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Clinical Implementation of Comprehending Dentin on a Microscopic Level

Implementación Clínica de la Comprensión de la Dentina a Nivel Microscópico

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SUMMARY: The aim of the present in vitro study is to visualize dentin to get an in-depth knowledge of the nature of dentin that could provide useful information regarding conditioning dentinal substrate when treating dentinal lesions. Forty-nine extracted human third molars were obtained and prepared to produce artificial dentinal lesions through demineralizing with acetic acid for 7 and 14 days, or lactic acid for 7 days. The teeth were divided into groups and treated with either NaOCl, pepsin, trypsin, or phosphoric acid. To obtain information on the morphology of the treated dentinal surfaces, all samples were visualized under high resolution field emission scanning electron microscope. With high magnification reaching x50000 dentin was clearly visualized together with its constitutes. The effect of various demineralization approaches and various treatment protocols were demonstrated clearly. The relationship between the conditioning procedure steps and the subsequent bond strength was discussed. To our best knowledge, there is no previous clear highly magnified scanning electron microscope images for dentin, and dentinal components and constitutes with and without various treatments. The current in vitro study suggests the complexity nature of dentin as a substrate that should be treated carefully especially with technique sensitive procedures such as adhesive restorations.

KEY WORDS: FE-SCM; Dentin; Collagen; Pepsin; Trypsin; NaOCl; Demineralization.

INTRODUCTION

The essential goal of any adhesive restoration is to achieve a durable restoration with adequate mechanical properties together with long-lasting seal to enamel and dentin (Perdigao, 2020). While bonding to enamel by micromechanical interlocking of resin tags within the array of microporosities in acid-etched enamel can be reliably achieved and can effectively seal the restoration margins against leakage, bonding effectively and durably to organic and humid dentin is a challenging task in adhesive dentistry (Perdigao, 2020).

Tooth enamel is a complex mineralized tissue consists of 96 % inorganic and 4 % organic and water content, comprising of long and parallel apatite crystals configured into decussating enamel rods (Pandya & Diekwisch, 2019). On the other hand, dentin is a more complex dental substrate consists of 70 % inorganic phase in which carbonated calcium phosphate micro-crystals are located within an organic matrix that forms 20 % of dentin, the remainder 10 % is water (Lee *et al*., 2022).

Dentin crystallites are well known to be smaller (Nikiforuk, 1985; ten Cate, 2001) and less systematically oriented (LeGeros, 1990) than enamel crystallites, resulting in an increased surface area and rapid dissolving rate under acid attacks (Ostrom, 1980). Organic Dentin material contains 90 % collagen and 10 % non-collagenous compounds (NCC) (Heinrich-Weltzien & Kneist, 2001). Collagen type I is the predominant collagen in dentin (89 %), type I trimer is 11 % and 1 % is of types III, V, VI (Heinrich-Weltzien & Kneist, 2001). Dentin collagen which constitutes 90 % of dentin organic matrix forms a fibrous three-dimensional network which remineralizes to provide the fundamental building blocks of dentin (Balooch *et al*., 2004). Dentin cellular and extra-cellular proteins are synthesized, controlled and secreted by the odontoblasts (Goldberg & Smith, 2004). Those odontoblastic cells located at the periphery of the dental pulp possess odontoblastic processes arise from the cell body of the odontoblasts and penetrates into the mineralized dentine (Arana-Chavez & Massa, 2004). The odontoblastic processes traverse dentin

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contained in dentinal tubules, in which these dentinal tubules decrease in diameter and number in the direction of the amelodentinal junction away from the pulp (Ostrom, 1980; Heinrich-Weltzien & Kneist, 2001). Dentin that lines the inner walls of the tubules is called intra-tubular dentin or peri-tubular dentin, while dentin in between these dentinal tubules is called inter-tubular dentin (Luukko *et al*., 2011).

Carious process is initiated by demineralization of the mineral phase followed by the breakdown of the organic matrix (Abou Neel *et al*., 2016). The organic material in dentin is protected by the hydroxyapatites so that neither the bacterial nor the endogenous proteolytic enzymes have access to the organic matrix without dissolving the apatite crystals first (Dung *et al*., 1994). As mentioned earlier the organic matrix of dentin is composed of 90 % collagen and 10 % non-collagenous compounds (NCCs).

Bonding of restorative material to dentin and its subsequent durability and success is highly affected by several factors such as the tooth substrate to be restored, the restorative material, patient-factors, and operator-factors.

The aim of this *in vitro* study to visualize dentin under Field Emission Scanning Electron Microscope (FE-SEM) to get closer knowledge that could provide us with useful information regarding conditioning dentinal substrate when treating dentinal lesions.

MATERIAL AND METHOD

Sample Preparation: Fourty-nine extracted human third molars were obtained and used within 8 months of extraction. After extraction teeth were immediately stored at room temperature in Ringer solution to which sodium-azide was added to prevent bacterial growth. All teeth were clinically sound and they were carefully observed for caries, abrasions or any mechanical traumas. Teeth were cleaned with a tooth brush aided sometimes with a scalpel to remove the periodontal ligament and inter-crestal bone remnants and rinsed under running tap water.

The roots were cut 1.5-2.0 mm below the cementoenamel junction. The teeth were then embedded individually in transparent cold-curing methylmethacrylate (Technovit 4004, Kulzer GmbH, Wehrheim, Germany). To expose mid-coronal dentin each tooth was sectioned parallel to its long axis into two halves using a slow speed water-cooled diamond saw (Isomet, Beuhler, Illinois, USA), the occlusal part of each half was then removed by a cut perpendicular to the long axis of the tooth and 1.5-3.0 mm away from the pulp. Each half was divided into four slices parallel to the long axis of the tooth and extends from the middle of the tooth to the outer enamel with a thin saw microtome (Leica SP 1600, GmbH, Nußloch, Germany) under tap water.

Except for twenty halves (five teeth), all other dentin exposed surfaces were polished flat with water proof silicon carbide abrasive paper (P500-grit) with a Leco VP 100 (GmbH, Neuss, Germany) device. Subsequently they were polished using wet polishing paper with silicone paste of polycrystalline diamonds of size 9 mm (DAP-7, Struers, Copenhagen, Denmark).

Dentin surfaces together with the surrounding Technovit were covered with two coats of nail varnish to avoid the penetration of the solutions' molecules in any marginal gaps that could exist between the tooth and the acrylate, leaving one window of exposed mid-coronal dentin per slice. Adhesive paper was cut into 1x1.5 mm∑ pieces and attached to the dentin surfaces before applying the nail varnish to standardize the window. Two of the four exposed surfaces were perpendicular to the dentinal tubules and the other two were parallel to the tubules. The samples were then kept in air for about fifteen minutes to allow the nail varnish to dry. After the removal of the adhesive strips each window was etched for 20 s with 37 % phosphoric acid gel (Total etch, Ivoclar Vivadent GmbH, Schaan, FL) to remove the smear layer, except for sixteen slices (two teeth) perpendicular to the dentinal tubules were kept without etching. Successive steps of the preparation of the slices are schematically shown in Figure 1.

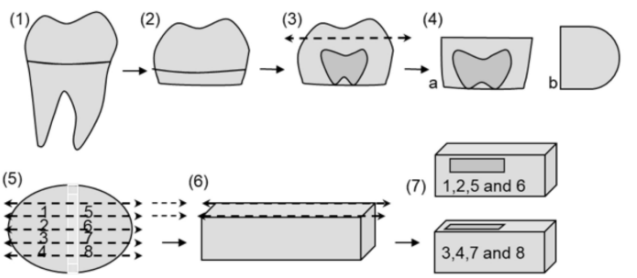


Fig. 1. A schematic drawing illustrating the slice preparation. (1) Caries free molar. (2) The root was abraded short below the cemento-enamel junction and the tooth was cut longitudinally from the middle. (3) The occlusal part of each half was removed to expose deep dentin. (4) a. longitudinal section of one half of the crown after the occlusal part was removed, b. cross section. (5) Each half was sliced into four slices (1, 2, 3, 4) and (5, 6, 7, 8). (6) Each slice of dentin was prepared separately in which the close related slices had comparable windows. (7) One window per slice was prepared parallel to the dentinal tubules (slices 1, 2, 5 and 6) and slices 3, 4, 7 and 8 were prepared with the windows perpendicular to the dentinal tubules.

Lesion formation: 256 slices (32 teeth) were immersed in 80 ml of 0.5 M acetic acid (pH 5) for 7 days (n= 216), and 14 days (n= 40). The demineralization process was always with agitation of the solution with 150 rpm at 37°C (Forma Orbital Shaker, Thermo Electron Corporation, Ohio, USA) with 20 ml per slice. The demineralizing solutions were refreshed each three days to avoid changes of the solution's pH of more than half a pH unit.

64 slices (8 teeth) were immersed in 80 ml of 1.0 M lactic acid (pH 4) for 7 days with agitation of the solution as in the acetic acid demineralization. The purpose of this particular lactic acid demineralization protocol is to expose singular naked collagen fibers without extrafibrillar mineralization.

Various treatments: 32 slices (4 teeth) were treated with aqueous Sodium Hypochlorite (NaOCl) after 7 days acetic acid demineralization: Each slice was incubated in 1.5 ml of 2.5 % of sodium hypochlorite for 5 min at 37 °C with agitation 150 rpm. The slices were then washed with distilled water at 4 °C.

32 slices (4 teeth) were treated with 1.5 ml of pepsin (pepsin from hog stomach, 77152, Fluka, Biochemika, Sigma-Aldrich, Steinheim, Germany) (1 mg/1 ml 0.01 M HCl pH 2) for 5 min, at room temperature, after 7 days lactic acid demineralization. Only half of the slices (n= 16) were then rubbed with a micro brush. The slices were then washed with 0.2 M HCl tris buffer pH 8.6 at 4 °C to stop the reaction and then washed with distilled water at 4 °C.

32 slices (4 teeth) were treated with 1.5 mg trypsin (trypsin from hog pancreas, 93614, Fluka, Biochemika, Sigma-Aldrich, Steinheim, Germany) in 1.5 ml of 50 mM HEPES buffer (50 mM HEPES, 5 mM CaCl2.H2O, 0.15 M KCl, 5 mM sodium azide), pH 7.4, at 37 °C, with 150 rpm, for 10 min, at room temperature, after 14 days acetic acid demineralization. The slices were then washed with distilled water at 4 °C.

32 horizontal slices (4 teeth) were not demineralized and they were etched with 37 % phosphoric acid gel (Total etch, Ivoclar Vivadent GmbH, Schaan, FL) for 20 seconds. Four slices were then rubbed after etching with micro brush and the other four were kept without brushing to serve as control.

Field Emission-Scanning Electron Microscope (FE-SEM): To obtain information on the morphology of the demineralized dentin surfaces, a high-resolution FE-SEM was used. Samples were fixed in 0.25 M Glutaraldehyde in 0.1 M Cacodylatebuffer pH 7.4 for 1 h, washed with 0.1 M Cacodylateuffer pH 7.4, then immersed in 50 % alcohol for 20 min, subsequently in 70 %, 80 % and 90 % alcohol, each for 20 min, and they were kept finally overnight in 96 % alcohol. According to Perdigao *et al*. (1995) samples were put in Hexamethyldisilazane for 10 min and air dried at room temperature. Each sample was then fixed with carbon paste on the SEM sample holder. Gold sputtering was done for 1 min, with 1.0 kV, 0.3 mbar and 40 mA (Edwards Sputter Coater S15OB, Sussex, UK) and the pictures were then made with a Leo FE-SEM (Leo DSM 982, Carl-Zeiss NTS GmbH, Oberkochen, Germany).

Throughout the whole experimental procedure care was taken to avoid dentin desiccation particularly after the lesion was formed to avoid collapsing of the demineralized collagen fibrils. Exposing the demineralized fibrils to air drying before critical point drying would lead to collapsed collagen fibrils and proteinaceous precipitates which would mask the accurate morphological appearance of the surface.

Two teeth (16 slices) were intentionally kept dry after lesion formation to examine it under FE-SEM to visualize collapsed dentin collagen fibers.

RESULTS

Fractured dentin surface: As shown in Figures 2a and 2b the fractured dentinal surfaces which are perpendicular to dentinal tubules and Figures 2c and 2d in which the fractured dentinal surfaces are parallel to dentinal tubules, all figures demonstrate typical sound dentin structure composed of mineralized inter-tubular dentin in which hydroxyapatites completely cover the collagen network fibers. Dentinal tubules are patent with peri-tubular dentin lining the inner walls of the tubules scattered within dentin.

Demineralized dentin surface: One-week acetic acid demineralized dentinal surfaces (horizontal slices) which are perpendicular to dentinal tubules as shown in Figures 3a to 3d demonstrate partial demineralized dentin. Dentinal tubules are patent and lack peritubular dentin which was dissolved and demineralized. Hydroxyapatites scatters covering the collagen network are clearly visible. Intact collagen fibers are shown clearly in some areas which were totally deprived of inter-tubular mineralization, in which extrafibrillar and intrafibrillar mineralization protected collagen fibers structural integrity from denaturation and destruction (Fig. 4). Surface precipitates which are thought to be remnants of dissolved minerals with NCPs (non-collagenous proteins) are scattered on the dentinal surfaces. Although no attempts were made to qualify these precipitates

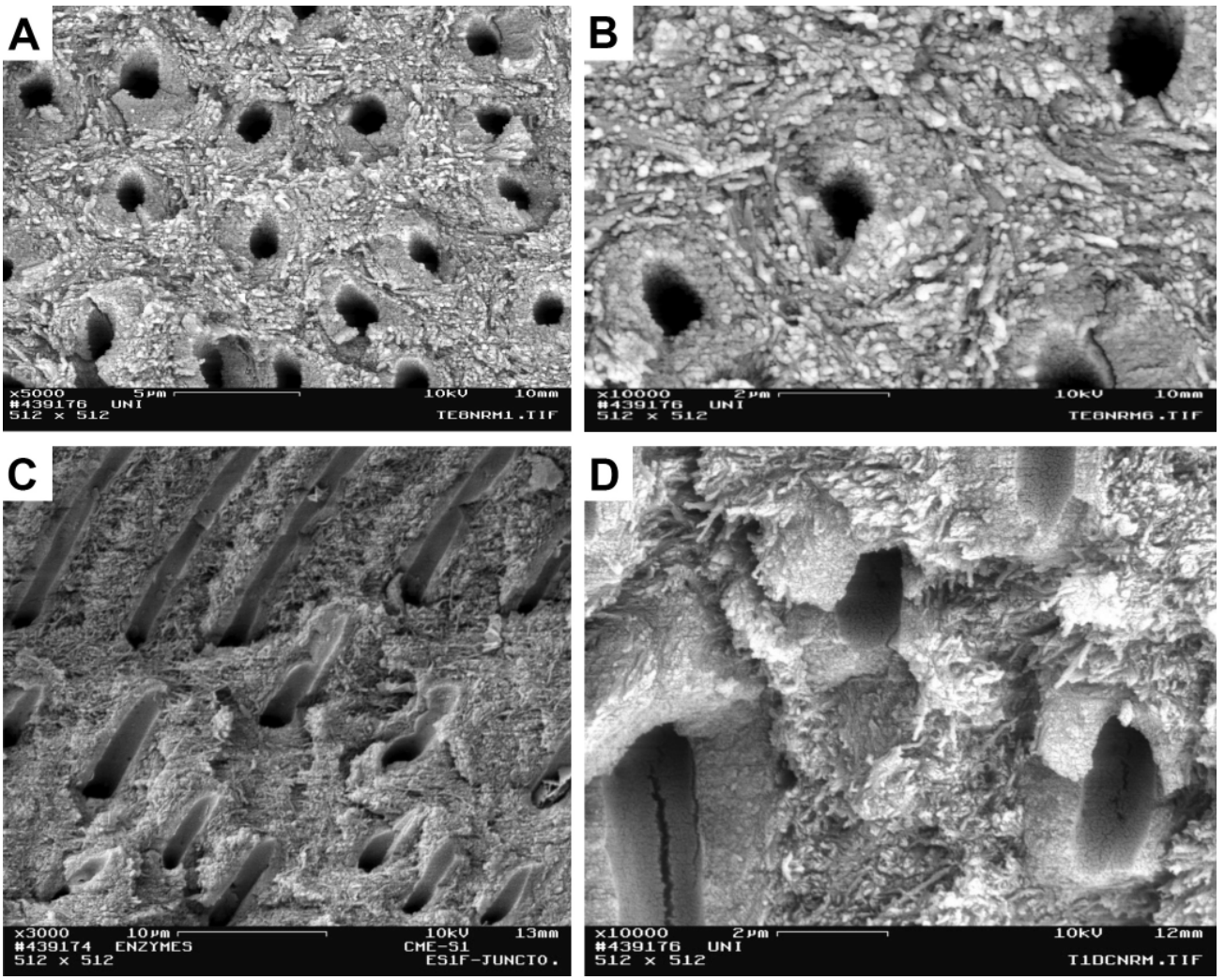


Fig. 2. (A) The horizontal fractured surface of sound dentin (x5000). (B) The horizontal fractured surface of sound dentin (x10000). (C) The vertical fractured surface of sound dentin (x3000). (D) The vertical fractured surface of sound dentin (x10000).

in the present study, Figures 5a to 5d demonstrate the similarity of these precipitates in comparison to enamel hydroxyapatite at a higher magnification.

There was no difference in the morphological appearance of the lesions which were formed perpendicular to the dentinal tubules and those which were parallel to the dentinal tubules. However, the intermolecular cross-links banding with periodicity of 62 nm were clearly visible in the slices where windows were made parallel to the dentinal tubules because the exposed deep inter-tubular collagen between the cut tubules lacked the heavy precipitates that covered the superficial inter-tubular collagen (Fig. 6). Naked inter-tubular collagen fibers at the edge of the cut deprived of surface precipitates were also clearly visible (Figs. 7a and 7b).

Odontoblastic processes were clearly visible projecting from dentinal tubules and traversing them in most of the slices as demonstrated in Figures 8a and 8b.

Contaminated demineralized dentin surface: As shown in Figures 9a to 9d sound dentinal surfaces could be easily contaminated during preparation procedures with various cocci bacteria. No attempts were made to classify those bacteria.

Components of partially demineralized surface: Non unform demineralization was amazingly demonstrated on the surface of some samples in which all components of dentin substrate were clearly visible in a magnificent sharp clear unique image (Figs. 10a to 10c). Areas of hydroxyapatite crystals completely covering collagen fibers

network adjacent to areas where the collagen network is uncovered and lack inter-tubular crystallized minerals. Intact single collagen fibers which are enclosed with extrafibrillar minerals with other intact collagen fibers that lack extrafibrillar minerals but contain intra-fibrillar crystals. Denaturated collapsed collagen fibers were torn outand odontoblastic processes projecting from dentinal tubules.

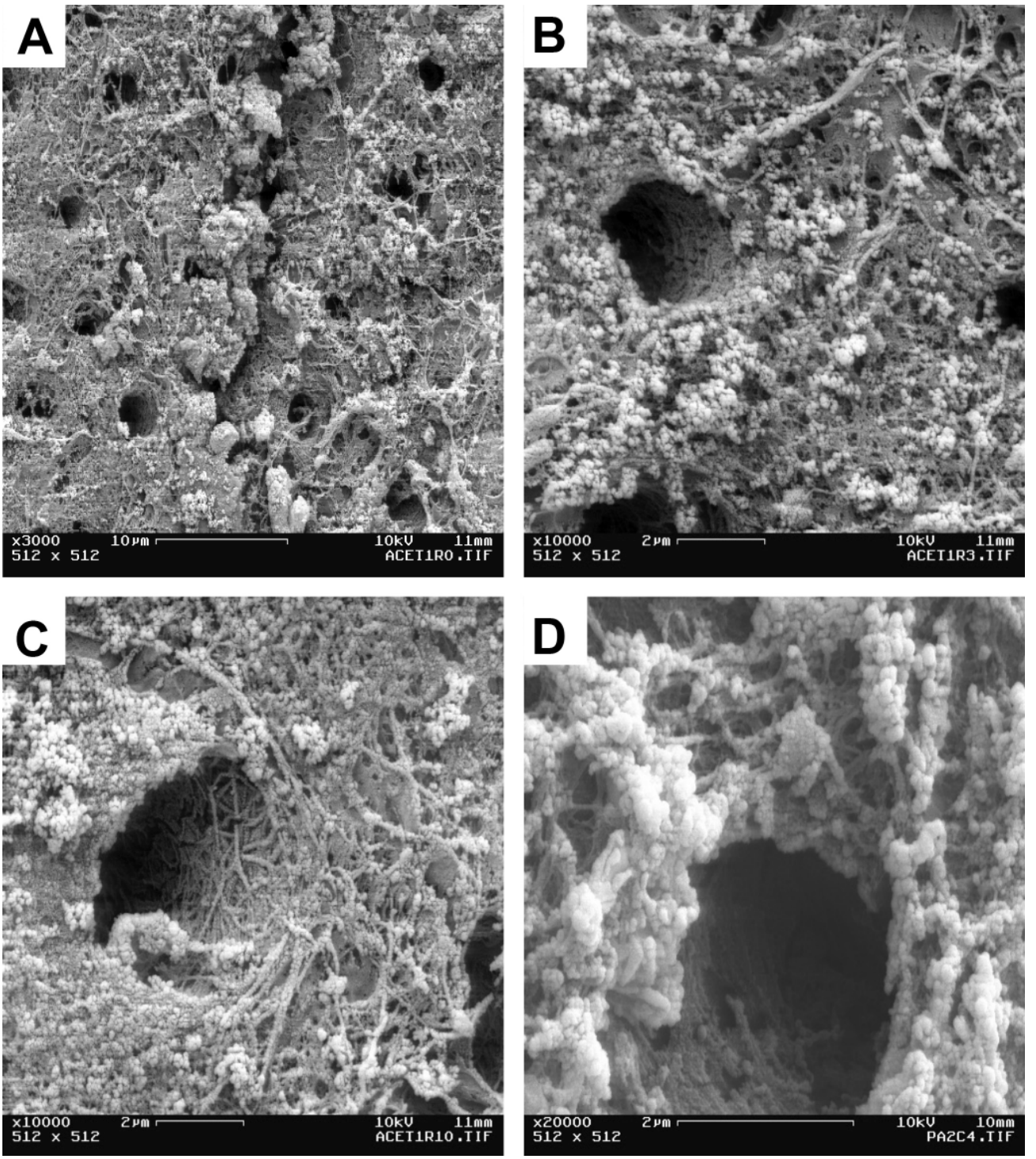


Fig 3. (A) The horizontal partially demineralized dentinal surface (x3000). (B,C) The horizontal partially demineralized dentin (x10000). (D) (x20000).

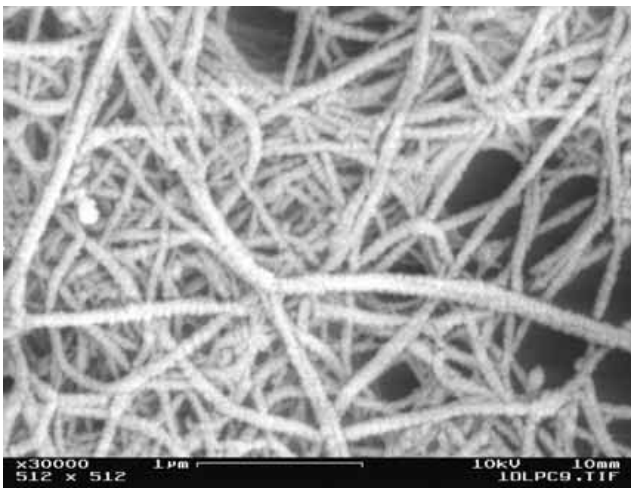


Fig. 4. Intact collagen fibers deprived of inter-tubular mineralization, in which extrafibrillar and intrafibrillar mineralization protected collagen fibers structural integrity from denaturation.

Collagen fibers exposure after lactic acid demineralization: In an attempt to completely demineralize dentin and expose collagen fibers, lesions were produced with lactic acid for two weeks. Completely demineralized peritubular and inter-tubular dentin was clearly demonstrated as naked collagen fibers were shown deprived of any extra and intrafibrillar apatite crystals (Figs. 11a to 11c).

Collapsed collagen fibers: On the other hand, collapsed demineralized collagen fibers were shown in Figure 12 as unidentified structure.

Sodium hypochlorite (NaOCl) treated surfaces: Slices which were treated with 2.5 % of NaOCl for 5 min showed different surface properties in which all surface precipitates and demineralized collagen fibers were digested. Partially demineralized dentin fibers were completely covered with inter-tubular dentin minerals and just the peritubular dentin was dissolved incompletely (Figs. 13a and 13b).

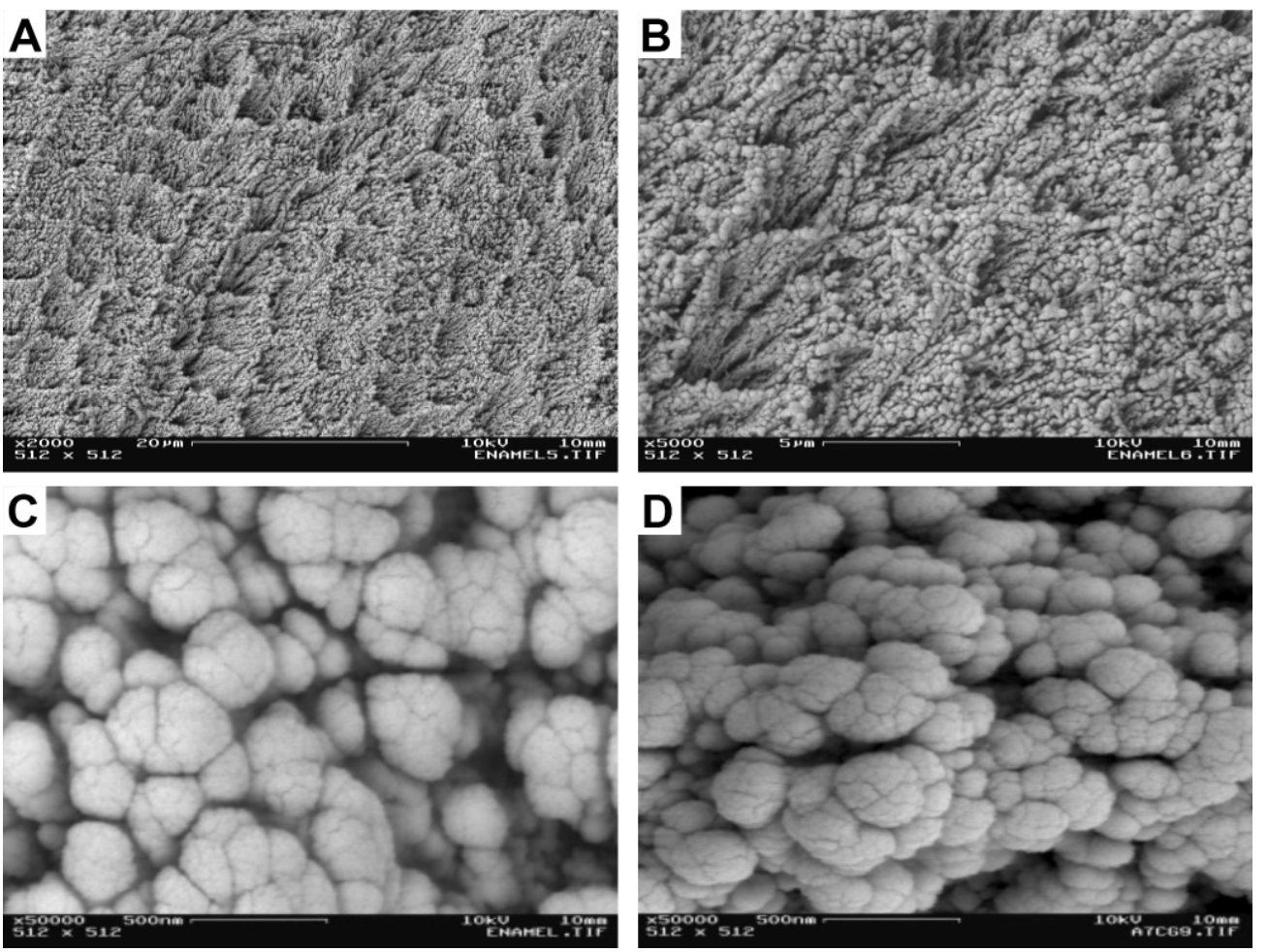
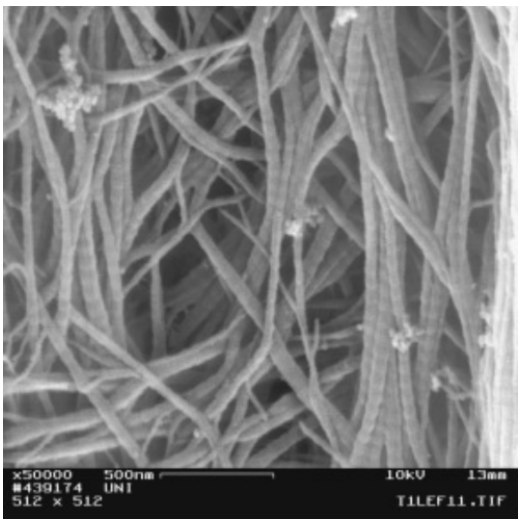


Fig. 5. (A,B) Demineralized enamel after 1 week of acetic acid demineralization (x2000) (x5000). (C) Demineralized enamel hydroxyapatite (x50000). (D) Precipitates at dentin surface after 1 week of acetic acid demineralization (x50000).



Pepsin treated demineralized dentin surface with and without brushing: After demineralizing dentin surfaces for 7 days with lactic acid, pepsin enzyme in its buffer was added for 5 min. As it is shown in Figures 14a to 14d surface precipitates completely covered the demineralized surface. No attempts were made to qualify these precipitates. Half of the slices (n= 16) were then rubbed with a micro brush for several seconds after the pepsin treatment and as Figures 15a to 15d showed the surface precipitates were completely digested and removed leaving behind partially demineralized dentin surface. Dentinal tubules were patent and inter-tubular dentin was semi-permeable where collagen fibres were covered with mineral hydroxyapatites.

Fig. 6. The banding pattern was clearly visible at the collagen fibers when the surface layer was removed (x50000).

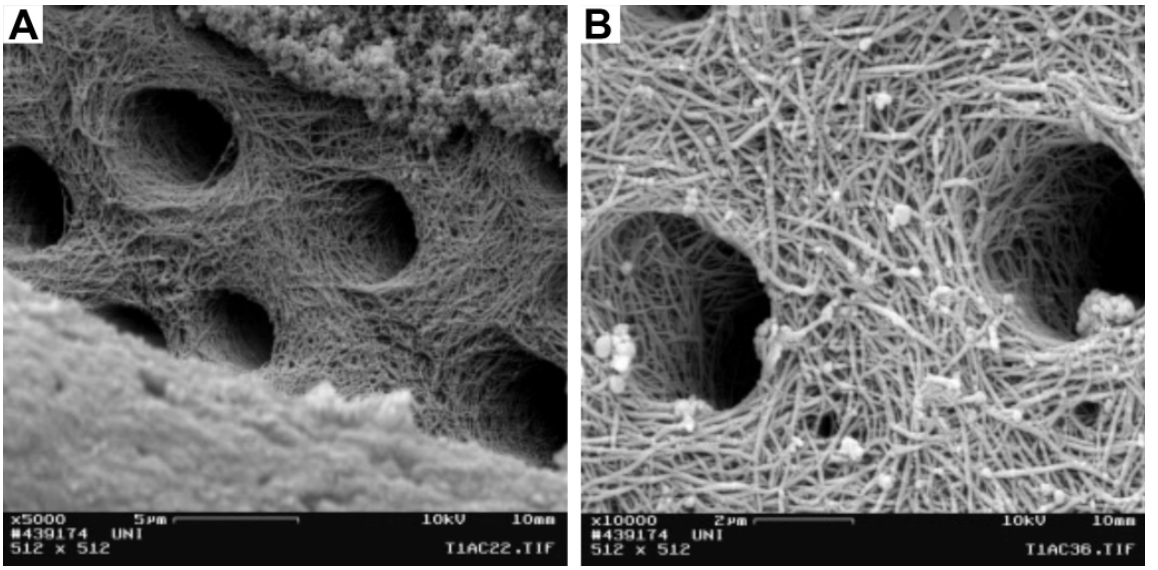


Fig. 7. (A) At the edge of the lesion and under the surface precipitate the demineralized collagen fibers were clearly visible (x5000). (B) A higher magnification of the demineralized peri- and inter-tubular dentin (x1000).

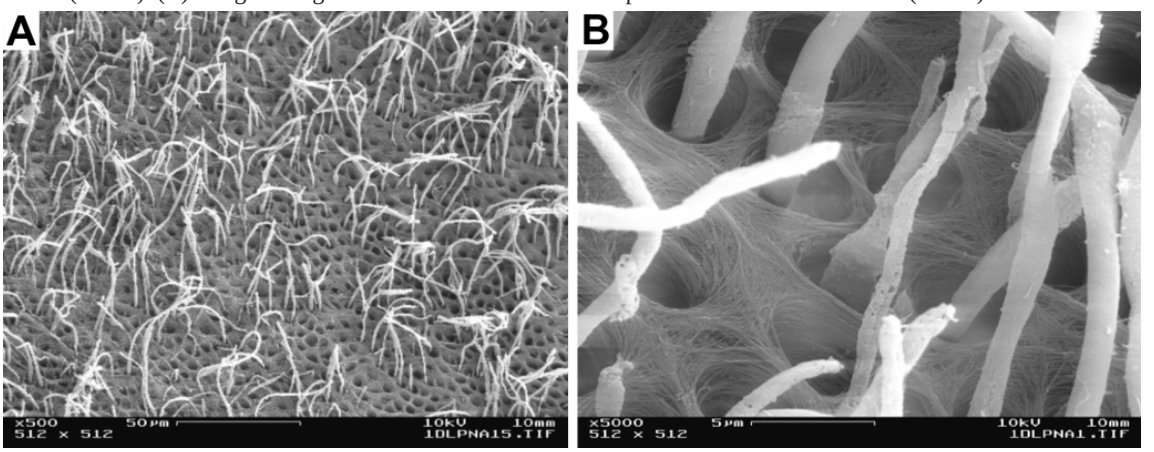


Fig. 8. (A) Odontoblastic processes emerging from demineralized dentinal tubules (x500). (B) Demineralized peritubular and inter-tubular dentin exposing collagen fiber network with odontoblastic processes clearly visible emerging from patent dentinal tubules (x5000).

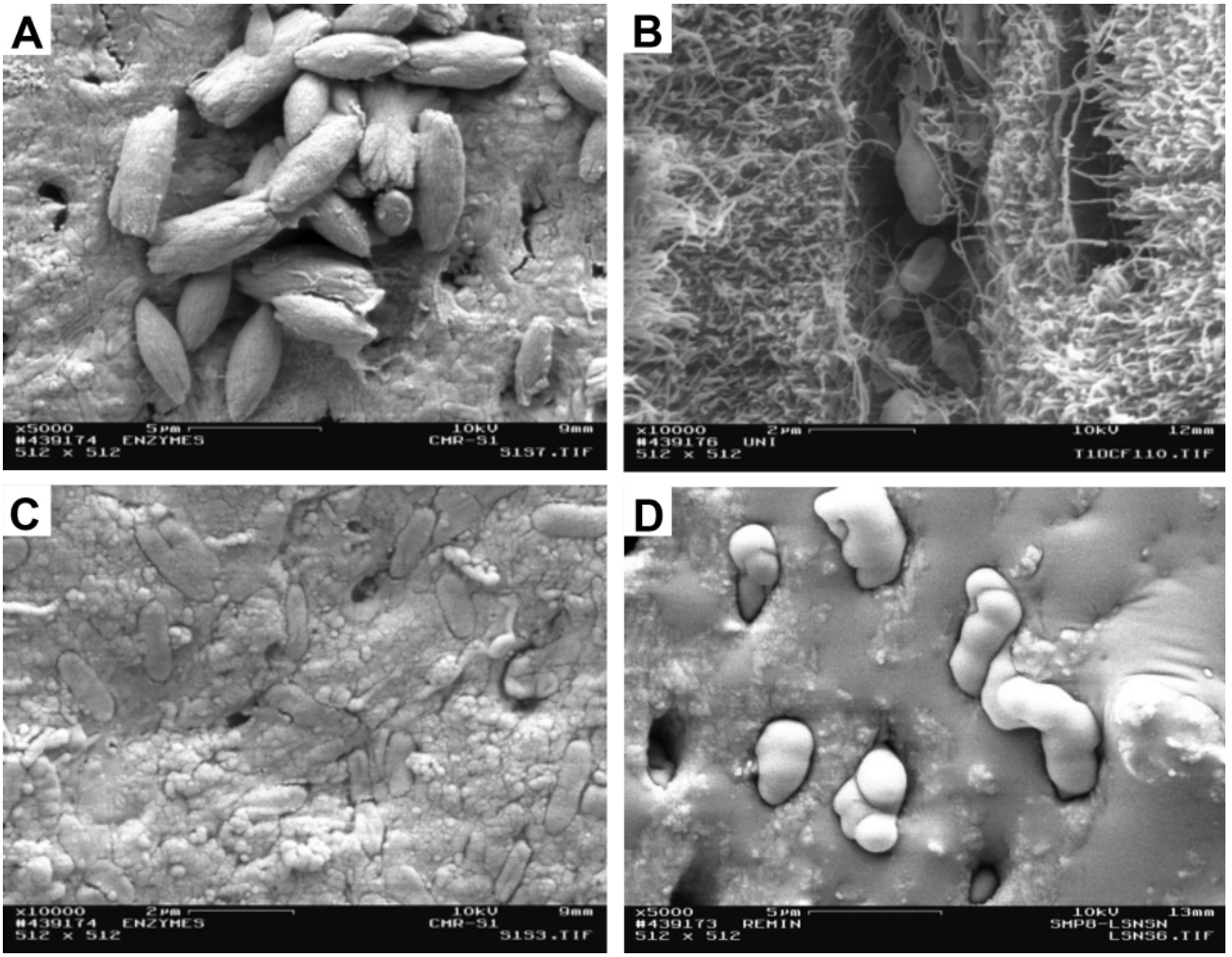


Fig. 9. (A) Bacterial contamination throughout the experimental procedures (x5000). (B) Another specimen contamination (x10000). (C) Bacteria embedded in inter-tubular dentin (x20000). (d) Bacterial contamination during remineralization experiment (x5000).

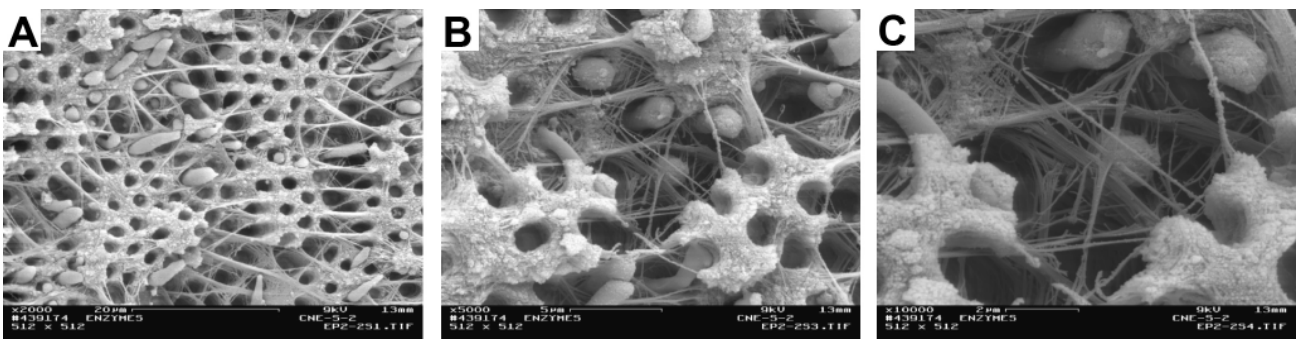


Fig. 10. (A) Partially demineralized dentin surface with all components of dentin substrate visible (x2000). (B) (x5000). (C) (x10000).

Trypsin treatment after 14 days demineralization: After demineralizing dentin surfaces for 14 days with acetic acid, trypsin enzyme in its buffer was added for 10 min. As it is shown in Figures 16a to 16d patent dentinal tubules with dissolved peritubular dentin and projecting odontoblastic processes were demonstrated clearly. Partially demineralized inter-tubular dentin with minerals covering most of the collagen fiber network were also clearly visible, with some collagen fibers either deprived of extrafibrillar mineralization or covered in a singular pattern with extrafibrillar mineralization.

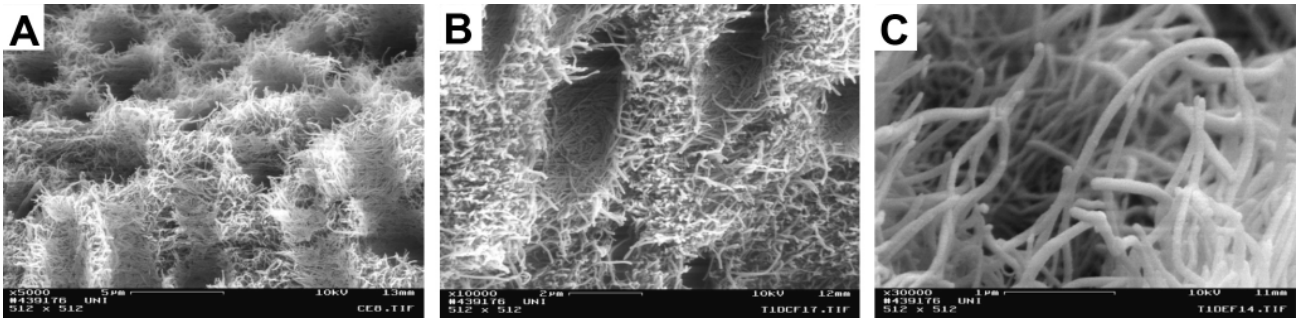


Fig. 11. (A) Cross sectional completely demineralized peritubular and inter-tubular dentin, naked collagen fibers deprived of any extra and intrafibrillar apatite crystals (x5000). (B) Longitudinal sectional completely demineralized peritubular and inter-tubular dentin (x10000). (C) Naked collagen fibers deprived of any extra and intrafibrillar apatite crystals (x30000).

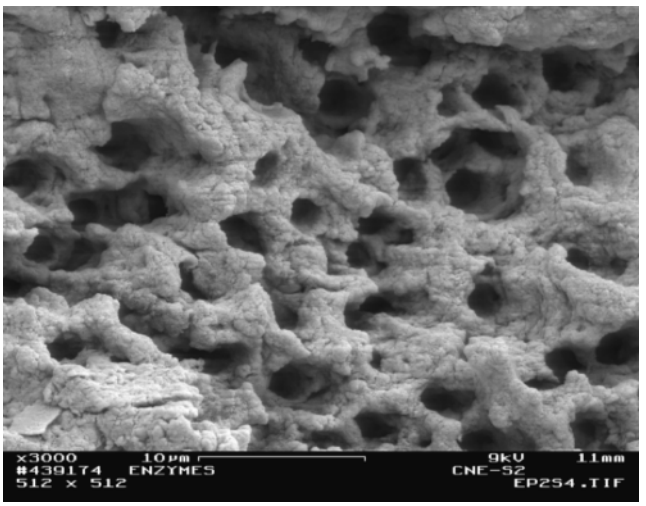


Fig. 12. Collapsed dentin collagen fibers in partially demineralized dentin surface which was kept dry after lesion formation (x3000).

Non-etched and etched surfaces with and without brushing: Slices which were not etched, the smear layer was covering the dentin surface together with the dentinal tubules and are represented by Figures 17a and17b. Etched surfaces which were treated with 37 % phosphoric acid gel for 20 sec without brushing the surface are represented by Figure 17c in which some of the dentinal tubules are patent and some are covered with smearing, with surface smearing partially presented covering inter-tubular dentin. Etched surfaces which were agitated during the 20 sec using micro brush are represented by Figure 17d in which dentinal tubules are patent and inter-tubular dentin is exposed and uncovered with smear layer.

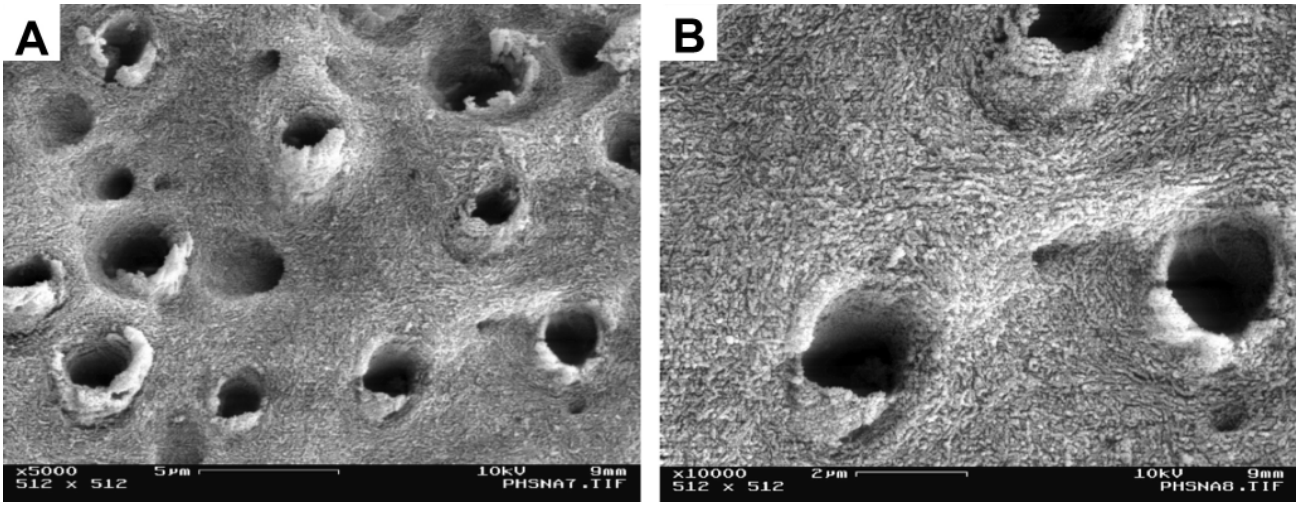


Fig. 13. (A) Partially demineralized dentin covered with inter-tubular dentin minerals and just the peritubular dentin was dissolved when treated with 2.5 % NaOCl treated surfaces for 5 min (x5000). (B) (x10000).

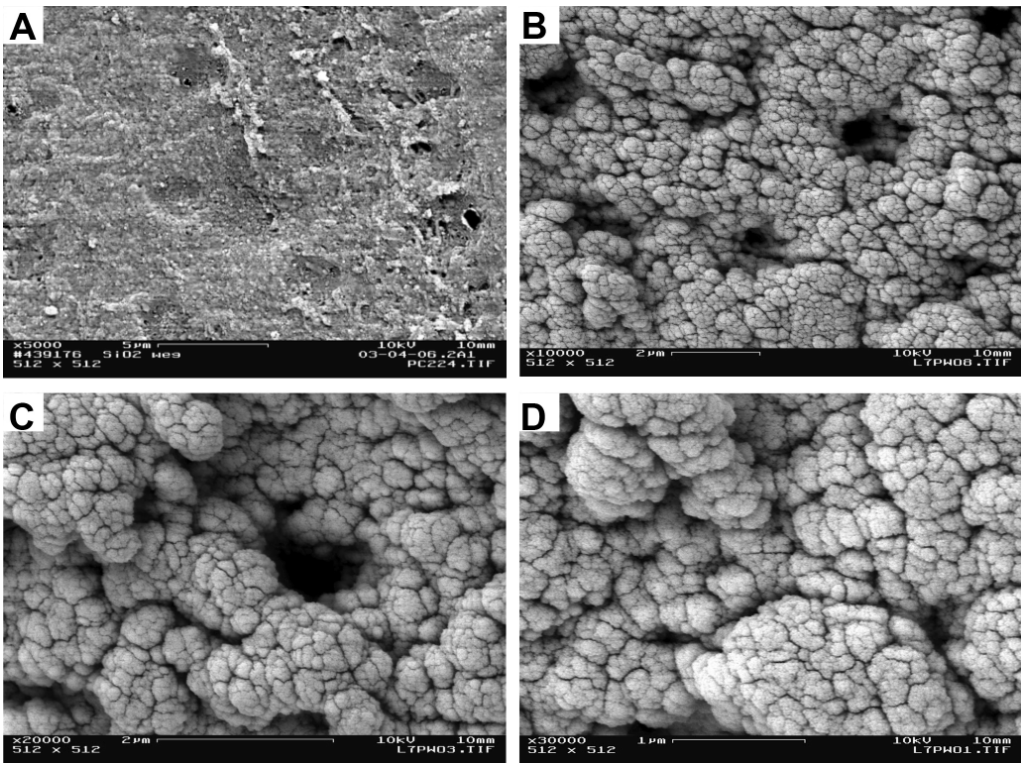


Fig. 14. (A) Pepsin treated surface of lactic acid demineralized dentin with surface precipitates completely covering the demineralized treated surface (x5000). (B) (x10000). (C) (x20000). (D) (x30000).

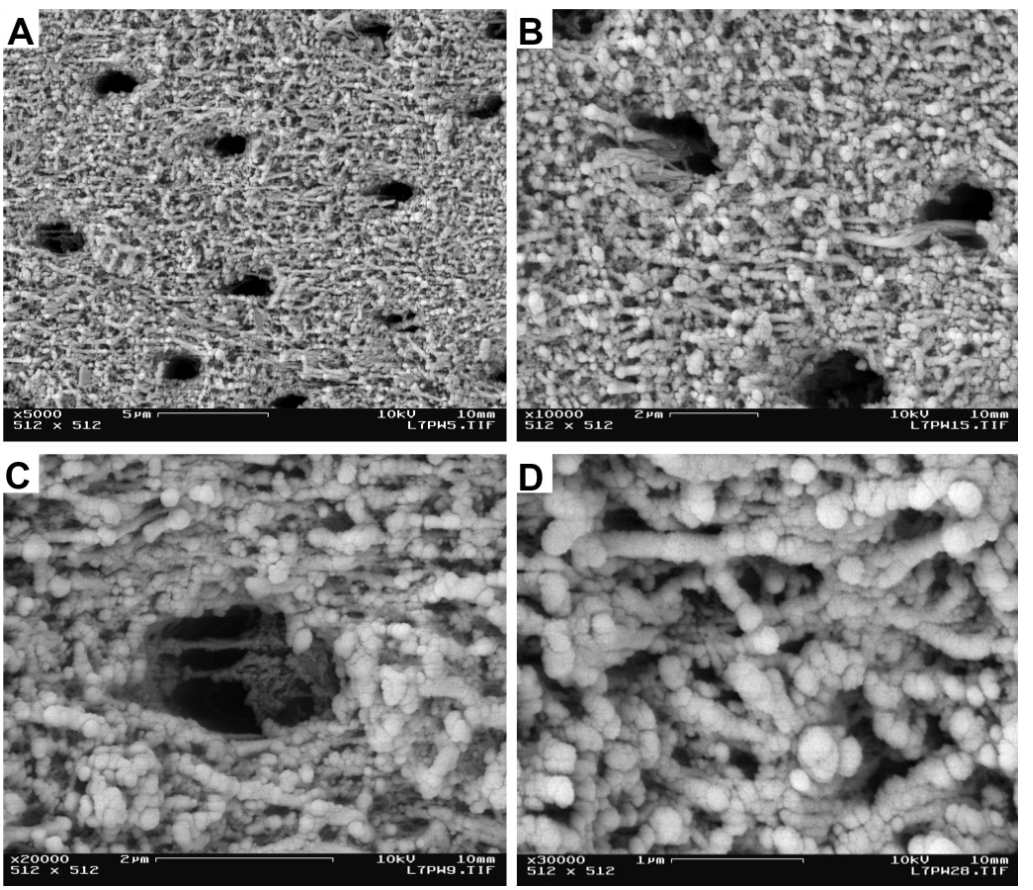


Fig. 15. (A) Rubbing the surface after pepsin treatment of lactic acid demineralized dentin removed surface precipitates exposing demineralized intertubular dentin with patent dentinal tubules (x5000). (B) (x10000). (C) (x20000). (D) (x30000).

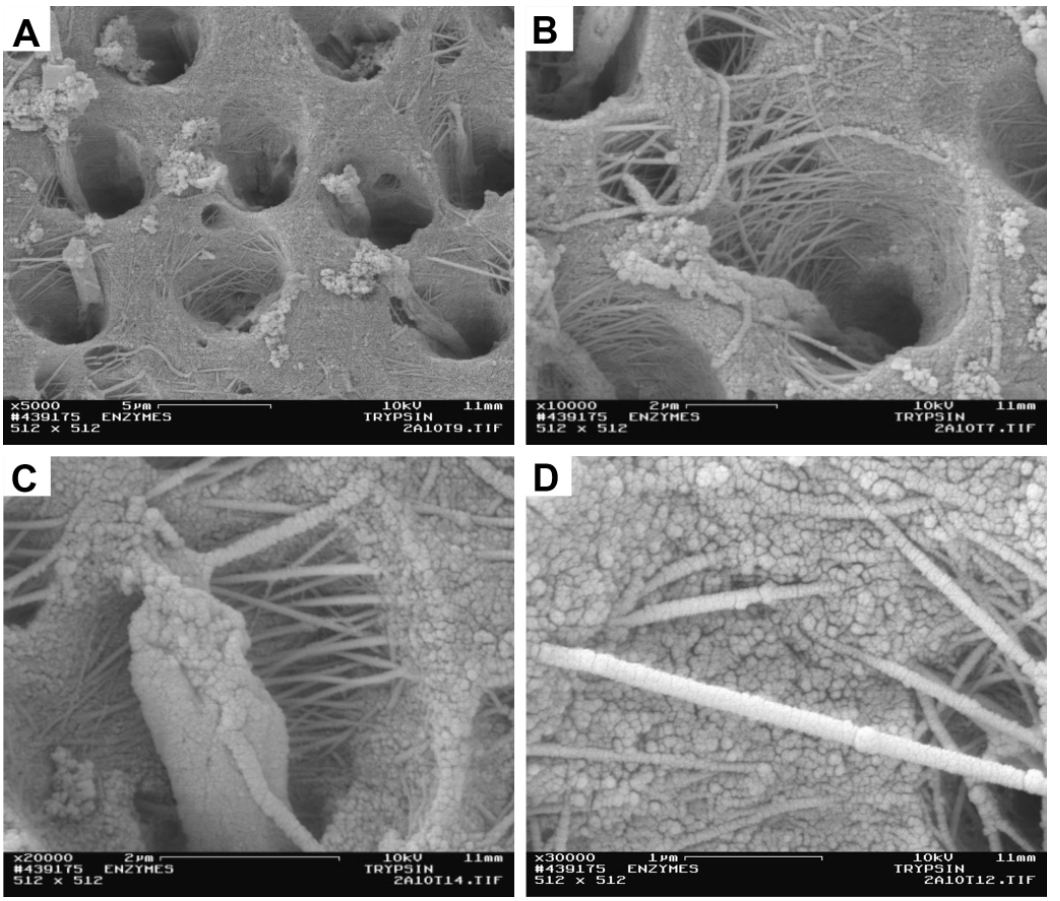


Fig. 16. (A) Trypsin treated demineralized dentin surface with partially demineralized inter-tubular dentin and completely demineralized peritubular dentin, with singular collagen fibers covered with extrafibrillar mineralization showing in some areas (x5000). (B) (x10000). (C) (x20000). (D) (x30000).

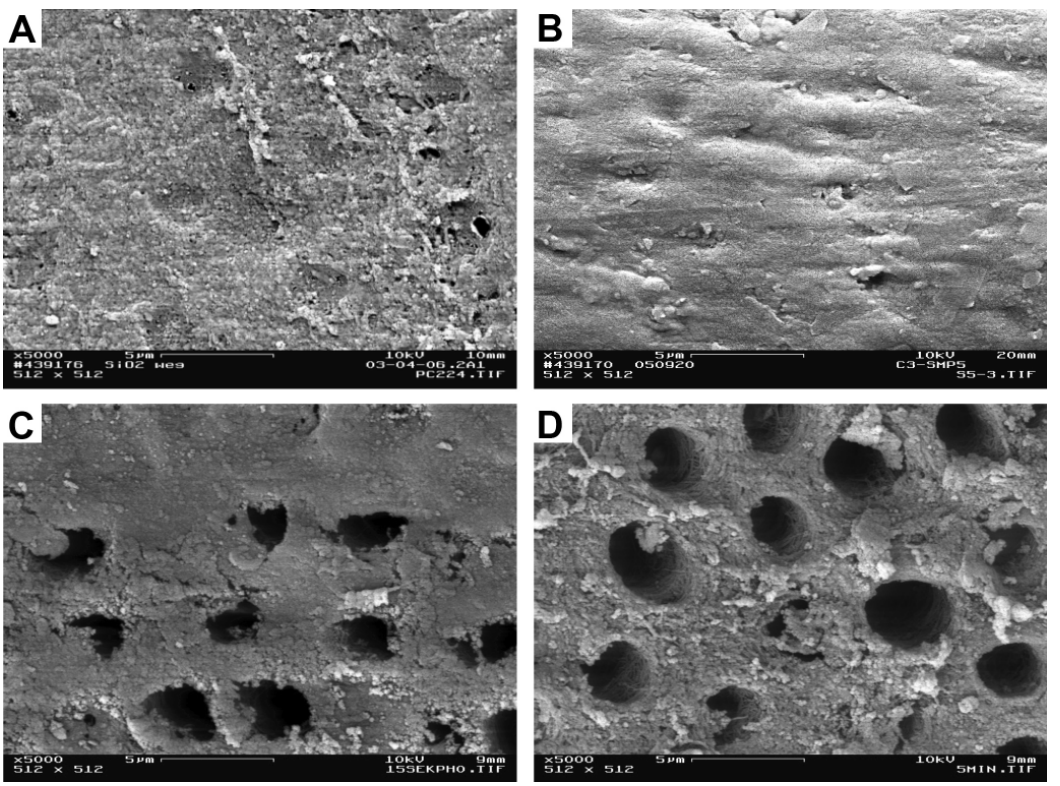


Fig. 17. (A,B) Smear layer covers dentin surface (x5000). (C) Etched surface with 37 % phosphoric acid gel without brushing the surface partially covering the dentin surface (x5000). (D) Etched surface with agitation during the etching period using micro brush exposed dentinal tubules and inter-tubular dentin (x5000).

DISCUSSION

In the present study we aimed to visualize dentinal lesions to get closer knowledge that could provide us with useful information regarding conditioning dentinal substrate when treating dentinal lesions.

It is now well documented that dentinal carious lesions consist of two layers, that differ in their microscopic structure and biochemical and physiological characteristics (Shimizu *et al*., 1981). Superficial infected layer which should be removed completely due to several reasons which were described in details in literature, and inner affected layer which could be left behind during excavation before restoring the tooth due to multiple factors which can be summarized as follows:

1. The inner layer was considered as a partially demineralized but otherwise morphologically intact dentin that demonstrated similarity between collagen fibers of this layer and sound dentin with characteristic cross-links and regularly arranged fibrils.
2. The numbers of viable microbial cells in this affected layer are less than 0.1 % of those in the infected one.
3. This layer is physiologically re-mineralizable due to the following structural, biochemical and physiological characteristics:

1. Collagen fibers in this layer are demineralized but not denaturated in which the extrafibrillar minerals that lie within the interstitial spaces separating the fibrils were dissolved. These minerals are suggested to form the major portion of the mineral phase. On the other hand, the intrafibrillar minerals which are confined within or immediately adjacent to the gap junctions of the collagen were not affected. Consequently, the internal structure of the fibers was not destructed or degenerated.
2. The phosphophoryns are in the phosphorylated insoluble bounded form and covalently cross-linked to the collagen, so they are able to bind calcium and initiate crystal nucleation and hydroxyapatite formation.
3. Residual crystals and calcium ions are found in this layer.
4. Odontoblastic processes which contribute to recalcification are living.

Through imaging dentin utilizing FE-SEM with high magnifications we could visualized sound healthy dentin clearly, so we can use it as a baseline reference to other images during modeling artificial lesions with both acetic and lactic acids, with various treatments.

Acetic acid is a weak organic acid that is incapable of solubilizing dentin collagen (Van Strijp *et al*., 1992). Lactic acid is a stronger organic acid (Featherstone & Rodgers, 1981), although it did not show capability of denaturating human dentin collagen after short incubation periods (Dung *et al*., 1994). However, it is suggested that dentin collagen is likely to denaturate during long-term acid exposure (Klont & ten Cate, 1991; Kleter *et al*., 1998). Both acids are well known to be produced in major proportions by dental plaque and play an important role in carious dentin (Featherstone & Rodgers, 1981; Hojo *et al*., 1991).

The surface of the demineralized human dentin samples after 7 days of demineralization, as shown in the SEM pictures, were similar for both acetic and lactic acids. Due to its lower content of collagen, the peritubular dentin is harder than inter-tubular dentin and therefore is more quickly dissolved in acid than inter-tubular dentin, thus enlarging the orifices of dentinal tubules (Luukko *et al*., 2011).

Inter-tubular collagen fibers were partially demineralized and surface precipitates were formed. The triple-helix of collagen fibrils is resistant to most proteases and its degradation is initiated by cleavage of the three forming polypeptide chains (Brodsky & Ramshaw, 1997) as a result of proteolytic enzyme activity. The triple-helix of the undenaturated collagen is considered to be resistant to degradation by enzymes other than collagenases (Klont *et al*., 1991). Therefore pepsin, which is a carboxylic protease, is expected to act only on the non-helical and denaturated collagen segments (Kleter *et al*., 1997; Tonami & Ericson, 2005). Our results, as shown in the SEM pictures, indicated the capability of pepsin to remove a lot of the surface precipitates. Thus, large proportions of these precipitates are thought to be denaturated collagen fibers. Our hypothesis is supported by previous observations that the same enzyme preparation was incapable of removing these surface precipitates when the demineralizing pre-treatment was acetic acid and not lactic acid. It is known that acetic acid lacks the potential to solubilize either dentin collagen (Van Strijp *et al*.,1992) or dentin phosphorylated phosphoprotein (Klont & ten Cate, 1991). Consequently, we suggest that the surface layer precipitates at the lactic acid demineralized dentin surfaces is formed due to two steps. First the acid diffuses in an un-uniform pathway leaving remnants of hydroxyapatite at the surface together with calcium and phosphate reprecipitations (Moreno & Zahradnik, 1974; Featherstone *et al*., 1983; Fejerskov *et al*., 2003). Second the degraded denaturated collagen fibres together with the released NCCs remain in the demineralized tissue (Klont & ten Cate, 1990). Moreover, it was argued that mineral precipitates from the demineralizing solutions could also form some of these

surface precipitates due to the high similarity between the morphology of the surface precipitates and the morphology of demineralized enamel hydroxyapatites as shown in our SEM images in the current study.

Trypsin was used in comparison to pepsin, it is a serine protease that is also able to digest denaturated collagen, but at a neutral pH 7.4 (Kleter *et al*., 1997). Again, peritubular dentin was dissolved and inter-tubular dentin was partially demineralized with extrafibrillar covered collagen fibers remained intact without degradation (Brodsky & Ramshaw, 1997) resisting the proteolytic enzyme activity (Klont *et al*., 1991).

NaOCl is a nonspecific proteolytic agent which is used widely in various dental procedures (Marshall *et al*., 2001). It was introduced as a chemomechanical method to remove carious dentin after mixing it with three amino acids in the CarisolvTM system. It was reported that these amino acids are responsible for the selective carious dentin tissue removal observed with the Carisolv system in comparison to the pure NaOCl (Tonami *et al*., 2003). In our study 2.5 % of pure NaOCl was chosen to study the extent of its effect at both demineralized and denaturated dentin. Our results were in agreement with Hannig (1999). NaOCl effectively removed both the denaturated and demineralized dentin layers for demineralized surfaces. There were no single mineralized collagen fibers at the surface after NaOCl treatment. These results suggest that extrafibrillar mineralization could not resist the NaOCl action.

An exhausting literature studies assume that elimination of the harmful microbial mass at the lesion surface would permit the underlying layer to heal gradually through the biological properties of the tissue after sealing it with a restorative material, at the same time they preserve the remineralizable tissue, maintain the pulp vitality by avoiding its exposure and arrest caries progression (Ostrom, 1980; Nikiforuk, 1985; McComb, 2000; Heinrich-Weltzien & Kneist, 2001; Fejerskov *et al*., 2003). However, the restorative material interacts with the tooth tissue in many different ways in which sealing properties are considered to be the most important properties in preventing caries progression or/and recurrent caries (Ostrom, 1980; Mertz-Fairhurst *et al*., 1998; Heinrich-Weltzien & Kneist, 2001; Maltz *et al*., 2002; Fejerskov *et al*., 2003; Kidd, 2004). It is worthwhile to mention here that although secondary caries is the most common cause of restorative failure (Splieth *et al*., 2003), the imperfect seal is claimed to be the most important etiological factor behind recurrent caries (Bjorndal *et al*., 1997; Heinrich-Weltzien & Kneist, 2001; Fejerskov *et al*., 2003; Kidd, 2004).

Our study through clearly visualizing dentin under several conditions using high magnification FE-SEM images emphasize the followings regarding adhesive dentistry:

1. When etching dentin surface, timing is very crucial sense too much demineralizing will negatively affect the subsequent bonding. The micro tensile bond strength tests showed lowered bond strength between resins and demineralized dentin (Fuentes *et al*., 2004; Hara *et al*., 2004; Nakajima *et al*., 2005). The bonding quality will be severely altered, due to lack of tag formation, loss of the mineral support resulted in low mechanical properties and collagen matrix collapsing and improper resin infiltration (Hara *et al*., 2004; Nakajima *et al*., 2005). Consequently, there will be no hybrid layer formation which is the basic mechanism of resin-dentin bonding through the resin and collagen fibril network molecular interlocking (Prati *et al*., 1999). Despite the extensive and abnormal interdiffusion at the resin-dentin interface (Hara *et al*., 2004) the collapsed collagen fibers in the inter- and intra-demineralized dentin prevented the monomers from penetrating and complete infiltrating within the dentinal layer (Fuentes *et al*., 2004; Piemjai *et al*., 2004). This has its vital clinical implication, in which the sealing quality is impaired and microleakage can take place due to the microscopic voids between the collagen fibrils which were left by the incomplete diffusion of the adhesive monomers (Fuentes *et al*., 2004; Hara *et al*., 2004; Piemjai *et al*., 2004). This leads to the so called nanoleakage (Prati *et al*., 1999; Hashimoto *et al*., 2004; Nakajima *et al*., 2005), which is exposed unprotected collagen beneath the resin-dentine zone which adds to the micro leakage (Piemjai *et al*., 2004). This naked unsupported collagen is a weak link at the bonding interface because it is liable for hydrolytic or/and proteolytic degradation by bacterial enzymes or/and host-derived matrix metalloproteinase (Fuentes *et al*., 2004). On the other hand, this apatite depleted collagen zone has a low modulus of elasticity; therefore, it undergoes more strain than the hybrid layer overlying it (Yang *et al*., 2005). This in return largely controls the strength, quality, durability and longevity of the bond (Prati *et al*., 1999; Fuentes *et al*., 2004; Hara *et al*., 2004; Piemjai *et al*., 2004; Nakajima *et al*., 2005; Yang *et al*., 2005).

2. The rubbing action using micro brush during the etching procedure can be highly effective due to its ability to detach the smear dissolved layer from the underlying demineralized layer which in return will make it easier to be removed during the washing phase. Eventually, this simple timeless step may significantly enhance bonding strength and hybrid layer formation through better infiltration and tag formation (Irmak *et al*., 2018; Kharouf *et al*., 2020; Saikaew *et al*., 2022).

3. The drying approach has a high impact on the success of the subsequent bonding. It is necessary to maintain etched dentin moist to preserve demineralized collagen interfibrillar spaces prior to hybridization (Stape *et al*., 2021). The inability of resin-solvent blends to re-expand dried collapsed collagen limits the dry-bonding approach (Pashley *et al*., 2007; Manso *et al*., 2008). Thus, dry bonding negatively affected resin-dentin bond strength and hybrid layer stability, and produce inferior outcomes in terms of micro tensile strength, nanoleakage, and hybridization compared to moist dentin approach (blot drying) to avoid collagen collapse (Pashley *et al*., 2007; Stape *et al*., 2021).

CONCLUSION

To our best knowledge, there is no previous clear highly magnified FE-SEM images for dentin, and dentinal components and constitutes with and without various treatments. The current *in vitro* study suggests the complexity of dentin as a substrate that should be treated carefully especially with technique sensitive procedures such as adhesive restorations.

AL-ASMAR, A. A. Implementación clínica de la comprensión de la dentina a nivel microscópico. *Int. J. Morphol., 41(1)*:195-209, 2022.

RESUMEN: El objetivo del presente estudio *in vitro* fue visualizar la dentina para obtener un conocimiento completo de la naturaleza de ella lo que podría proporcionar información útil sobre el acondicionamiento del sustrato dentinario en el tratamiento de lesiones dentinarias. Se obtuvieron 49 terceros molares humanos extraídos y se prepararon para producir lesiones dentinales artificiales mediante desmineralización con ácido acético por 7 y 14 días, o ácido láctico por 7 días. Los dientes se dividieron en grupos y se trataron con NaOCl, pepsina, tripsina o ácido fosfórico. Para obtener información sobre la morfología de las superficies dentinarias tratadas, todas las muestras se visualizaron bajo un microscopio electrónico de barrido de emisión de campo de alta resolución. Con un gran aumento que alcanzó x50000, la dentina se visualizó claramente junto con sus componentes. Se demostró el efecto de varios enfoques de desmineralización y varios protocolos de tratamiento. Se discutió la relación entre los pasos del procedimiento de acondicionamiento y la subsiguiente fuerza de unión. Hasta donde sabemos, no hay imágenes claras previas de microscopio electrónico de barrido altamente ampliadas para la dentina y los componentes y constituyentes de la dentina con y sin diferentes tratamientos. El estudio *in vitro* actual sugiere la naturaleza compleja de la dentina como sustrato que debe tratarse con cuidado, especialmente en los procedimientos sensibles a la técnica, tal como las restauraciones adhesivas.

PALABRAS CLAVE: FE-SCM; Dentina; Colágeno; Pepsina; Tripsina; NaOCl; Desmineralización.

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