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Alternative model for cathepsin K activation in human dentin

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ABSTRACT

Objective. To evaluate the protease activity in dentin matrices subjected to lactic acid (LA) in comparison to polyacrylic acid (PAA) challenge model at cathepsin K (CT-K) optimum pH 5.5 to assess effectiveness of inhibitors in dentin collagen degradation.

Methods. Dentin disks measuring 0.5 mm prepared from human molars were completely demineralized in 10% H_3PO_4. Demineralized dentin disks were challenged with 0.1 M LA, 1.1 mM PAA, artificial saliva (AS), or deionized water (C) for 24 h or 7-days. Dentin collagen properties were tested by measurement of %dry mass change, and ultimate tensile strength (UTS). Degradation of dentin type I collagen was measured by telopeptide assays measuring the sub-product release of C-terminal cross-linked telopeptides (ICTP) and C-terminal peptide (CTX) in the incubation media in relation to total protein concentration, which correlates with matrix metalloproteinases (MMPs) and CT-K activities.

Results. Gravimetric analysis showed statistically significant difference between C and other groups ($p < 0.04$) at 24 h. LA specimens showed significantly higher weight loss from 24 h to 7-days ($p = 0.02$). UTS revealed statistically significant difference between AS and LA at 24 h and 7-days. UTS at 24 h and 7-days for C and AS had significantly higher mean values compared to LA and PAA. Telopeptide assays reported that CTX_{tp} results showed that LA at 24 h had significantly higher mean values compared to C and AS.

Significance. LA has the ability to activate endogenous CT-K in dentin as measured by the release of CTX (CT-K specific telopeptide). This LA based model has the potential application for further investigations on the activity and possible inhibitors of CT-K in human dentin.

1. Introduction

Cysteine cathepsins (CT) are papain-like enzymes that belong to C1 (papain) family of the clan CA of cysteine peptidases, the largest and the most characterized family of cysteine peptidases [1–3]. There are 11 human cysteine cathepsins; cathepsin B, C, H, F, K, L, O, S, V, X and W [4]. Of all cathepsins, cathepsin K (CT-K) is the only one with pronounced collagenase activity [5]. It cleaves collagen at various sites within the triple helical region of the tropocollagen [6]. CT-K is activated and functional in low pH, with optimal function at around pH 5.5 [7].

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The lysosomal cysteine proteases such as CT-B, -K and -L have been detected in human dentin, and their activities have been suggested to be comparable to matrix metalloproteinases (MMPs) [8]. An earlier study evaluated the activity of cysteine cathepsins at various depths in dental carious lesions. They reported increase in CT-B activity with increased depth of carious lesion, which highlights the significance of pulp-derived CT in active carious lesions [7]. This observation also stressed the possibilities of targeting CTs to inhibit caries progression. Although CT-B is found in higher fractions in carious dentin, CT-K is the one that truly demonstrated a triple helical collagenase activity [6]. The capacity of CTs to simultaneously activate MMPs in dentin have also been reported [9]. It has been demonstrated that CT-K cleaves and activates Pro-MMP-9 in acidic environments such as in tumors and bone resorption [7,10,11].

Several studies have assessed the feasibility of *in vitro* dentin demineralization models to simulate the effect of bacterial biofilm and dentin matrix degradation in carious lesions [12–14]. Application of 0.1 M lactic acid for 7 or 14 days has been widely used to produce artificial carious lesions in bovine teeth *in vitro* [12,15]. This is likely due to the production of lactic acid, as the major end-product of glycolysis by the cariogenic bacteria under the conditions of excess sugar or low environmental pH [16].

Previously the effect of polyacrylic acid (PAA) was evaluated in comparison to phosphoric acid (PA) model on MMPs and CT-K activities from demineralized dentin beams. It was reported that the application of 10% PAA to demineralized dentin resulted in stronger CT-K activation compared to PA groups as measured by CTX (C-terminal cross-linked telopeptide of type I collagen) release [17]. In spite of selected studies that assessed the effect of protease inhibitors, there is limited number of studies that determined the actual CT-K activation protocols in dentin [17,18]. Thus, the aim of the current study was to evaluate the protease activity in dentin matrices subjected to lactic acid in comparison to polyacrylic acid challenge model at a CT-K optimum pH 5.5. The null hypothesis tested is that there were no differences in the protease activity between lactic acid and polyacrylic acid challenge models.

2. Materials and methods

2.1. Preparation of demineralized dentin samples

Extracted sound human third molars were collected according to the local research ethics protocol (protocol # 33369). The teeth were sterilized by gamma radiation at a dosage of 2.5 MRad [19]. The enamel and superficial dentin were removed by horizontal sectioning at 1 mm below the central fissures using a slow-speed diamond saw under continuous water-cooling (Isomet, Buehler Ltd, Lake Bluff, IL, USA). Dentin disks were prepared by cutting 0.5 ± 1 mm from mid-coronal dentin under water-cooling. The prepared dentin disks were submerged in 10% phosphoric acid (H_3PO_4) (LabChem, Zelienople, PA, USA) for 40 h at room temperature under agitation using the hematology chemistry mixer (Model 346; Fisher Scientific Co., Toronto, ON, Canada) at a speed of 12 rpm to completely demineralize the dentin. Following complete demineralization, dentin disks were thoroughly rinsed in deionized water for 5 min. To confirm complete demineralization of dentin disks, qualitative digital radiograph and quantitative micro CT (eXplore Locus Ultra, General Electric Healthcare, London, ON, Canada) were performed in random samples ($n = 6$). For the latter analysis, five random grey scale values from each image of the pre and post demineralization samples were compared. Following complete demineralization of dentin, samples were prepared for circular dichroism (CD) spectra (JASCO J-810 CD Spectrometer, Easton, MD, USA) to confirm that structural characteristics of the dentin collagen were not altered. Crushed powder obtained using the SPEX Sample Prep Freezer/mill machine (SPEX Sample Prep, Metuchen, NJ, USA) from demineralized dentin disks were analysed. 0.1 mg of dentin powder suspended in 1 ml of phosphate buffered saline (PBS) and an aliquot of the solution was subjected to CD spectra test.

2.2. Dentin matrices low pH challenge

A total of 120 collagen disks were incubated in the following groups according to the acidic challenge applied ($n = 10$), deionized water was used as control (C): (i) 10 mL deionized water, 24 h storage (C); (ii) 10 mL deionized water, 7 days storage (C7); (iii) 10 mL artificial saliva (pH 7.4); 24 h storage (AS); (iv) 10 mL artificial saliva; 7 days storage (AS7); (v) 10 mL lactic acid 0.1 M, pH 5.5; 24 h storage (LA) (Bio Basic Canada Inc., Markham, ON, Canada); (vi) 10 mL lactic acid 0.1 M, pH 5.5; 7 days storage with daily change of media (LA7); (vii) 10 mL polyacrylic acid 1.1 mM, pH 5.5; 24 h storage (PAA) (Sigm-Aldrich, Oakville, ON, Canada); and (viii) 10 mL polyacrylic acid 1.1 mM, pH 5.5; 7 days storage with daily change of media (PAA7).

2.3. Gravimetric measurement of challenged dentin matrices

The dry weight change of dentin matrices was determined before and after acidic challenges ($n = 10$). The initial (W_1) and final dry weights (W_2) of each sample ($n = 15$) were measured using an analytic balance (0.1 mg resolution) (Denver Instrument, Bohemia, NY, USA). The percentage mass change (W_{mc}) was calculated using the following formula: $W_{mc}\ (\%) = (W_2 - W_1)/W_1 \times 100$.

2.4. Measurement of ultimate tensile strength (UTS) of challenged dentin matrices

Demineralized dentin beams ($0.5\ mm \times 1\ mm \times 6\ mm$) were subjected to ultimate tensile strength ($n = 10$). The specimens were glued to a custom jig using cyanoacrylate adhesive system and mounted on a microtensile testing machine (Micro Tensile Tester, Bisco, Inc, Schaumberg, IL, USA). Samples were subjected to tensile force at a crosshead speed of 1 mm/min [20]. The microtensile strength (MPa) was calculated by dividing the force (N) by the surface area (mm^2).

2.5. Total protein and solubilized telopeptide assays

After the acid challenge step, the dentin matrices were washed with deionized water for 5 min and buffered in calcium and

zinc containing media at 37 °C for 7 days [17]. Aliquots of the buffer media were used for the total protein and solubilized telopeptide analysis ($n = 3$). Total protein concentrations were measured using the Pierce Bicinchoninic Acid assay (Thermo Scientific 23225, Waltham, MA, USA) at 562 nm. The matrix degradation caused by MMPs was determined by measuring the quantity of solubilized type I collagen C-terminal cross-linked telopeptides (ICTP) in the incubation medium using the ICTP ELISA assay (MyBioSource, Inc., San Diego, CA, USA) ($N = 3$; $n = 3$) [18]. The matrix degradation by cathepsins was determined by measuring the quantity of C-terminal peptide (CTX) in the incubation medium using the Serum CrossLaps ELISA assay (MyBioSource, Inc., San Diego, CA, USA) ($N = 3$; $n = 3$) [18]. The ratios of ICTP and CTX concentration in relation to total protein concentration ($ICTP_{tp}$ and CTX_{tp}) were calculated [21].

2.6. Scanning electron microscopic (SEM) analysis of challenged dentin matrices

A random sample from each group following acid challenge was examined under SEM. Briefly, dentin disks were completely dried using serial dilution of ethanol (critical point drying). Dentin disks were then gold coated, mounted on stubs with carbon adhesive tape and colloidal silver paint, and were examined under SEM (JEOL-SEM 6400, Peabody, MA, USA) with high vacuum mode under 5000× magnification.

2.7. Statistical analysis

Statistical analysis was performed with SPSS version 24 (2016 IBM Software, USA). For all tests, the assumptions of equality of variances and normal distribution of errors were checked and satisfied. One-way ANOVA and Tukey *post hoc* tests were used within each test and time point to compare statistical differences among groups. For each group and test, Student *t*-test was used to compare 24 h and 7 days mean values. Level of significance was set at 0.05.

3. Results

3.1. Demineralized dentin samples characterization

The digital radiographs and micro CT test confirmed complete demineralization of the selected samples (Fig. 1). Table 1 shows the average grey scale values as measured from the micro CT images. The average grey scale (AGS) values before demineralization was comparable to compact bone indicating mineralization of dentin. Following demineralization, the average grey scale was comparable to soft tissues confirming the complete demineralization of the samples. CD spectra analysis showed that dentin collagen structure was unchanged after demineralization and was comparable to the structural characteristics of folded type I collagen (Fig. 2).

3.2. Gravimetric and ultimate tensile strength

Table 2 shows the gravimetric and UTS results. Statistical analysis of gravimetric measurements showed that at 24 h, the group C specimens had the lowest weight loss ($p < 0.04$), with no significant difference among groups AS, LA and PAA ($p > 0.05$). No statistically significant difference was observed among all groups after 7 days ($p = 0.10$). Of all groups, the LA specimens were the only ones to show significantly higher weight loss from 24 h to 7-day time point ($p = 0.02$). The UTS at 24 h and 7 days for C and AS groups had significantly higher mean values compared to LA and PAA groups, which did not differ from each other ($p = 0.82$ and $p = 0.10$, baseline and 7-day respectively). Only specimens from LA group showed significantly lower UTS at 7-day time point ($p = 0.047$).

3.3. Solubilized telopeptides

Tables 3 and 4 show data normalized to total protein ICTP ($ICTP_{tp}$) and CTX (CTX_{tp}) results. In the $ICTP_{tp}$ analysis, the 24 h results showed that PAA had significantly higher mean values than groups C and AS. The LA group did not differ significantly from C, AS or PAA ($p > 0.05$). At 7 days, no statistically significant differences were observed among groups ($p > 0.05$). No significant individual changes in $ICTP_{tp}$ values were also observed at 24 h and 7-day time points ($p > 0.05$). Table 4 shows the data obtained from the CTX_{tp} analysis. The CTX_{tp} results showed that the LA group at 24 h had significantly higher mean values than groups C and AS. The PAA group did not significantly differ from C, AS or LA groups ($p = 0.01$). At 7 days, no statistically significant differences were observed among the tested groups ($p = 0.09$). While no significant changes were observed for AS and PAA from 24 h to 7-day time point, the C and LA groups showed significantly lower CTX_{tp} values from 24 h to 7-day time point ($p < 0.01$).

4. Discussion

According to the results of this study, the initially proposed null hypothesis was accepted. Earlier studies have used 10% phosphoric acid to demineralize dentin and subsequently evaluate the type I collagen or dentin organic matrix properties [8,21,22]. The Micro CT and radiographic image analyzes confirmed the complete demineralization of dentin disks as AGS values after demineralization ranged from −46 to −140 [23]. Moreover, in order to confirm the effect of demineralizing dentin with 10% H_3PO_4 on collagen structure, selected dentin samples were subjected to CD spectra analysis, which indicated that the dentin collagen triple helical structure was not affected, while it was comparable to the structure of a properly folded type I collagen [24]. SEM images showed complete demineralization of dentin, open dentinal tubules and exposed dentin collagen. The SEM images of control and acidic groups of demineralized dentin confirmed demineralization of dentin and lack of peritubular dentin. The application of LA and PAA resulted in loss of the superficial layer of exposed demineralized dentin suggesting that exposure to acidic challenges result in continuous loss of the superficial layer reducing the thickness of dentin samples (Fig. 3).

The mechanical property of dentin disks was determined using UTS measurements [21,25]. The UTS results of the control group is consistent with the previous studies in which UTS ranged from 6 to 18 MPa [21,25,26]. Our results showed

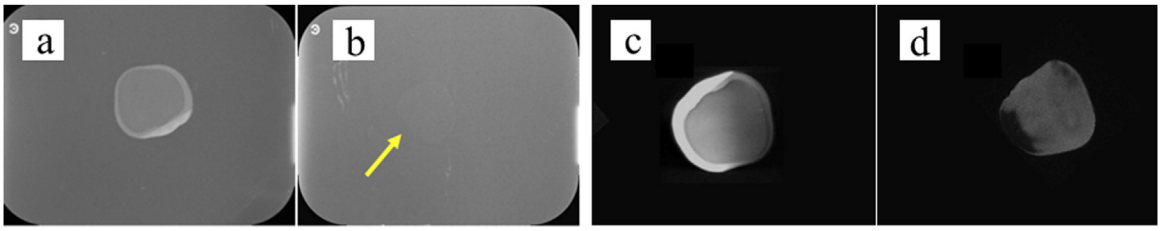

Fig. 1 – Digital radiographs (a, b) and micro CT images (c, d) of dentin discs showing no radiopacity after demineralization (b, d).

Table 1 – Average grey scale (AGS) values and standard deviations (s.d.) measured from micro CT images before (b) and after (a) the demineralization protocol.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	b	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*	*b*	*a*
AGS	2385	−110	2204	−71	2153	−46	2233	−50	1948	−140	2184	−64
±s.d.	±204	±52	±120	±18	±93	±24	±85	±26	±29	±33	±181	±17

Reference Values: Bone: 2700–3000; Water: 0; Air: -100 (Brooks & Chiro 1976); s.d.: standard deviation.

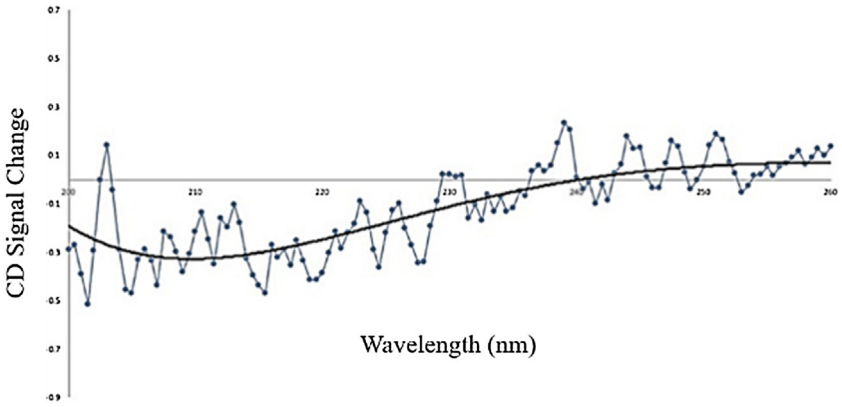

Fig. 2 – Representative CD spectra analysis of dentin collagen structure after demineralization in 10% phosphoric acid. Collagen had a weak positive maximum absorption band at 230 nm, suggesting a typical triple helical conformation of type I collagen.

Table 2 – Mean gravimetric change and mean (s.d.) ultimate tensile strength (MPa) results.

Storage media	Weight loss (%)		Microtensile strength	
	24 h	7 days	24 h	7 days
Deionized water (C)	9.1 (2.4)Aa	13.2 (1.9)Ac	17.6 (3.7)Aa	17.1 (2.4)Aa
Artificial saliva (AS)	12.7 (3.4)Bb	16.9 (2.3)Bc	16.6 (3.3)Ba	14.8 (3.5)Ba
Lactic acid (LA)	15.5 (0.8)Cb	16.8 (1.7)Dc	10.8 (4.2)Cb	4.05 (1.2)Db
Polyacrylic acid (PAA)	14.9 (3.5)Eb	15.91 (6.2)Ec	9.80 (2.3)Eb	6.90 (2.0)Eb

s.d.: standard deviation; Within each test and time-point, same lower case letters indicate no significant difference among groups; same upper case letters indicate no significant difference between same group/test at different time points.

that the acidic challenged groups (LA, PAA) had significantly lower UTS values compared to C and AS. The pH of LA and PAA used were 5.5, which has been shown to be the optimum pH for cathepsin K activity [6]. The results of UTS did not differ significantly at 24 h or 7 days for the same group except for LA, which decreased significantly after 7 days incubation. This can be explained by the lower molecular weight of LA (MW = 96.01), which would facilitate enhanced diffusion through collagen, resulting in pronounced activation of CT-K by LA when compared to PAA (MW = 100,000).

The percentage of weight loss was significantly lower in the control group compared to other groups at 24 h only (9.1%). The average weight change in the control group (demineralized dentin without further acidic challenge) reported in previous studies ranged from 6 to 28% [17,27,28]. Ozcan et al. [17] reported less weight change compared to our results.

Table 3 – Average (s.d.) total protein (TP) concentration (mg/mL) and normalized to total protein ICTP ($ICTP_{tp}$; ng/mg) results.

Storage media	24 h		7 days	
	TP	$ICTP_{tp}$	TP	$ICTP_{tp}$
Deionized water (C)	0.50 (0.20)	3.35 (1.20)Aa	0.39 (1.90)	2.37 (1.20)Ac
Artificial saliva (AS)	0.32 (0.08)	3.64 (0.70)Ba	0.62 (0.30)	2.85 (1.00)Bc
Lactic acid (LA)	0.15 (0.01)	6.46 (1.41)Cab	0.19 (0.05)	4.85 (0.40)Cc
Polyacrylic acid (PAA)	0.10 (0.01)	7.46 (0.76)Db	0.28 (0.01)	3.84 (0.57)Dc

s.d.: standard deviation; Within each time point, same lower case letters indicate no significant difference among groups; same upper case letters indicate no significant difference between same group at different time points.

Table 4 – Average (s.d.) total protein concentration (mg/mL) and normalized to total protein CTX (CTX_{tp}; ng/mg) results.

Storage media	24 h		7 days	
	TP	CTX_{tp}	TP	CTX_{tp}
Deionized water (C)	0.25 (0.03)	0.49 (0.10)Aa	0.84 (0.30)	0.13 (0.04)Bc
Artificial saliva (AS)	0.18 (0.02)	0.65 (0.08)Ca	0.32 (0.15)	0.42 (0.21)Cc
Lactic acid (LA)	0.10 (0.03)	1.48 (0.37)Db	0.49 (0.08)	0.28 (0.04)Ec
Polyacrylic acid (PAA)	0.11 (0.03)	1.27 (0.38)Fab	0.23 (0.09)	0.66 (0.30)Fc

s.d.: standard deviation; Within each time point, same lower case letters indicate no significant difference among groups; same upper case letters indicate no significant difference between same group at different time points.

In that study the acidic challenge was applied for 20 s after which samples were placed in buffer to neutralize the effect of the acid [17]. Seseogullari-Dirihan et al. [27] reported similar weight change of the control group (18%) after 7 days. Tezvergil-Mutluay et al [28] reported higher weight loss when dry weight loss was measured after 30 days compared to our results. It has been reported that the maximum protease activity of CT-K usually happens within the first 24 h [29]. Interestingly, AS group showed significantly more weight loss compared to C group and was similar to other acidic groups. MMPs are zinc-dependent enzymes that can be activated by the disturbance of zinc–cystine interaction (known as cysteine switch theory) [30]. The ions in the artificial saliva can disrupt this interaction resulting in the release of active MMPs. Our results showed that there was significantly more weight loss in LA group after 7 days as compared to 24 h. This is attributed to the continuous release of degraded collagen sub-products with time. When the results from the weight change and UTS experiments were compared a trend was observed in which samples with higher degree of weight loss had the reduction in UTS values.

Enzymatic degradation of dentin collagen by MMPs and CT-K as measured by $ICTP_{tp}$ and CTX_{tp} telopeptide release in the solution were less than the quantities noted in previous studies [17,18,31]. This is likely because of the sub-product release into the buffer solution after incubation of the demineralized dentin disks in the challenge media, according to the caries simulation models. The detection of the initial release of ICTP and CTX was not possible because of the acidic pH. It is speculated that in acidic pH, the conjugated bond between the biotinylated antibody and the enzyme might be broken, as is the case with the acidic stop solution used in the assay. The pH values are noted to influence the protonation of ionizable amino acids in protein–protein interactions [32]. Thus the binding of antibody to its antigen at low pH would be affected by their stability in acidic pH [33].

The only source of ICTP fragments in the solution is from the degradation of type I collagen by MMPs, whereas the only source of CTX fragments is from the degradation of type I collagen by CT-K [34]. Therefore, findings from this study demonstrated that there was no significant difference in the proteolytic degradation by MMPs and CT-K between LA and PAA. However, our results showed significantly higher release of $ICTP_{tp}$ telopeptides following incubation in PAA as compared to C and AS groups at 24 h only. The release of $ICTP_{tp}$ in LA was not statistically significant from the C and AS groups. After 7 days, the release of $ICTP_{tp}$ in the acidic solutions (LA and PAA) was more than C and AS groups, yet the difference was not significant. Our data showed a non-significant reduction in the release of $ICTP_{tp}$ from all the tested groups after 7 days.

The release of CTX was significantly higher in LA compared to C and AS groups at 24 h. The results from the proteolytic degradation in the current study coincided with the weight change and UTS values, indicating higher rate of proteolytic degradation in the presence of acidic media. The results showed that there was significant reduction in the release of CTX in C and LA after 7 days compared to 24 h. This finding corresponded with a previous study that highlighted maximum release of CTX and ICTP telopeptides within the first 24 h and decreased values with time [29]. In conclusion, the findings from this study confirmed that LA has the ability to activate endogenous CT-K in human dentin as measured by the release of CTX (CT-K specific telopeptide) and CT-K activity, as well as confirmed by the weight change and UTS. This LA based model has the potential application for further investigations to assess the activity and possible inhibitors of CT-K in human dentin.

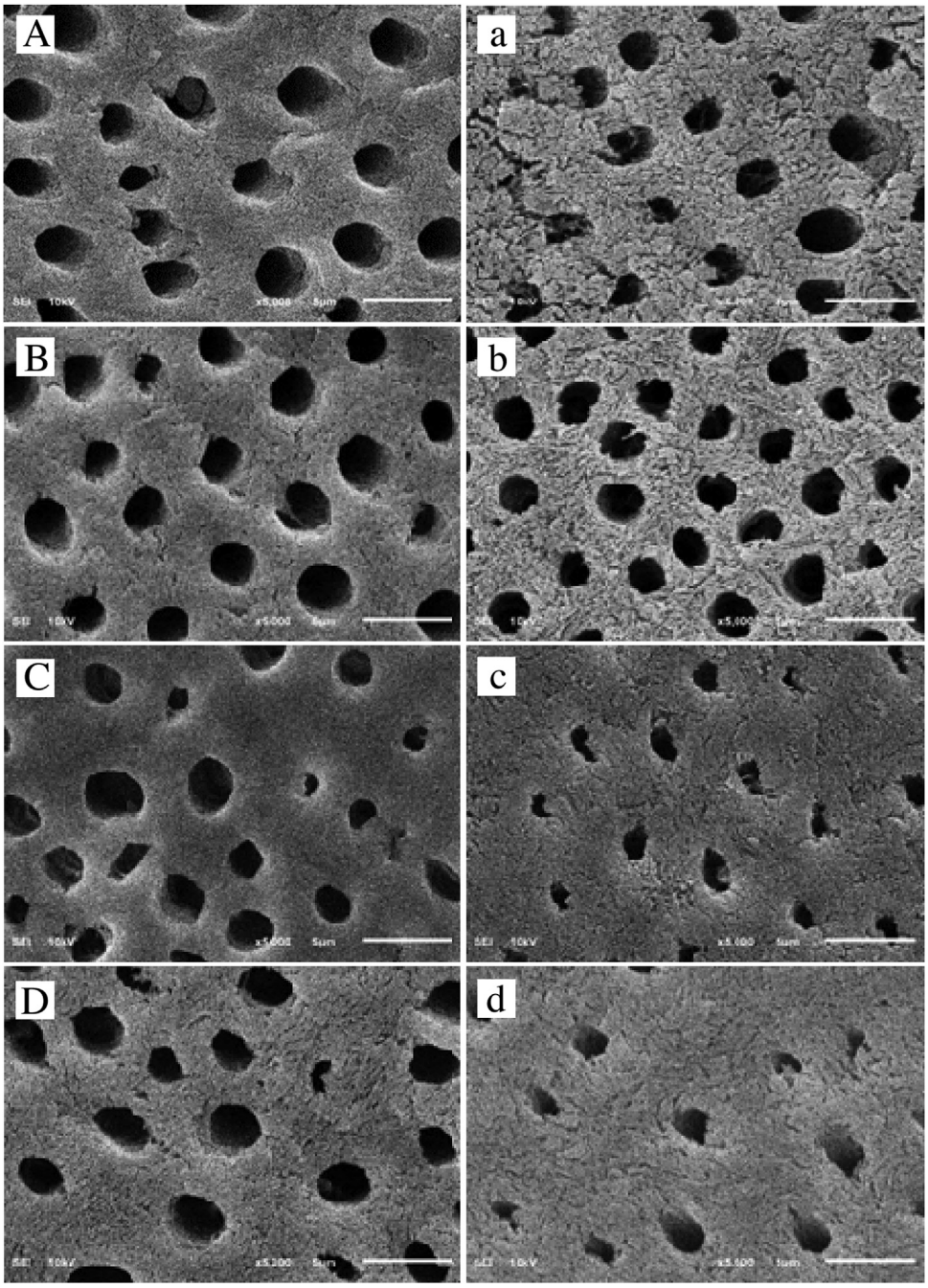

Fig. 3 – Representative SEM micrographs of dentin samples incubated in the different media for 24 h (upper case letters) and 7 days (lower case letters); A,a: Deionized water, B,b: Artificial saliva; C,c: Lactic acid; D,d: Polyacrylic acid.

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