PROTEOLYTIC ACTIVITY, DEGRADATION, AND DISSOLUTION OF PRIMARY AND PERMANENT TEETH

Short title: Degradation of primary and permanent teeth

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D.L.S.S., J.A.C., L.M.A.T., D.H.P., C.A.S.C. and J.H. designed the study. D.L.S.S., J.A.C., L.M.A.T., R.H.S., D.G.S. and F.G.B. collected the data. D.L.S.S. and J.H. was responsible for statistical analysis of the data. D.L.S.S., L.M.A.T., J.H. and C.P.P. prepared the first draft of the paper. All authors contributed to data interpretation, writing, revising of the manuscript, and approved its final version.

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PROTEOLYTIC ACTIVITY, DEGRADATION, AND DISSOLUTION OF PRIMARY AND PERMANENT TEETH

Background: Primary and permanent teeth composition may influence dissolution and degradation rates. **Aim:** To compare the dissolution and degradation of primary and permanent teeth. **Design:** Enamel and dentin powders were obtained from primary molars and premolars and incubated within different pH buffers. Calcium and inorganic phosphate release was quantified in the buffers by atomic absorption and light spectrophotometry. A colorimetric assay was used to assess the MMP activity of primary (PrD) and permanent dentin (PeD). Collagen degradation was assessed by dry mass loss, change in elastic modulus (*E*), ICTP and CTX release. Data were submitted to ANOVA and Tukey tests (α=0.05). **Results:** Similar dissolution was found between PrD and PeD after 256 h. At pH 4.5 enamel released more minerals than dentin while at pH 5.5 the inverse result was observed. MMP activity was similar for both substrates. PrD showed higher dry mass loss after 1 week. In general, greater reduction in *E* was recorded for PrD. Higher quantities of ICTP and CTX were released from PrD after 1 week. **Conclusions:** Primary and permanent teeth presented similar demineralization rates. However, collagen degradation was faster and more substantial for PrD.

Keywords: collagen; dentin; enamel; dissolution; proteases

1. Introduction

Dentin and enamel inorganic phase consist of phosphate minerals, mostly in the form of hydroxyapatite (Hap) crystals[1,2,3] which are constantly susceptible to loss (demineralization) or gain (remineralization) of ions due to pH variations.[1] Demineralization of dental substrates occurs mainly by acids from diet (foods and drinks),[1] bacteria[4] or dental restorative procedures such as acid etching.[5,6]

Despite the high mineral concentration (~96 wt.%), enamel has a very low organic content (~1 wt.%) predominantly concentrated in the inner region of this substrate.[7] The post-eruptive enamel organic matrix contains little percent of type IV and VII collagen, proteolyzed fragments and matrix metalloproteinase (MMP)-20[7] also known as enamelysin. On the other hand, dentin has approximately 20% of organic component of which 90% is type I collagen and the remaining 10% is non-collagenous molecules such as glycosaminoglycans, proteoglycans, growth factors and proteases.[8]

In mineralized dentin, collagen fibrils are protected from degradation by Hap crystals. However, when dentin is demineralized, the collagen network is exposed and become susceptible to enzymatic cleavage and destruction mediated by endogenous proteases such as matrix metalloproteinases (MMPs) and cysteine cathepsins (CCs) released from the mineralized matrix.[8-11] By degrading the dentin matrix those proteases play an important role in caries progression[11] and accelerate the enzymatic-mediated hydrolysis of exposed collagen in hybrid layers, compromising the quality and stability of resin-dentin bonds.[9,10]

Hybrid layers produced in primary teeth seem to be especially susceptible to degradation.[9,12,13] The lower mineral content and higher reactivity to acids of primary dentin culminates in deeper demineralization by etchants what aggravates the incomplete hybridization of demineralized collagen, resulting in thick and structurally porous hybrid layers[12] with a significant zone of exposed collagen.[13] The exposed collagen is highly susceptible to proteolytic degradation interfering on resin-dentin bond quality and stability over time.[9,10]

Although the literature provides considerable amount of information on the activity of proteases in permanent teeth[10,14,15] there are few data available for primary teeth. Chibinski et al.[16] showed that in caries-affected primary dentin, MMPs-2 and -9 are concentrated around the dentinal tubules, while MMP-8 could be found throughout the matrix. However, it has not been shown whether

primary and permanent dentin matrices are similarly degraded by these proteases over time.

Differences between primary and permanent teeth in terms of demineralization rate, proteolytic activity and matrix degradation may influence caries prevention, selection of restorative materials and restorative protocols. Thus, the aim of this study was to compare the rate of demineralization of primary *vs.* permanent enamel and dentin, and to monitor the proteolytic activity and degradation of primary and permanent dentin matrices over time. The null hypotheses tested were that there is no difference between the dissolution of primary and permanent mineralized tissues, and there is no difference between MMP activity and collagen degradation rates of primary and permanent dentin.

2. Material and Methods

The teeth used in this study were collected from the Human Tooth Bank of Araraquara School of Dentistry after approval by the Research Ethics Committee of Araraquara School of Dentistry, Sao Paulo State University, Brazil (Protocol #15/08).

2.1 Dissolution of mineralized tissues

2.1.1 Enamel and dentin powder preparation

The dissolution rates of enamel and dentin from primary and permanent teeth were tested using 24 sound teeth (12 premolars and 12 primary molars). In order to minimize age-related mineralization differences, the selected premolars were in function in oral cavity between 36 and 72 months and were stored in a 0.1% thymol solution at 4^{o}C for no longer than 3 months.

Teeth roots were removed with a metallographic diamond saw blade (ISOMET 1000, Buehler, Lake Bluff, IL, USA). The crowns were dehydrated in an oven at 90º C for 24 hours. Thereafter, they were manually powdered using a metal pestle. The obtained mixed powders of dentin and enamel were fractionated by a set of sieves and only the particles between 0.074 to 0.105 mm were used.

Enamel and dentin powders were separated from each other according to the method proposed by Asgar,[17] which is based on the density difference between these tissues. The powders from primary or permanent teeth were

placed in two different separatory funnels containing 7 mL of separation solution [92 mL of bromoform and 8 mL of acetone (both from Sigma-Aldrich, St Louis, EUA); d=2.7 g/mL] for each 1 g of powder. After 1 hour, enamel (d=2.95 g/mL) was collected from the bottom of the funnel while dentin (d=2.14 g/mL) was removed from the supernatant. Then, the solvents were eliminated by evaporation in a vented hood. Separated powders were rinsed several times with deionized water until a clear supernatant was obtained. The powders were dehydrated in an oven at 50° C for 2 h.

2.1.2 Dissolution of enamel and dentin powders

Two mL of 0.1 mol/L acetate buffer solutions with pH 4.5; 5.0; 5.5 or 6.5 were placed in microcentrifuge tubes containing 1 mg of dentin or enamel powder. The tubes (n=6) were kept under agitation at room temperature for 4, 8, 16, 32, 64, 128 or 256 h. Then, the tubes were centrifuged for 6 min at 13,000 RPM and the solubility of each substrate was estimated by the concentration of calcium (Ca) and inorganic phosphorus (Pi) found in the supernatant. Ca was quantified by atomic absorption spectrophotometry and phosphate by light spectrophotometry. For each analysis (dental tissue x buffer x time) six replicates were tested in duplicates and the values averaged in such a way that 6 values were obtained for each group. After each time period, the final pH of each group was determined. The concentration of Ca *vs* Pi was calculated, and the data presented in μmol/mL/mg.

2.2 Dentin matrix degradation

2.2.1 Dentin matrix preparation

Five sound human third molars and five primary second molars were obtained from 11 to 21-year-old patients. The teeth were stored in a 0.1% thymol solution at 4°C for no longer than 3 months. The roots were removed, and mid-coronal dentin was exposed by removing enamel and superficial dentin by means of a diamond disk (Isomet, Buehler Ltd., Lake Bluff, IL, USA) under water cooling. One 1 mm-thick dentin disk was obtained from each tooth; afterwards twenty-four dentin beams (average of 3 beams per disk) measuring 1×1×4 mm were sectioned from the disks (n=12 for each substrate). Beams with any defect such as pulp horn projection were discarded.

2.2.2 Total MMP activity

To determine the total MMP activity in primary and permanent dentin (n=12), the beams were completely demineralized in 10% phosphoric acid at 4°C for 18 hours, and then rinsed in deionized water for 2 hours. The demineralized primary and permanent dentin beams were immediately placed in the wells of a 96-well plate containing 300 µl of a generic MMP substrate (SensoLyte Generic MMP colorimetric assay kit, 10 mM, 100 µl, AnaSpec Inc., Lot# 131-029, Fremont, CA, USA) at 25℃ for 1 h. Then the beams were removed from the wells, and the total MMP activity of each specimen was determined by measuring the absorbance of each well at 412 nm in a plate reader (Synergy HT microplate reader, BioTek Instruments, Winooski, VT, USA) against blanks.[18] Twelve individual values were obtained for each primary and permanent dentin. When degraded by MMPs the SensoLyte substrate releases a sulfhydryl group which reacts with Ellman's reagent and forms 2-nitro-5-thiobenzoic acid, a compound of yellowish color that represents the proteolytic activity of MMP-1, 2, 3, 7, 8, 9, 12, 13 or 14. The higher the absorbance values, the higher the activity of these proteases.

2.2.3 Elastic modulus (E) and dry mass

Before analyzing the MMP activity for both primary and permanent dentin, the initial elastic modulus of each demineralized beam (n=12) was determined by three-point flexure testing. An aluminum testing jig with a 2.5 mm span between supports was fixed to the bottom of a glass Petri dish. Specimens were tested under compression with the use of a 100 g load cell (Transducer Techniques, Temecula, CA, USA) at a cross-head speed of 1 mm/min, while immersed in deionized water (Vitrodyne V1000, Liveco Inc., Burlington, VT, USA) at room temperature. Load-displacement curves were converted to stress-strain curves, and the apparent modulus of elasticity was calculated at 15% strain.[18] Subsequently, the beams were completely dried in a desiccator containing calcium sulfate for 48 h, and weighed on a microanalytical balance (XP6 Microbalance, Mettler Toledo, Columbus, OH, USA) to obtain their initial dry mass. The beams were rehydrated in deionized water for 1 h and stored in 300 µL of a simulated body fluid (SBF composition in mM/L: KCl, 13; KSCN, 2:

Na_2SO_4-$10H_2O$, 2.4; HEPES, 5; $CaCl_2.2H_2O$, 1.5; $NaHCO_3$, 7.5; $ZnCl_2$, 0.02; 0.02% sodium azide) at 37°C for up to 2 months. Dry mass and elastic modulus values were reevaluated after time intervals of 1 week, 1 month and 2 months following the same procedures described above. Dry mass loss (Δm) and changes in E (ΔE) were calculated by subtracting the initial dry mass from the final dry mass, and the initial E from the final E.

2.2.4 Quantification of type I collagen fragments (ICTP and CTX)

Five out of the 12 samples stored for elastic modulus and dry mass assessment were randomly used for quantification of type I collagen fragments (n=5 for each substrate). The simulated body fluid in which the beams were stored was collected and used for assaying solubilized carboxyterminal telopeptides (ICTP and CTX) after each storage time interval. These specific fragments are the degradation products of collagen cleaved by matrix metalloproteinases (ICTP) and by cysteine cathepsins (CTX).[19]

After time intervals of 1 week, 1 month and 2 months of storage, collagen degradation by MMPs was determined by the presence of ICTP fragments using an ELISA kit (ELISA TSZ, Framingham, MA, USA; Cat. # HU9655), while the degradation mediated by cysteine cathepsins was measured by identifying CTX fragments using the Serum CrossLaps ELISA kit (Immunodiagnostic System, Scottsdale, AZ, USA). The concentration of both fragments was calculated based on specific standard curves for each assay.

2.3 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 8 software (GraphPad Software, La Jolla, CA, USA). Datasets from all the response variables, dentin and enamel dissolution (Ca *vs* Pi µmol/mL/mg), total MMP activity (absorbance), changes in elastic modulus (MPa), dry mass loss (%) and release of ICTP and CTX fragments (ng/mg of dentin) were normally distributed and variances were homogeneous. The effect of time on substrates dissolution was analyzed by simple linear regressions ($p<0.05$). A two-way analysis of variance (ANOVA) with one repeated measure complemented by a post-hoc test for two by two comparisons were applied to all data, except for

total MMP activity. For this response variable, a non-paired t-test was used. Statistical decisions were made using the level of significance of 5%.

3. Results

3.1 Dissolution of primary and permanent mineralized tissues

Data obtained from the longest equilibration time (256 h) were submitted to two-way ANOVA. That time period was chosen in order to ensure that the maximum dissolution was reached for each tissue. Enamel and dentin dissolution caused similar changes in acetate buffer pH in such a way that the average final pH of each solution irrespective of substrate and time were 4.6; 5.1; 5.6; 6.4 and 6.9.

Interaction was found between the factors “substrate type” and “pH” (Table 1). No difference was observed between primary and permanent hard tissues (dentin or enamel) regardless of pH. Primary and permanent dentin released similar amounts of minerals at pH 4.5; 5.0 and 5.5 while primary and permanent enamel showed higher demineralization at pH 4.5, followed by pH 5.0. Although enamel showed highest dissolution at pH 4.5 when compared to dentin, at pH 5.0, enamel and dentin behaved similarly, and at pH 5.5 the initial scenario was inverted with dentin releasing significant more minerals than enamel (Table 1). The same pattern was also observed at all the other periods (Figure 1). The dissolution rate at pH 6.0 and 6.5 was similar for all hard tissues, and was significantly lower, when compared to pH 4.5 or 5.0.

3.2 Total MMP Activity

The total MMP activity was similar between primary (0.254 ± 0.069) and permanent (0.252 ± 0.053) dentin (p=0.9160).

3.3 Elastic Modulus (E) and dry mass

Regarding elastic modulus, the two-way ANOVA revealed no interaction between the variables “time” and “substrate” (p=0.6165). However, both factors “time” (p<0.0001) and “substrate” were significant (p=0.0002). The initial E of

primary and permanent dentin matrices were statistically different (p=0.0124). No difference was observed after one week in SBF (p>0.9999). After 1 and 2 months, there were significantly lower stiffness values measured for primary dentin (0.48±0.21 MPa and 0.47±0.16 MPa) compared with permanent dentin (0.97±0.54 MPa and 0.98±0.36 MPa) (Figure 2). The analysis of dry mass loss (%) by the two-way ANOVA test showed interaction between the factors "time" and "substrate" (p=0.0009). For both dentin matrices, there was a progressive and significant loss of dry mass over time. The loss of dry mass for primary dentin (-33.03±3.7%) was statistically greater than that observed for permanent dentin (-23.52±1.6%) after 7 days. However, after 1 and 2 months, primary (-16.58±5.9% and -8.38±1.0%) and permanent (-15.89±4.9% and -6.91±3.3%) dentin showed similar mass loss (Figure 3). The accumulated mass loss for primary dentin was 58%, and 46.3% for permanent dentin after 2 months in SBF.

3.4 ICTP and CTX releasing

The release of ICTP (ng/mg of dentin) after 1 week of incubation (2.9±1.3 ng/mg for primary vs. 1.5±1.4 ng/mg for permanent dentin) differed significantly from the values observed after 30 days (6.9±1.0 ng/mg for primary vs. 2.6±1.5 ng/mg for permanent dentin) for both hard tissues. However, when primary and permanent dentin specimens were compared within the same storage periods, ICTP release from primary dentin matrices was always significantly higher (Figure 4a). Finally, assessment of CTX in the storage medium (ng/mg of dentin) showed the opposite result (Figure 4b). A higher concentration of CTX was observed for both hard tissues after 7 days in SBF. After 7 days, primary dentin (59.2±14.3 ng/mg) released significantly more CTX than permanent dentin (34.1±10.8 ng/mg) while after 1 month of incubation, similar CTX concentrations were found for both hard tissues (25.3±9.8 ng/mg for primary vs. 23.2±16.0 ng/mg for permanent dentin). Within 2 months of incubation, the amount of both ICTP and CTX telopeptides released into the incubating solution was below ELISA detection limits.

4. Discussion

Despite the differences in mineral content, similar behavior of chemical and mechanical properties of primary and permanent dentin have been reported in the literature.[20] However, Mejàre and Stenlund[21] reported a caries rate 2-3 times higher in second primary molar than in first permanent molar. The present study contributes to elucidate clinical and laboratory findings regarding caries progression rates and resin-dentin bond degradation in primary and permanent teeth by pointing differences and similarities between the dissolution and organic matrix degradation of those substrates.

During tooth aging process calcium ions from Hap structure may be substituted by sodium, magnesium or potassium while hydroxyl may give way to fluoride, chloride or carbonate, being this last one also a substitute to phosphate. Those changes significantly influence Hap solubility making the substrate more susceptible (carbonate) or more resistant (fluoride) to dissolution.[1] Therefore, the premolars selected for this study were in function in the oral cavity for at least 36 months in order to minimize age-related solubility differences between primary and permanent teeth.

In general, after 256 h of incubation dentin maximum dissolution rates were observed at pH 4.5 and sustained even at higher pH (5.0 and 5.5). The greater susceptibility of dentin to demineralization (critical pH ~ 6.5) may be explained by its elevated carbonate content (5-6%) when compared to enamel (3%).[1] Besides that, the crystals in dentin are plate-like and smaller (60 nm length and 2-5 nm thick) when compared to the long spaghetti shaped crystals in enamel (widths and thicknesses ranging from 20 to 60 nm) providing a higher surface area of dentin in contact with the acidic solutions.[22] Enamel released more minerals than dentin at pH 4.5, due to its higher mineral amount in both primary and permanent teeth. The significant dissolution decrease observed for enamel at pH 5.0 and 5.5 may be explained by its fewer ions substitutions[23] and larger better oriented crystals (68.3 width; 26.3 thick[2]) that favor mineral stability and reduce its solubility.[22] The similar dissolution of enamel and dentin at pH 5.0 suggests a solubility equilibrium. All those chemical differences between enamel and dentin may also explain the inversion in the solubility of these tissues as seen in Figure 1.

Studies have shown that primary enamel has higher carbonate[24] and lower mineral content compared to permanent enamel,[3] what contributes to the

faster demineralization of primary teeth.[24,25] As enamel, primary dentin also has lower mineral content and higher reactivity to acidic solutions than permanent dentin.[5] These characteristics may influence the progression of carious lesions in primary substrates[21] as well as their interaction with restorative materials.[5] Despite those differences the present study showed similarity between the dissolution of primary and permanent substrates after 256 h of incubation. It requires the acceptance of the first null hypothesis tested that there is no difference between the dissolution of primary and permanent mineralized tissues. The absence of significant differences among the dissolution rates of primary and permanent powdered enamel and dentin points to the importance of tissue morphology on tooth demineralization, and not only its chemical composition. Not powdered primary enamel has higher diffusion coefficients for mineral ions[26] and a lower overall mineral density in the outermost layers compared to permanent enamel.[27] While primary dentin has a higher number of tubules, which leads to a reduced area of intertubular dentin,[28] lower buffer capacity and increased diffusion of acidic solutions.[5] These structural differences were minimized by powdering the teeth.

It has been shown that primary dentin surfaces release more calcium when acid-etched with 35% phosphoric acid for the same period of time as permanent dentin surfaces[5] which leads to deeper demineralization during restorative procedures.[13] The thickness of demineralized zone has been related to the porosity and instability of resin-dentin bonds due to the lack of complete monomer infiltration and collagen exposure within the hybrid layer.[12,13] Collagen exposed zone is more significant in primary teeth[13] and is susceptible to rapid degradation over time.[11]

Resin-dentin bond degradation has been correlated with the activity of host-derived proteases such as MMPs -2, -8 and -9, and cathepsin B and cathepsin K. Those enzymes are capable of cleaving type I collagen within the hybrid layer.[9] This study used 10% phosphoric acid to demineralize the dentin beams, this moderate acid concentration mixed with dentin presents a favorable pH for MMPs activation and subsequent collagen degradation. When 10% phosphoric acid is compared to 37% and 1% phosphoric acid, it shows the highest expression and activity of extracted MMP-2 as well as the highest HYP release.[6] The present study pointed out the similarity of the MMP activity in both

substrates after 1 h which was also observed after 7 days when the ICTP concentration was measured. It requires the partial acceptance of the second null hypothesis. ICTP fragments are produced by MMPs during collagen degradation, and reflect the total activity of these proteases.[19] The amount of ICTP accumulated in the medium from the 7th to the 30th day was higher for both types of dentin than the amount observed for the first week. This suggests a prolonged release of ICTP during the first month of degradation. After 30 days, primary dentin released significant more ICTP, indicating that although primary and permanent dentin had similar initial MMP activities, primary dentin produced a higher rate of MMP-mediated degradation over time. Thus, the second null hypothesis was partially rejected.

The opposite phenomenon was observed for CTX release, which was higher for primary dentin after 7 days of incubation, and significantly decreased for both substrates in the course of time. CTX fragments are produced by collagen degradation mediated by CCs.[19] Thus, these findings suggests that the activity of CCs, especially in primary dentin, was more intense during the first days after demineralization. A significant release of ICTP and CTX fragments, as well as a higher concentration of CTX within the first week of incubation was also observed by Turco et al.[29] for permanent teeth. According to those authors, CTX could easily diffuse out collagen networks due to its smaller-sized molecule, based on the size-exclusion phenomena. To the best of our knowledge, the present study is the first to analyze ICTP and CTX release from demineralized primary dentin. For both substrates, dentin matrix hydrolysis seemed to drastically decrease after 1 month since no ICTP or CTX fragments were identified in the time interval of 2 months.

Although the literature describes similarity in the organic content of primary and permanent dentin[20] as well as in the molecular arrangement of the organic matrix after acid etching,[30] the present study recorded lower initial elastic modulus for primary dentin. That may be explained by morphological differences between those substrates. The higher density of tubules and reduced intertubular dentin in the primary substrate[28] seem to influence not only dentin demineralization, but also facilitate primary organic matrix deformation. Over time, the stiffness and dry mass of dentin matrix are adversely affected by collagen fibril degradation, resulting in gradual reduction of its elastic modulus

and increase in mass loss. Significant decrease in elastic modulus was concentrated in the first week for permanent dentin, however it continued for up to 1 month for primary dentin. These findings suggest that primary dentin is susceptible to collagen deterioration for a longer period than permanent dentin. Primary dentin released 2.37 times more ICTP after 1 month than it did within the first week, while permanent dentin released only 1.7 times the quantity in the same period. Therefore, it could be pointed that MMPs in primary dentin play an important role in collagen degradation not only in the first days after demineralization, as observed for permanent dentin,[29] but also within the first month.

Primary dentin lost significantly more mass after 7 days of incubation than permanent dentin. This could have been caused by a sharper performance of CCs in the hydrolysis of primary dentin matrix. CTX analysis strengthened the possibility that CCs were responsible for a more significant initial degradation of primary dentin since this telopeptide fragment concentration was higher for primary specimens in the first week than it was for permanent specimens.

Although primary and permanent dentin presented similar initial MMP activity, they behaved differently over time. Solubilization of primary dentin matrix happened significantly faster, when compared with permanent dentin matrix. Considering that host MMPs and CCs are intensely involved in caries progression in human dentin[15] future studies should investigate the relevance of matrix degradation and proteolytic activity rates on caries progression on primary and permanent dentin and how the inhibition of those enzymes could interfere on caries development. Furthermore, the results point to the importance of specific protocols that promote stability of resin-dentin bonds in primary dentin, such as the use of protease inhibitors[9] and reduced acid-etching times.[5,13]

While the present study has shown that primary and permanent substrates have similar demineralization in acidic solutions over time. However, the results are limited in generalizability since tooth three-dimensional morphology was not considered once the teeth were powdered and a limited pH

range was tested (4.5-6.5). This study also demonstrated that primary dentin is more susceptible to degradation than permanent dentin, but highly demineralized specimens were used in order to enable the tests. In a clinical setting the demineralization for bonding procedures is superficial. Future researches could investigate the effectiveness of cross-linking agents able to increase collagen resistance to hydrolysis[18] on resin-primary dentin bonds. Additional studies are also needed to clarify and quantify the role of CCs in collagen degradation of primary dentin and its impact on pediatric dental practice.

5. Conclusion

Primary and permanent substrates showed similar degrees of demineralization when powdered and stored in acidic solutions over time. Although primary and permanent dentin presented similar initial MMP activity, collagen degradation was faster and more significant for primary dentin over time.

Why this paper is important to paediatric dentists

- Primary and permanent enamel and dentin powders have similar degrees of demineralization stored in acidic solutions over time.
- Dentin matrix degradation is faster and more significant in primary dentin.

Conflict of interest

The authors declare that there is no conflict of interest.

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TABLE 1 Dissolution of primary and permanent hard tissues (Ca *vs* Pi μmol/mL/mg) after 256 h in 0.1 mol/L acetate buffer.

pH	Hard Tissue			
	Dentin		Enamel	
	Primary	Permanent	Primary	Permanent
4.5	6.07 (0.99) ^{A}	6.11 (2.60) ^{A}	9.82 (0.58) ^{B}	10.08 (2.13) ^{B}
5.0	6.62 (1.50) ^{A}	6.71 (1.74) ^{A}	6.16 (1.96) ^{A}	6.68 (2.22) ^{A}
5.5	6.12 (1.14) ^{A}	5.42 (1.69) ^{A}	1.98 (1.20) ^{C}	1.19 (0.62) ^{C}
6.0	1.63 (0.56) ^{C}	1.91 (0.51) ^{C}	0.28 (0.16) ^{C}	0.20 (0.13) ^{C}
6.5	0.21 (0.07) ^{C}	0.27 (0.10) ^{C}	0.04 (0.01) ^{C}	0.07 (0.04) ^{C}

Numbers are mean (standard-deviation), n=6. Letters should be used to compare means in any direction, regardless pH and substrate (Interaction $p<0.0001$; 2-way ANOVA). Different letters represent statistical difference between groups ($p<0.05$).

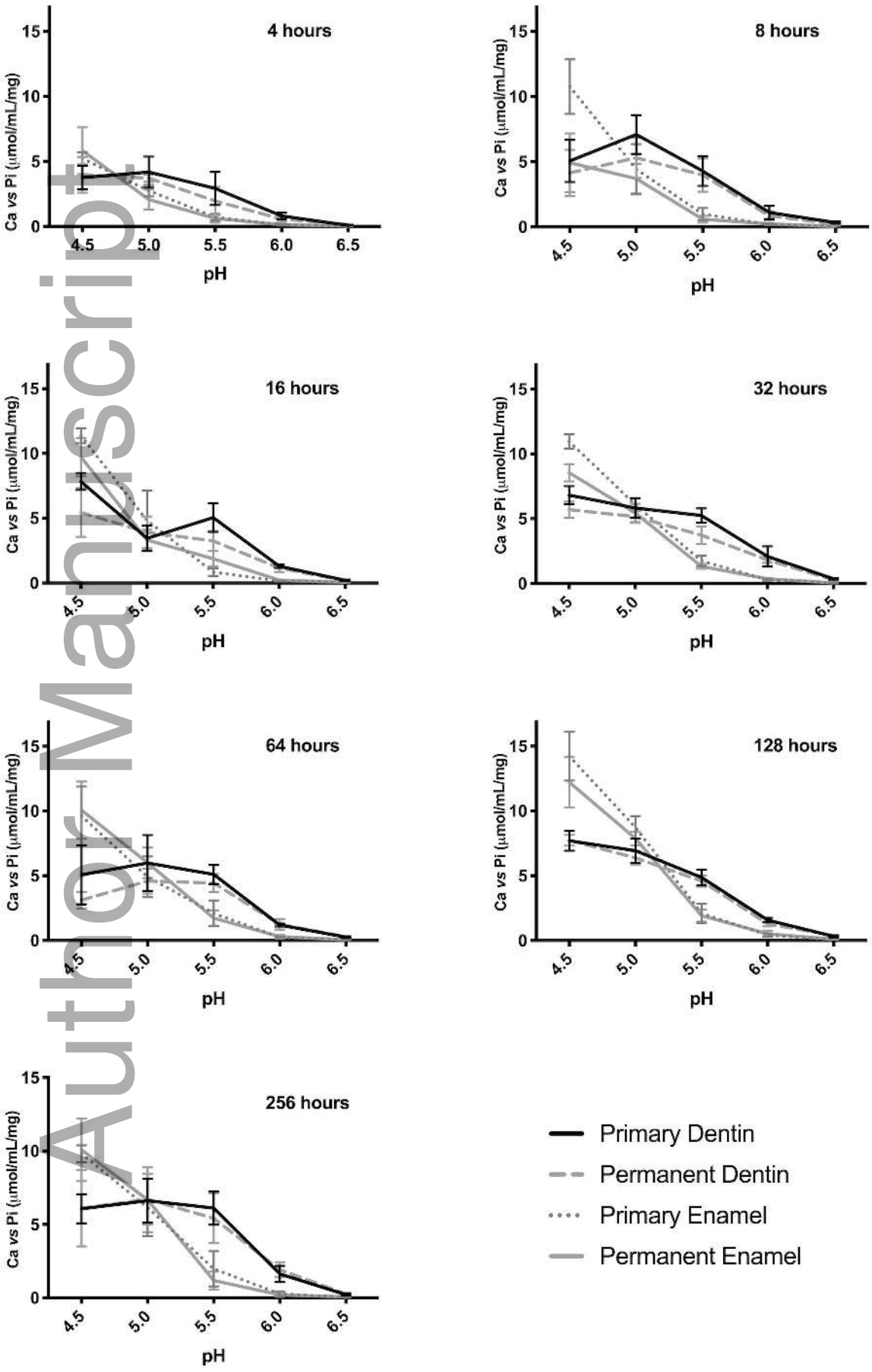
Figure legends

FIGURE 1 The charts show Ca *vs* Pi rates against pH after each evaluated period. Points represent means and error bars the standard deviations (n=6). Error bars shorter than the height of the symbol were not drawn. Lines represent the rates of dissolution for each substrate.

FIGURE 2 Elastic modulus (E) of primary and permanent dentin matrices after up to 2 month in simulated body fluid at 37°C (n=12). The chart columns represent the mean values and the error bars represent standard deviation. Within each type of substrate (primary or permanent) the same letter shows no statistical difference (Tukey, $p > 0.05$). Within the same time interval (initial, 7 days, 1 or 2 months), the columns connected with an asterisk (*) show significant difference (Tukey, $p < 0.05$).

FIGURE 3 Dry mass loss (%) of primary (n=12) and permanent (n=12) dentin matrices after storage in simulated body fluid at 37°C for 1 week, 1 month and 2 months. The chart columns represent the mean values and the error bars represent standard deviation. Equal letters show no statistical difference (Tukey, $p > 0.05$).

FIGURE 4 ICTP (A) and CTX (B) type I collagen fragments released from primary (n=5) and permanent (n=5) dentin matrices after storage in body simulated fluid at 37°C after 7 days and 1 month. The chart columns represent the mean values and the error bars represent standard deviation. Within each type of substrate (primary or permanent) the same letter shows no statistical difference (Tukey, $p > 0.05$). Within the same period (7 days or 1 month), columns connected with an asterisk (*) show significant difference (Tukey, $p < 0.05$).



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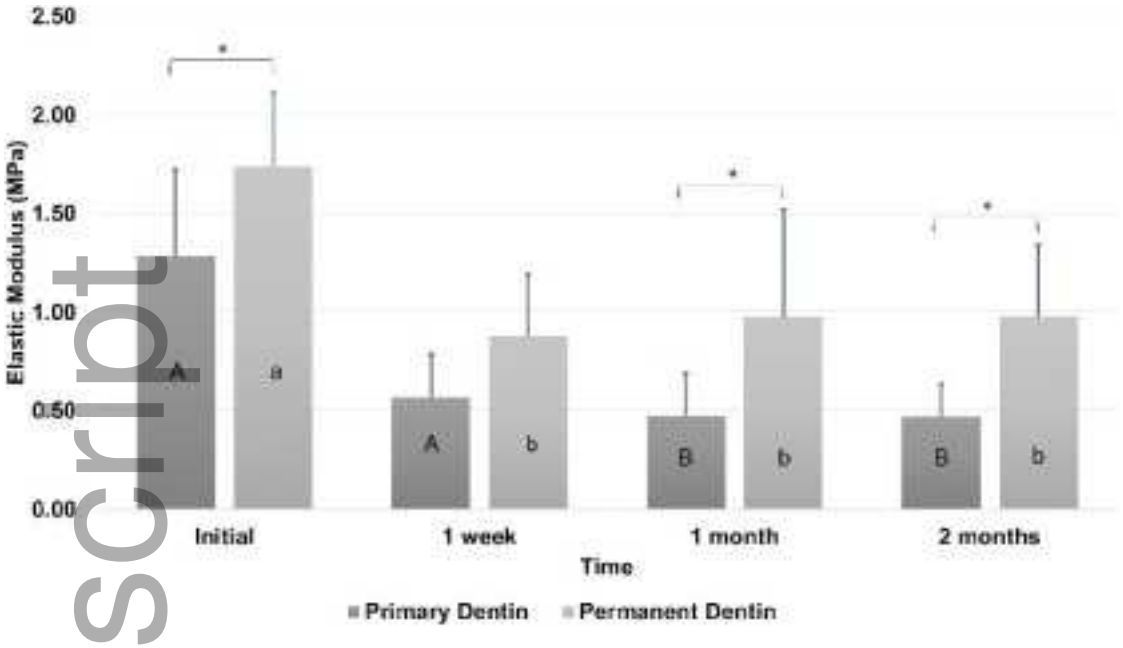

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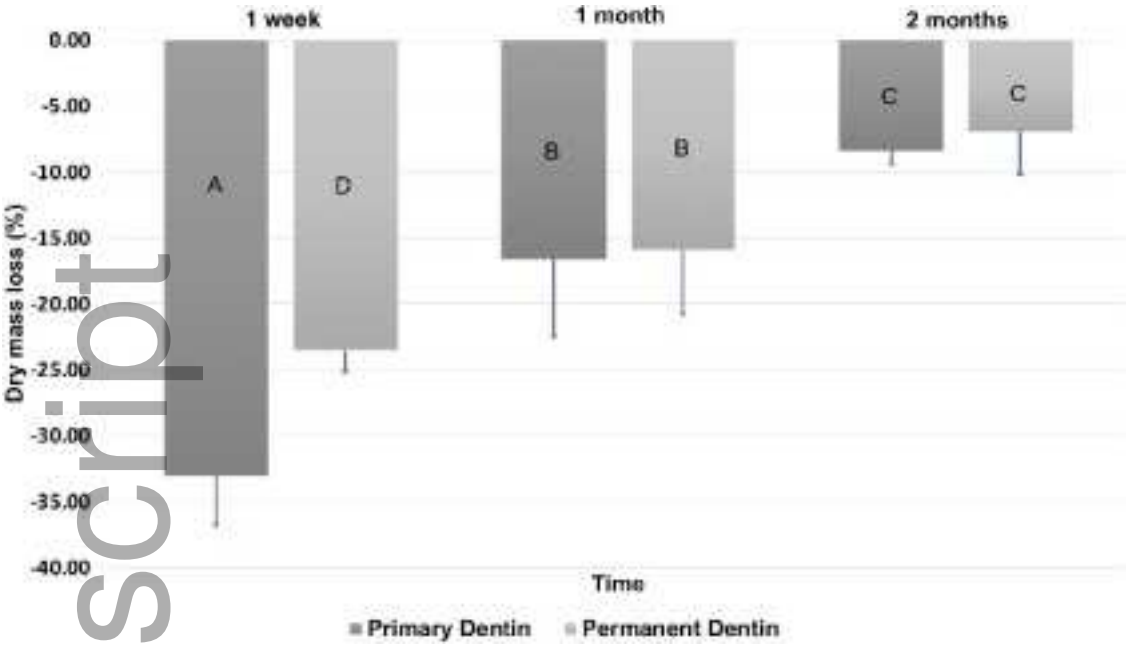

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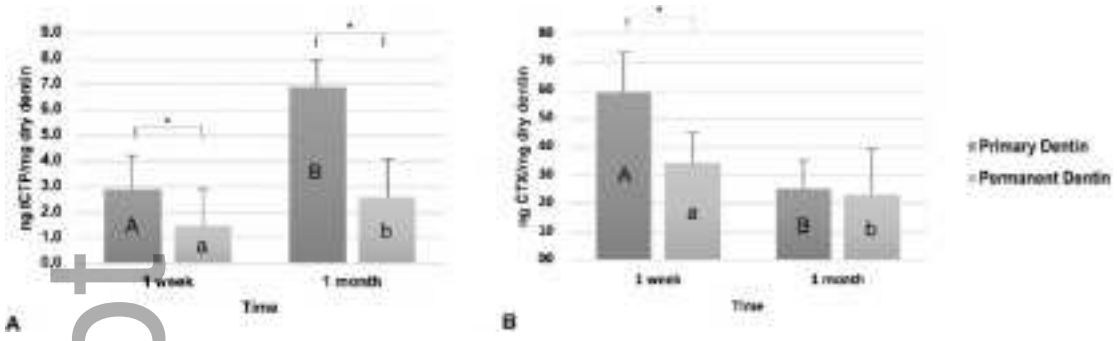

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