contain little or none of this amino acid³⁰. Measurement of HYP was determined by quantitative analysis, as described by Jamall *et al.*³¹. Briefly, all residues of dried hydrolyzates (test specimens and HYP standards) were resolubilized with 1.2 mL of 50% isopropanol and 0.2 mL of 0.56% buffered chloramine-T solution (pH during this oxidation step was between 6.4 and 6.6). After a 10 min interval, 1.0 mL of p-dimethylanilimobenzaldehyde dissolved in perchloric acid (Ehrlich's reagent) was added to give a final volume of 2.4 mL. The chromophore was developed by incubating the specimens at 50 °C for 90 min. The absorbance of each specimen was spectrophotometrically read at 558 nm (Shimadzu UV-a 180, Tokyo, Japan) against a blank. A calibration curve was established by plotting the absorbance of the HYP standards against the amount of HYP in these standards ($R^2=0.99$). The absorbance values exhibited by the hydrolyzates were calculated based on the regression equation generated by the calibration curve. The resulting amount of HYP (mg/mL) was used to estimate the percentage of solubilized (degraded) collagen assuming that 90% of the dry mass of demineralized dentin beams consisted of type I collagen and that 10 mass% of collagen is HYP³⁰.

The moduli of elasticity and the percentage of collagen solubilization of EDTA- and PA-demineralized dentin specimens were separately analysed using two-way repeated measures ANOVA with incubation time and specimen treatment mode (with or without pretreatment with CHX) as main factors, and with the specimen as the factor of repetition. Comparisons of the moduli of elasticity or the percentage of collagen solubilization between EDTA- versus PA-demineralized were performed by other two-way repeated measures ANOVA tests with the incubation media being analyzed separately. The modulus of elasticity data was transformed into square root in order to achieve normality. All post hoc multiple comparisons were performed using Holm-Sidak test. Statistical significance was preset at $\alpha=0.05$.

Ultrastructural evaluation

Four EDTA-demineralized and four PA-demineralized dentin beams that had not been subjected to 3-point bending were used for transmission electron microscopy (TEM). Half of the beams were treated with CHX in the manner previously described and the other half were left untreated. The beams were immersed in AS for 4 weeks. They were removed from the AS, thoroughly rinsed in sodium cacodylate buffer, fixed in Karnovsky's fixative and then post-fixed in 1% osmium tetroxide. The beams were then dehydrated in an ascending ethanol series (50%-100%), transferred to propylene oxide as a transitional medium and subsequently embedded in epoxy resin, according to the protocol described by Tay *et al.*²³. Ninety nanometer thick sections were prepared and stained with 1% phosphotungstic acid and 2% uranyl acetate for examination with a transmission electron microscope (TEM) (JEM-1230, JEOL, Tokyo, Japan) operated at 80 kV.

RESULTS

Three-point bending results - modulus of elasticity

Changes in the E (i.e. stiffness) of the EDTA- and PA-demineralized dentin beams are shown in Figs. 1A and 1B, respectively. For all the specimens observed, straining of the demineralized dentin specimens did not produce much stress until it reached a strain about 4% to 5%. Thereafter, the stress increased rapidly with strain (data not shown). A progressive and statistically significant decrease in the stiffness of both EDTA- and PA-demineralized dentin specimens was observed in control specimens that were incubated in AS without chlorhexidine pretreatment ($p < 0.05$), but not in specimens that were pretreated with 2% chlorhexidine ($p > 0.05$). Both EDTA- and PA-demineralized dentin matrices became, respectively, 26% and 24% less stiff after 1 week of incubation in AS, when compared to the baseline values ($p < 0.05$). The lowest stiffness was achieved after the fourth week of incubation in AS, when the E values of EDTA- and PA-demineralized dentin specimens were about 35% lower in comparison with the baseline values ($p < 0.05$; Figs. 1A and 1B). For all specimens (EDTA- and PA-demineralized dentin beams) incubated in AS without chlorhexidine pretreatment, statistically significant decreases in the stiffness were obtained between the first and fourth weeks and between the second and fourth weeks ($p < 0.05$; Figs. 1A and 1B). In general, EDTA-demineralized dentin beams exhibited E values that were significantly higher than those of PA-demineralized dentin beams, regardless of the incubation period and whether chlorhexidine pretreatment was performed (Figs. 2A and 2B) ($p < 0.05$).

Extent of collagen degradation

Percentage of collagen degradation after the incubation of EDTA- and PA-demineralized dentin specimens are shown in Figs. 3A and 3B, respectively. The highest percentages of collagen degradation derived from the EDTA- and PA-demineralized matrices occurred when the specimens were incubated in AS, regardless of the period of incubation (Figs. 3A and 3B). After the first week of incubation, the percentages of collagen degradation derived from EDTA- (1.3%) and PA-demineralized dentin beams (3.7%) were significantly higher when compared to those observed for the corresponding group of specimens pretreated with 2% CHX (i.e. 0.03% for EDTA-demineralized and 1.6% for PA-demineralized dentin specimens) ($p < 0.05$; Fig. 3A). A significant increase in the percentage of collagen degradation was observed for EDTA-demineralized dentin specimens incubated in AS with or without CHX pretreatment after the fourth week of incubation ($p < 0.05$). For these specimens, collagen degradation without CHX pretreatment (2.05%) was significantly greater than those pretreated with CHX (0.38%) ($p < 0.05$; Fig. 3A). In contrast, the percentage of collagen degradation for PA-demineralized dentin beams after the fourth week of incubation was significantly lower than after the first week ($p < 0.05$; Fig. 3B). For these PA-demineralized specimens, the percentage of degradation for beams incubated in AS without CHX pretreatment (2.95%) was significantly greater than beams that were pretreated with CHX (0.03%) ($p < 0.05$; Fig. 3B). In general, the percentage of collagen degradation derived from PA-demineralized dentin beams was significantly greater than that derived from EDTA-demineralized dentin beams, regardless of the incubation period and whether CHX pretreatment had been performed (Figs. 3C and 3D). An exception was observed when these two substrates (EDTA- and PA-demineralized dentin) were pretreated with CHX before incubation in AS for 4 weeks. For this pretreatment mode-time interval, there was no difference in the percentage of collagen degradation between the two substrates ($p > 0.05$; Fig. 3D).

Ultrastructural features

The morphological aspects of EDTA- and PA-demineralized dentin beams are shown in Figures 4A and 4B, respectively. They revealed that the typical ultrastructure of collagen fibrils in the matrices demineralised by EDTA vs. PA both showed cross-banded collagen fibrils due to differential heavy metal staining. However, the PA-demineralized matrices (Fig. 4B) showed some fibril shrinkage that was not seen in the EDTA-demineralized specimens.

DISCUSSION

The results of this study do not support the full acceptance of the null hypothesis that there are no differences in the mechanical properties, collagen solubility and morphological features of dentin matrices demineralized with EDTA or PA when they are pretreated in water *versus* chlorhexidine and then incubated in $\text{CaCl}_2/\text{ZnCl}_2$ -containing artificial saliva. In a relatively short period of time (4 weeks), it was possible to verify significant changes in the stiffness, solubility and morphological features of EDTA- and PA-demineralized dentin matrices aged in AS containing Ca^{2+} and Zn^{2+} , both ions that are needed to activate collagenases. In contrast, a very low percentage of collagen degradation was associated with the relatively stable stiffness and ultrastructural features of EDTA- and PA-demineralized dentin matrices when those specimens were pretreated with CHX before incubating in AS. We speculate that the loss of matrix stiffness and the solubilization of collagen peptides in control specimens were due to the hydrolytic activity of intrinsic MMPs. We further speculate that the lack of significant change in stiffness and the significantly lower release of collagen peptides in specimens pretreated with 2% CHX were due to the inhibition of intrinsic MMPs by CHX.

Incubation of EDTA-demineralized dentin specimens in calcium- and zinc-containing artificial saliva showed both a loss of stiffness and collagen solubilization of the dentin matrices. These results suggest that our previous reports of the lack of change in the stiffness of EDTA-demineralized dentin^{32,33} was due predominantly to the absence of the Ca^{2+} and Zn^{2+} in the media rather than to the inhibitory effect of residual EDTA. In the current study, EDTA-demineralized dentin received prolonged rinsing (i.e. 48 hours) and long incubation in $\text{Ca}^{2+}/\text{Zn}^{2+}$ -containing medium would have been sufficient to reactivate the collagenolytic activity of EDTA-demineralized dentin matrix.

The lack of proportionality between the loss of matrix stiffness over time and the degree of solubilization of collagen is probably due to the fact that to solubilize a collagen peptide fragment, the MMP-induced cleavage sites would have to be between covalent cross-links^{34,35}. It is likely that there were many peptide chains that were cleaved at least once along their lengths. Presumably, this would reduce the stiffness of the matrix without allowing solubilization of the peptide fragments from the insoluble, cross-linked matrix. It is likely that collagen peptide fragments would only solubilize from the surface of these specimens. Soluble peptide fragments deep within the specimen may have more difficulty reaching the surface. Thus, it is not surprising that the proportion of solubilized collagen was lower than the percent decrease in stiffness. Interestingly, the PA-demineralized dentin specimens consistently exhibited E values that were significantly lower than those derived from EDTA-demineralized dentin (Figs. 2A and 2B). It is possible that the more aggressive nature of PA demineralization or low pH of phosphoric acid could have altered the biochemical nature of the intrapeptide and interpeptide cross-links. This, in turn, may result in a larger decrease in stiffness in the PA-demineralized specimens. These issues have to be investigated in further studies.

The observation that more collagen was solubilized from the control and experimental groups of PA-demineralized dentin relative to the EDTA-demineralization specimens (compare Figs. 3A and 3B) suggests that 12 hrs of exposure of normal dentin beams to 10% phosphoric acid may partially denature the collagen, making it more soluble in the CHX-pretreated experimental group, but especially in the control (AS) group.

The modulus of elasticity values obtained in the current study ranged between 4-6 MPa. In a study using a similar-sized specimens of EDTA-demineralized dentin beams, measured as cantilever, Maciel et al.³⁶ reported values between 6-9 MPa values that are not significantly different from those reported in the current study. In 3-point flexure or cantilever flexure, the strain is concentrated at the fulcrum and tends to give lower stiffness values than when similar specimens are tested in tension, where there is a more uniform strain distribution, which results in higher moduli of elasticity. When measured in tension, the modulus of elasticity of EDTA-demineralized dentin has been reported to have a mean value of 58 MPa³³. Similarly, in another study, the modulus of elasticity of EDTA-demineralized dentin measured in tension was 60 ± 17 MPa³².

The ultrastructural examination of collagen fibrils in the matrices that were completely demineralized in EDTA or phosphoric acid were done primarily to show that 12 hr of demineralization in 10% phosphoric acid at 25 °C did not denature the collagen fibrils. Ultrastructural criteria for denaturation include the loss of 67 nm cross-banding caused by differential uptake of heavy metal stains, and conversion of 50-100 nm wide fibrils that maintain in linear configuration, to 5 nm wide random coils of gelatin. Since we saw no difference between EDTA or phosphoric acid demineralized collagen ultrastructure in specimens immediately after demineralization (Figs. 4A vs. 4B), we conclude that there is no ultrastructurally identifiable denaturation caused by exposure of dentin matrix to 10% phosphoric acid for 12 hr at 25 °C. The slight shrinkage of the diameter of collagen fibrils may have been due to contraction of the matrix induced by low pH.³⁷

Demineralization of dentin in 10% PA may have activated procollagenase MMPs and made them more active than was found in the EDTA group. In pilot experiments using dentin beams demineralized in either 0.5 M EDTA or 10% PA, the beams were preincubated with 2 mM amino-phenylmercuric acetate (APMA pH 7.1) for 1 hr to determine if that would increase the collagen degradation. The results indicated no significant difference in the collagenolytic activity of beams incubated with or without AMPA. We interpreted this to mean that the collagenolytic activity that we measured was due to active enzymes that did not require AMPA-activation. Perhaps the relatively high amount of collagen solubilization in the PA group was due both to some slight chemical denaturation caused by the 12 h demineralization process plus some activation of collagenolytic enzyme activity over the first week of incubation. The collagenolytic activity was significantly reduced by pretreatment with 2% CHX.

This *in vitro* model is powerful in that it permits mechanical, biochemical and ultrastructural correlates to be made on the same specimens. By slightly increasing the medium volume, it should be possible to run PAGE gels to identify $\frac{1}{4}$ - $\frac{3}{4}$ fragments and specific MMPs. Preliminary Western blots indicate that MMPs-2 and 9 are present on solubilized collagen. These MMPs are known to be inhibited by chlorhexidine [28].

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A

EDTA-demineralized dentin

B**PA-demineralized dentin** **Figure 1.**

Changes in the modulus of elasticity of completely demineralized normal human coronal dentin beams over 4 weeks of incubation. **A.** 0.5 M EDTA (pH 7.4) demineralized beams. AS - control beams preincubated in water and then incubated in artificial saliva (AS). 2% CHX - experimental beams pretreated with 2% chlorhexidine digluconate and then incubated in AS. **B.** 10% phosphoric acid (PA) demineralized beams. Abbreviations are the same as in **A.** Height of bars signifies the mean value, the brackets $\pm$ SD, $n = 10$ in each subgroup. Bars identified by different lower case letters are significantly different ($p < 0.05$).

A**EDTA vs PA - AS**

B**EDTA vs PA - 2% CHX** **Figure 2.**

Changes in the modulus of elasticity of completely demineralized normal human coronal dentin beams for 4 weeks of incubation. **A.** Same data as Fig. 1 but grouped to compare EDTA- vs. PA-demineralized specimens at each time-point in the control (AS) group. **B.** Same data as in Fig. 1 but grouped to compare EDTA- vs. PA-demineralized specimens in the experimental (2% CHX pretreatment group). Symbols have same meaning as in Fig. 1.

Figure 3.

Percent of the total collagen that was solubilized from dentin beams after 1 and 4 weeks of incubation. **A.** 0.5 EDTA-demineralized beams. The media from both groups was removed at 1 and 4 weeks, hydrolyzed in HCl and analyzed for hydroxyproline, that in turn, premixed calculation of solubilized collagen. More collagen was solubilized after 4 weeks than after 1 week in both control (AS) and experimental (CHX) specimens. Symbols without SD brackets indicates that ± 1 SD was smaller than the size of the symbol. Points identified by different lower case letters are significantly different ($p < 0.05$). $N = 10$ in all subgroups. **B.** Specimens completely demineralized in 10% phosphoric acid. Note that significantly more collagen was solubilized at 1 week compared to 4 weeks in both groups but especially in the control (AS) group. **C.** Same data as in Fig. 3A and B but grouped to permit comparison of the two types of demineralization (EDTA vs. PA) in the control (AS) group at 1 and 4 weeks. **D.** Data grouped to permit comparison of the two types of demineralization (EDTA vs. PA) in the experimental (CHX) group at 1 and 4 weeks.

Figure 4.

Transmission electron micrographs of demineralized dentin beams. **A.** A EDTA-demineralized beam immediately after 6 days of demineralization at 25°C. Notice the morphological integrity of collagen fibrils. The granular material on the surface (arrow) may represent residual smear layer material. **B.** A PA-demineralized beam immediately after demineralization. Although there was a thin (0.5 μm) zone of surface degradation with defibrillation of collagen fibrils, the collagen fibrils below the surface maintained their cross-banding and heavy metal uptake.