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Abstract:	Introduction: This study aimed to characterize the effectiveness of dentin-conditioning with bio-mineralizable chitosan-hydroxyapatite precursor (CS-HA) nanocomplexes alone or associated with tricalcium silicate sealer (TCS/CS-HA) on the mechanical property and antibiofilm efficacy in root dentin. Methods: Flow tests were performed following ISO6876:2012-specifications. Solubility was measured. Micromorphology was assessed using Scanning Electron Microscopy (SEM). Nanohardness/elastic modulus were also determined. Fracture resistance was determined on lower premolars that were prepared, and randomly distributed among 7-groups (n =8/group), including the control, CS-HA dentin-conditioning and root canal filled groups. Similar canal preparation/distribution procedure was followed to test the antibacterial effect on Enterococcus faecalis -infected roots. Descriptive statistic was used to report SEM findings. Flowability results were analyzed using Paired t-test. Multiple comparisons from solubility, fracture and antibacterial assays were assessed by one-way ANOVA-Tukey's tests. Results: TCS/CS-HA showed optimal flow and no effect on solubility after immersion for 4 weeks (p >.05). TCS/CS-HA significantly increased nanohardness and elastic modulus (210±11.3MPa, 7.9±0.9GPa) compared to TCS (44.5±7.8MPa, 2.1±0.3GPa, p<. 05). SEM revealed needle-shaped mineralized structures resulting in fewer voids and a well-adapted sealer-dentin interface in the TCS/CS-HA groups. NaOCl-EDTA irrigation resulted in reduced fracture resistance (444.34N) while CS-HA dentin-conditioning alone (928.28N, p <.05) and CS-HA dentin-conditioning plus CS-HA/TCS resulted in higher fracture resistance (1134.06N, p <.05). CS-HA dentin-conditioning also reduced bacteria by 2.04 log (4.50±0.43) from the initial bacterial load (6.54±0.07, p <.05). There was further bacterial reduction when CS-HA-conditioned root canals were filled with TCS or TCS/CS-HA (0.00 to 0.98±0.57, p >.05). Conclusion: Dentin modification with CS-HA increased the fracture resistance of root dentin, and decreased the residual bacterial burden. TCS/CS-HA potentiated the nanomechanical and physical properties of TCS.

Impact of dentin conditioning and sealer modification with chitosan-hydroxyapatite nanocomplexes on the antibacterial and mechanical characteristics of root dentin

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Statement of clinical relevance

Chitosan-hydroxyapatite nanocomplexes employed as dentin conditioning agent improved the fracture resistance as well as antibiofilm efficacy in root dentin. When mixed with TCS, they improved the physico-mechanical properties of TCS and fracture resistance of root dentin.

ABSTRACT

Introduction: This study aimed to characterize the effectiveness of dentin-conditioning with bio-mineralizable chitosan-hydroxyapatite precursor (CS-HA) nanocomplexes alone or associated with tricalcium silicate sealer (TCS/CS-HA) on the mechanical property and antibiofilm efficacy in root dentin. **Methods:** Flow tests were performed following ISO6876:2012-specifications. Solubility was measured. Micromorphology was assessed using Scanning Electron Microscopy (SEM). Nanohardness/elastic modulus were also determined. Fracture resistance was determined on lower premolars that were prepared, and randomly distributed among 7-groups (n=8/group), including the control, CS-HA dentin-conditioning and root canal filled groups. Similar canal preparation/distribution procedure was followed to test the antibacterial effect on *Enterococcus faecalis*-infected roots. Descriptive statistic was used to report SEM findings. Flowability results were analyzed using Paired t-test. Multiple comparisons from solubility, fracture and antibacterial assays were assessed by one-way ANOVA-Tukey's tests. **Results:** TCS/CS-HA showed optimal flow and no effect on solubility after immersion for 4 weeks (p>.05). TCS/CS-HA significantly increased nanohardness and elastic modulus (210±11.3MPa, 7.9±0.9GPa) compared to TCS (44.5±7.8MPa, 2.1±0.3GPa,p<.05). SEM revealed needle-shaped mineralized structures resulting in fewer voids and a well-adapted sealer-dentin interface in the TCS/CS-HA groups. NaOCl-EDTA irrigation resulted in reduced fracture resistance (444.34N) while CS-HA dentin-conditioning alone (928.28N, p<.05) and CS-HA dentin-conditioning plus CS-HA/TCS resulted in higher fracture resistance (1134.06N, p<.05). CS-HA dentin-conditioning also reduced bacteria by 2.04 log (4.50±0.43) from the initial bacterial load (6.54±0.07, p<.05). There was further bacterial reduction when CS-HA-conditioned root canals were filled with TCS or TCS/CS-HA (0.00 to 0.98±0.57, p>.05). **Conclusion:** Dentin modification with CS-HA increased the fracture

resistance of root dentin, and decreased the residual bacterial burden. TCS/CS-HA potentiated the nanomechanical and physical properties of TCS.

Keywords: Chitosan, dentin, nanocomplexes, hydroxyapatite, tricalcium silicate sealer.

INTRODUCTION

Chemo-mechanical preparation in root canal treatment altered the biomechanical response of endodontically treated teeth, and reduces the toughness of dentin, which may increase the risk of fractures in root-filled teeth (1-3). Currently restorative materials have been relied upon to enhance the mechanical integrity of endodontically treated teeth. This research sought to improve the mechanical characteristics of root dentin in endodontically treated teeth.

Chitosan (CS) is a biopolymer consisting of β-(1-4) glucosamine units. It is extensively used in the biomedical field due its biocompatibility, biodegradability and antibacterial properties (4). Phosphorylated-chitosan has been reported to induce dentin remineralization; its phosphate groups bind to calcium ions for hydroxyapatite nucleation (5). Nanoparticles are ultrafine particles of 1–100 nm in diameter, similar in size to many biomolecules. At nanometric range they demonstrate distinct interaction with prokaryotic cells and biomolecules, which provides significant therapeutic options (4). Chitosan nanoparticles has the ability to inactivate bacterial biofilm, inhibit biofilm formation at sealer-dentin interfaces (6, 7), enhance dentin surface resistance to collagenase, and improve dentin mechanical properties. These characteristics are favorable for hard tissue repair (8, 9).

Previous reports have demonstrated the ability of chitosan-hydroxyapatite precursor (CS-HA) nanocomplexes used as dentin-conditioner, induced intrafibrillar mineralization and improved intratubular penetration of tricalcium silicate sealer (TCS) (10, 11). The concept of biomimetic mineralization is to use natural/synthetic polymer additives to simulate the role of non-collagenous

proteins, which play a crucial role in biomineralization (11, 12). CS-HA nanocomplex mimic non-collagenous proteins, due to the carboxyl groups on chitosan backbone and the amorphous calcium phosphate, a hydroxyapatite precursor stabilized on CS-HA (10). Thus, CS-HA facilitated intra- and extra-fibrillar collagen biomineralization (12).

Generally, TCSs are considered to have good biocompatibility and sealer-dentin bonding properties (13). TCS produce calcium hydroxide as a reaction product of the cement hydration (14, 15), which in turn reacts to form hydroxyapatite deposits on the material surface at the tooth-material interface (16). The material's alkalinity results in a mineral infiltration zone formed by the permeation of high concentration of Ca(2+), OH(-), and CO(3) (2-) ions at the dentin-cement interface (17). However, this interfacial interaction was debated in other investigation (18). In the current study we hypothesized that CS-HA could (a) cause collagen biomineralization and increased fracture strength of root dentin; (b) enhance bacterial killing when used as dentin conditioner or associated with TCS. The purpose of this study was to characterize CS-HA-modified TCS and evaluate the effect of dentin conditioning with CS-HA nanocomplexes with/without CS-HA modified TCS on the mechanical and antibiofilm properties in root dentin.

MATERIALS & METHODS

All the chemicals used in this study were of analytical grade and were purchased from Sigma Aldrich (St Louis, MO, USA) unless otherwise stated. A previously published methodology was followed to synthetize the CS-HA nanocomplexes (12). EndoSequence BC sealer (Brasseler,

Savannah, GA, USA) alone or mixed with 30% CS-HA was tested in the study. The Institutional Ethics Board approved the study protocol (#38505).

Stage-1: Sealer characterization

1.1 Flow test

Flow was determined according to ISO 6876/2012 test method. Sealer alone (group 1) or sealer mixed with CS-HA (group 2) (0.05 mL) was placed between two 20 g. glass slabs and then a 100 g. weight was placed on the top slab for 10 min. The disc diameters were measured and the arithmetic means were determined (18) (*n*=10/group).

1.2 Solubility

Stainless steel ring moulds (*n*=10/group, 10 mm internal diameter, 1mm thickness) were filled with TCS or TCS/CS-HA and allowed to set for 7 days at 37°C and 100% humidity. A previous study methodology was followed (19). However, in the current experiment, simulated body fluid (SBF) (20) was used instead of distilled water (DW) and the samples were allowed to dry for 1 hour at room temperature followed by vacuum desiccator overnight. The specimens were then immersed in 60 ml of SBF, and stored in an incubator at 37°C for 24 hours. Subsequently, the discs were dried for 1 hour at room temperature followed by vacuum desiccator overnight, and the weight was recorded. The whole procedure was repeated after 1- and 4-weeks immersion in SBF.

1.3 Nanohardness and modulus of elasticity

Ten metallic rings (n=5/group, 6 mm internal diameter, 3 mm thickness) were filled with the two sealers and allowed to set as described above. A hand-press was used to control expansion during

cement setting since chitosan display swelling characteristics in presence of moisture. Nanohardness and elastic modulus were measured with a nanoindenter tester UNHT^3 (Anton Paar, Graz. Austria) equipped with a diamond Berkovich tip. Maximum load was 50 mN, which results in typical impression depths of 2-3 μm for TCS/CS-HA and 6-8 μm for the TCS samples. Force was loaded and unloaded linearly with time at a rate of 5 mN/s, and held at the maximum load for 10 seconds. Sixteen indentations were done on a square grid with 250 μm spacing to avoid interaction among impressions.

1.4 Morphology.

Fifteen plastic rings (*n*=5, 6 mm internal diameter, 10 mm thickness) were filled and allowed to set and dried as described above. The samples were fractured and one of the halves was polished while the other was left as it to avoid polishing the crystalline features. Samples were set over aluminum stubs and sputter-coated with gold/palladium at 20 mA. The surfaces morphologies were qualitatively examined using a Scanning Electron Microscope (SEM; Hitachi S-800, Tokyo) at 500, 2000 and 5000 times magnification.

Stage-2: Fracture resistance test

Fifty-six sterile lower premolar human teeth (*n*=8/group) with straight roots were decoronated to obtain 14 mm roots for experimentation. The roots were transilluminated and radiographed to discard teeth with cracks and multiple canals. The samples were accessed and instrumented to Protaper F3 (Protaper Gold, Dentsply Maillefer). The canals were irrigated with 2.5% NaOCl solution (total volume of 6 mL), 17% EDTA (1 mL) and/or CS-HA nanocomplex solution (1 mL). CS-HA nanocomplex solution was prepared by dissolving 2mg/mL of CS-HA nanoparticles in

1mL of DW. Seven experimental groups were prepared according to the different combinations of dentin conditioning/obturation protocols (Table 1). TCS and 30% CS-HA were mixed on a glass slab. TCS and TCS/CS-HA were placed into the root canal with a Protaper F3 gutta-percha cone using a gentle pumping motion.

The obturated teeth were allowed to set at 37°C and 100% humidity for two weeks. The specimens were embedded in self-curing resin (SR-Ivolen, Ivoclar-Vivadent, Lichtenstein) with a 0.2 mm silicone barrier (AquasilLV, Dentsply DeTrey GmbH, Germany) surrounding the roots to mimic the periodontal ligament. The samples were submitted to a compressive force with a 6.3 mm ball-indenter, along the long axis of the root at the crosshead speed of 1 mm/min until fracture (Instron, Canton, MA). The resistance to fracture was equal to the maximum compressive load recorded.

Stage-3: Antimicrobial assessment

Thirty-five roots (10 mm approx.) from mandibular premolars (*n*=5/group) were treated up to ProTaper F2 (Protaper Gold, Dentsply Maillefer) and irrigated as described above. Vertical grooves were prepared on the cylinders to facilitate splitting and the specimens were autoclaved. *Enterococcus faecalis* (American Type Culture Collection 29212) infection model was developed according to a previous protocol (7). After 21 days of incubation at 37°C in 1 mL brain heart infusion broth (BHI), which was refreshed every 48 hours, the specimens were washed with DW to remove nonadherent bacteria and instrumented with Protaper F3 and irrigated with NaOCl+EDTA (as described above) and/or CS-HA nanocomplex solution. Seven groups were prepared according to the dentin-conditioning/obturation protocol (Table 2). Later, the specimens were incubated for 14 days at 37°C and 100% humidity. After this setting phase, the samples were

split and gutta-percha was removed. One half of the specimen was analyzed under SEM, while the other half was cryopulverized in liquid nitrogen, resuscitated for 4 hours, serial diluted and spread onto BHI agar. CFU were counted after 48 hours of incubation.

Statistical analysis

Prisma 5.0 (GraphPad Software Inc, La Jolla, CA) was utilized as the analytical software. The flow results were analyzed using Paired t-test. The one-way ANOVA and Tukey's tests were used for multiple comparisons of data from solubility, fracture and antibacterial assays.

RESULTS

Flow test: TCS flow was uniform and was determined to be 22.09±1.23 (Mean±SD). Addition of CS-HA into TCS reduced the flow by ~2mm (Mean±SD=19.23±1.28. p=0.002). However, the flow met the ISO 6876 requirement (>17mm).

Solubility: The solubility (expressed in percentages) of TCS was 0.5% after 24 hours, 0.1% after 1 and 4-weeks. The solubility of TCS/CS-HA was 0.1% after 24 hours, 1 and 4-weeks intervals (p>.05).

Nanohardness and modulus of elasticity: Addition of CS-HA into TCS significantly improved the nanohardness values (201.0±11.3MPa) as compared to the unmodified sealer (44.5±7.8MPa. p<.05). The modulus of elasticity increased to ~6 GPa in TCS/CS-HA mix (7.9±0.9GPa), while lower values was measured for the TCS (2.1±0.3GPa. p<.05).

Morphology: SEM of the set TCS showed granular surface with pores (x500, Fig.1A-1B). At higher magnification (x5000,1C), heterogenous characteristics were confirmed. Incorporation of CS-HA into TCS resulted in crystal formation (x500, polished,1D). In x5000 (1E), crystal growth was observed within the TCS matrix (red arrows, polished, 1F). Interestingly, isolated needle-like crystal clusters were also observed on the external surface of the CS-HA samples (x2000,1G and x5000,1H) (Fig. 1).

Fracture resistance test

NaOCl-EDTA irrigation reduced the root fracture strength the most (444.34N), while CS-HA dentin-conditioning significantly enhanced fracture resistance (928.28N, $p<.05$) showing similar fracture load values to that of untreated root dentin samples ($p>.05$). Root obturation further increased the fracture strength of the experimental groups. The highest loads to fracture were exhibited in CS-HA dentin-conditioning groups regardless of using TCS/CS-HA (1134.06N, $p<.05$) (Table 3).

Antibacterial assessment

The initial bacterial load of 6.54±0.07 (SD) log was observed in the positive control group. CS-HA dentin-conditioning after NaOCl resulted in 4.50±0.43 log. A 2.04 log-reduction of bacterial load was measured when CS-HA conditioning was used ($p<.05$). The bacterial loads diminished to (2.78±0.92, $p<.05$) when CS-HA conditioning was applied after NaOCl+EDTA. Those bacterial loads further diminished significantly when CS-HA-conditioned root dentin was filled with TCS or TCS/CS-HA (0.00 and 0.98±0.57 respectively, $p>.05$) (Figure 2).

SEM analysis revealed biofilm structures on the positive control samples. Bacteria-free and opened dentinal tubules were seen in the negative control. Although NaOCl/EDTA irrigation disrupted biofilm structure, bacteria persisted around dentinal tubules (Figure 3A). Isolated bacterial conglomerates and strains were observed on samples treated with NaOCl+CS-HA, opened dentinal tubules were rarely seen in this group (Figure 3B). Bacteria were absent in NaOCl+EDTA+CS-HA dentin-conditioning groups; although intratubular bacteria was observed in some samples. NaOCl+EDTA irrigation followed by TCS obturation showed mostly bacteria-free sealer-dentin interfaces, with a few samples displaying bacteria near the dentin-sealer interface. Similar to sealer morphology characterization assay, TCS in root canal had a granulated surface with pores (Figure 3C (yellow arrows)). Dentin microcracks were observed on samples treated with NaOCl+EDTA. Microcracks (Figure 3D (red arrows)) and dentin-sealer interfaces gaps were mostly filled by mineral crystal when CS-HA was used for dentin-conditioning regardless of the application of TCS/CS-HA (Figure 3D (inset Da: showing S - sealer; C - crystals; DT - dentinal tubules). CS-HA dentin-conditioning and TCS/CS-HA association resulted in a homogenous well-adapted sealer-dentin interfaces (red arrows) and voids filled by crystals (Figure 3E (yellow arrows)). CS-HA dentin-conditioning followed by TCS/CS-HA, resulted in dentinal tubular mineralization, as well as mineral crystal formation on both, sealer and dentin (Figure 3F). At higher magnification, a well-organized interface filled with crystalline-mineral structures were noted (Figure 3F (inset Fa)).

DISCUSSION

Loss of dentin due to disease process and/or removal of dentin during root canal shaping procedures increase the risk of vertical root fractures (3, 21-23). Yan, *et al.* 2019 (24) reported that the strength of the root structure decreased after root canal treatment, which could be attributed to the compromised root dentin following root canal therapy. CS-HA based-biomimetic mineralization has been proposed as an method to restore mechanical properties of demineralized root dentin (10-12) by replacing dentin matrix components, via intrafibrillar mineralization and simultaneous encapsulation of collagen fibrils by CS (10). This study showed that the loads to fracture of specimens treated with CS-HA for dentin-conditioning was restored at the levels similar to that of the untreated root dentin. The fracture strength values increased further when the root canals were conditioned with CS-HA and filled with TCS or TCS/CS-HA.

The finding observed in the study could be explained due to the ability of CS-HA nanocomplexes to mimic the role of non-collagenous proteins, a key factor in dentin biomineralization (5). CS-HA conditioning may have reduced the interfacial energy between tissue fluid and dentin, resulting in a negatively charged surface on collagen. Given that nucleation and mineral formation is directly proportional to ions concentration (25), the interaction between TCS and dentin is positively modified, enhancing the bioactivity of TCS when associated with CS-HA (11), as it was corroborated by the results of the present study. Additionally, sealer characterization showed an improved nanohardness and elastic modulus when CS-HA was incorporated into TCS. This may justify the interfacial mineralization observed in the SEM analysis of the TCS/CS-HA group. In the same line, Hashmi, *et al.* (11) reported that dentin conditioning with CS-HA resulted in a chemically modified dentin substrate with an ion-rich layer of phosphate, calcium, calcium

phosphates, and chitosan that chemically modified the dentin surface/subsurface, which is critical for restoring chemical characteristics of iatrogenically demineralized dentin.

The sealer flow was maintained and the solubility for all materials tested was negligible. The method used to test solubility did not follow the ISO 6876 method. The use of water in the ISO method but rather in body fluids. The change in solution type has shown to be relevant in testing hydraulic sealer solubility (26). Calcium silicate cement produced tag-like structures at the sealer-dentin interface, since calcium ions reacted with phosphate molecules to form hydroxyapatite (17, 27). Precipitation of calcium phosphate salts (mainly hydroxyapatite) may occur when TCS is in contact with phosphate-containing body fluids (28). However, the structural disorder of poorly crystalline carbonated apatite from that reaction render them prone for dissolution when in contact with body fluids (29). CS-HA nanocomplexes present a highly stable, polyanionic surface charge in alkaline pH, which is important to establish higher ratio of ionized carboxyl groups and subsequent aggregation of dispersed nanocomplexes, inducing a gradual transition from a poorly-crystalline into ordered well-crystallized hydroxyapatite (30).

Cryopulverization technique was used for microbiological assessment in this study. This technique offers a more realistic determination of bacterial load from root dentin since bacteria from the main canal, lateral ramifications and dentinal tubules may be quantified by this technique (31). This study showed 2.04 and 3.76 log-reduction when dentin was conditioned with CS-HA after irrigation with NaOCl and NaOCl+EDTA. Thus CS-HA treatment may beneficially eliminate residual root canal bacteria following cleaning and shaping procedures. The bacteria were further reduced to 0 and 0.98 log after root canal filling with TCS or TCS/CS-HA. The interaction of the

biopolymeric polycationic CS with the negatively charged bacteria result in the leakage of intracellular components and cell death (32). This characteristic of CS, together with a CS-HA-modified dentin substrate prior to TCS application (11) could play an important role in killing or mineralizing bacteria / biofilm (33). Although the findings of the present study undoubtedly showed a significant antibiofilm action of CS-HA on root canal dentin when used as dentin-conditioning or associated to TCS, SEM did not show evidence of mineralized bacteria. However, microphotographs revealed entire surfaces covered by crystal-like structures, that filled up the gaps at the sealer-dentin interface and microcracks on dentin. The electrostatic interaction between CS and bacterial cell would have contributed significantly to bacterial reduction (32), while the scarce residual bacterial cells were entombed by HA crystals or mineralized by the supersaturation of calcium phosphate contained into TCS/CS-HA (34).

CS-HA dentin-conditioning created a hydrophilic and polyelectrolytic layer, containing carboxyl groups from chitosan and interstitial water from amorphous calcium phosphate. This modified surface allowed deeper intratubular penetration of TCS with more dentin wettability (10, 35). Considering the reported antimicrobial efficacy of TCS against well-stablished intracanal biofilm (36), the enhanced TCS intratubular penetration by CS-HA dentin-surface modification, may be consider as a contributing factor for the bacterial load reduction in the CS-HA dentin-conditioning groups filled with TCS or TCS/CS-HA. In summary, dentin substrate modification with CS-HA increased the fracture strength of root dentin and improved the antibacterial effect. Incorporation of CS-HA to TCS improved the physical and mechanical characteristics of TCS.

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FIGURE LEGENDS

Table 1: Experimental groups for fracture resistance test.

"x": Not used, "✓": Used.

NaOCl, sodium hypochlorite; EDTA, ethylenediaminetetraacetic acid; CS-HA, chitosan-hydroxyapatite precursor nanocomplexes; GP, gutta-percha; TCS, tricalcium silicate sealer.

Table 2: Experimental groups for antibacterial test.

"x": Not used, "✓": Used.

NaOCl, sodium hypochlorite; EDTA, ethylenediaminetetraacetic acid; CS-HA, chitosan-hydroxyapatite precursor nanocomplexes; GP, gutta-percha; TCS, tricalcium silicate sealer.

Table 3. Fracture resistance in Newton after root canal dentin conditioning with CS-HA and filling TCS or TCS/CS-HA.

1: Control, **2**: 2.5% NaOCl + 17% EDTA, **3:** 2.5% NaOCl + 17% EDTA + CS-HA solution, **4:** 2.5% NaOCl + 17% EDTA + TCS, **5**: 2.5% NaOCl + 17% EDTA + TCS/CS-HA, **6**: 2.5% NaOCl + 17% EDTA + CS-HA solution + TCS, **7**: 2.5% NaOCl + 17% EDTA + CS-HA solution + TCS/CS-HA

* Different superscript letters in each column represents significant differences ($p < 0.05$).

Figure 1. SEM morphology representative images of TCS in its original formulation (A-C) and associated to CS-HA (D-H).

TCS surface in x500 (A-B) and x5000 (C), different structure sizes are observed. TCS/CS-HA association (D-H). Needle-like crystal (x500, D) and square structures (x5000, E). Crystal between TCS (red arrows, F) and needle-like crystal clusters (x2000, G and x5000, H)

Figure 2. Bacterial log CFU/mg reduction expressed in mean (SD) after root canal dentin conditioning with CS-HA and filling TCS or TCS/CS-HA.

1: Control; **2**: 2.5% NaOCl + 17% EDTA; **3**: 2.5% NaOCl + CS-HA solution; **4**: 2.5% NaOCl + 17% EDTA + CS-HA solution; **5**: 2.5% NaOCl + 17% EDTA + TCS; **6**: 2.5% NaOCl + 17% EDTA + CS-HA solution + TCS; **7**: 2.5% NaOCl + 17% EDTA + CS-HA solution + TCS/CS-HA
* Different superscript letters in each column represents significant differences (p <0.05).

Figure 3. SEM representative images of *E. faecalis* infected samples after root canal dentin conditioning with CS-HA and filling TCS or TCS/CS-HA.

Bacteria around dentinal tubules after NaOCl/EDTA irrigation (X2000, A). Bacterial conglomerates/strains after NaOCl+CS-HA treatment, note the absence of opened dentinal tubules (X2000,B). TCS porous surface (yellow arrows) and dentin microcracks (red arrows) after NaOCl/EDTA (X500,C). Crystal formation inside microcracks (red arrows. X500,D) and sealer-dentin interface gaps when CS-HA dentin conditioning was done after standard irrigation regardless TCS/CS-HA association (X2000,Da. S: sealer; C: crystals; DT: dentinal tubules). Well adapted sealer-dentin interfaces (red arrows) and voids filled by crystals (yellow arrows. X500,E) after CS-HA dentin conditioning and TCS/CS-HA. In many cases, that protocol, resulted in crystal formation on both, sealer and dentin (X500,F & X2000,Fa).

Figure-1

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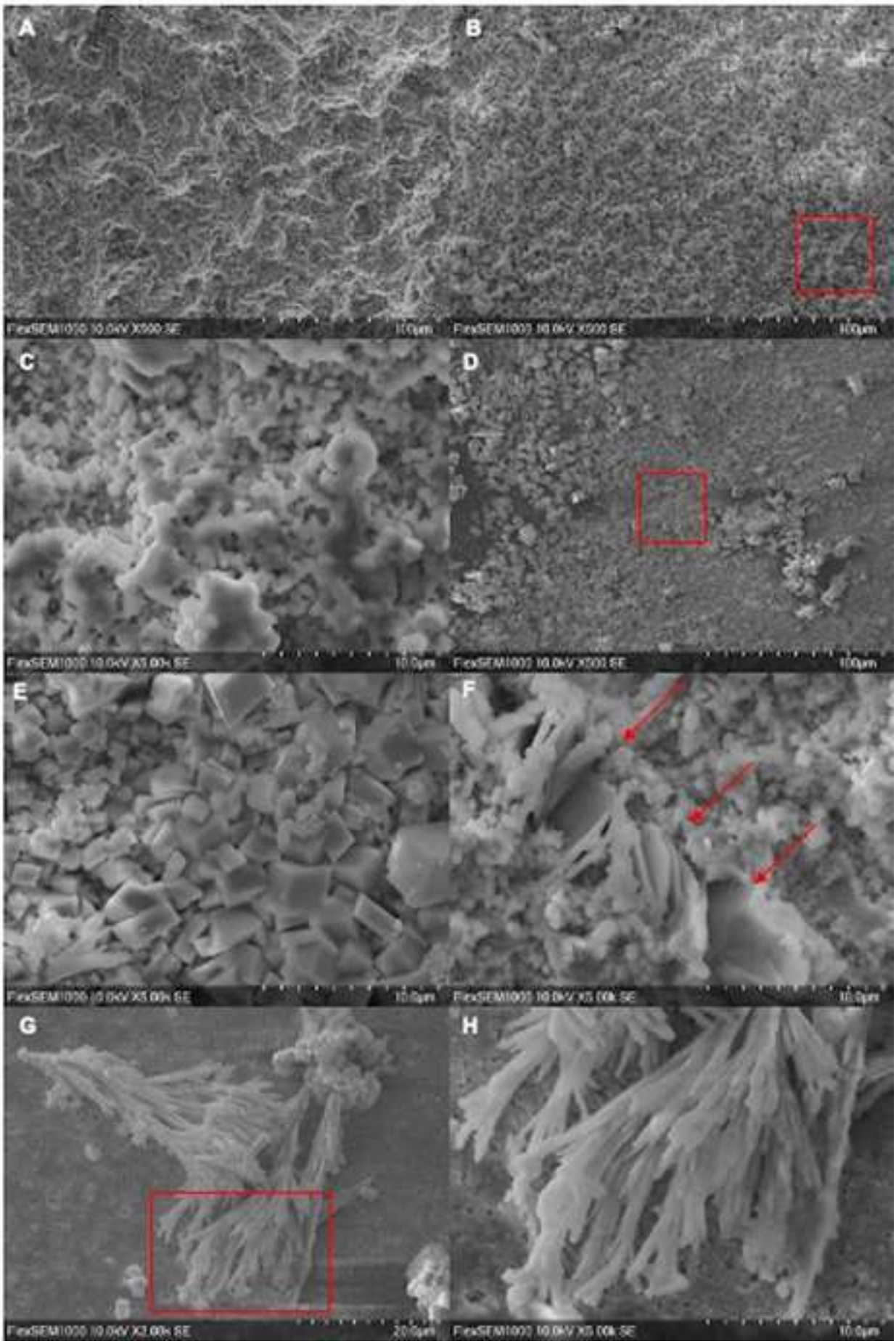

Figure-2

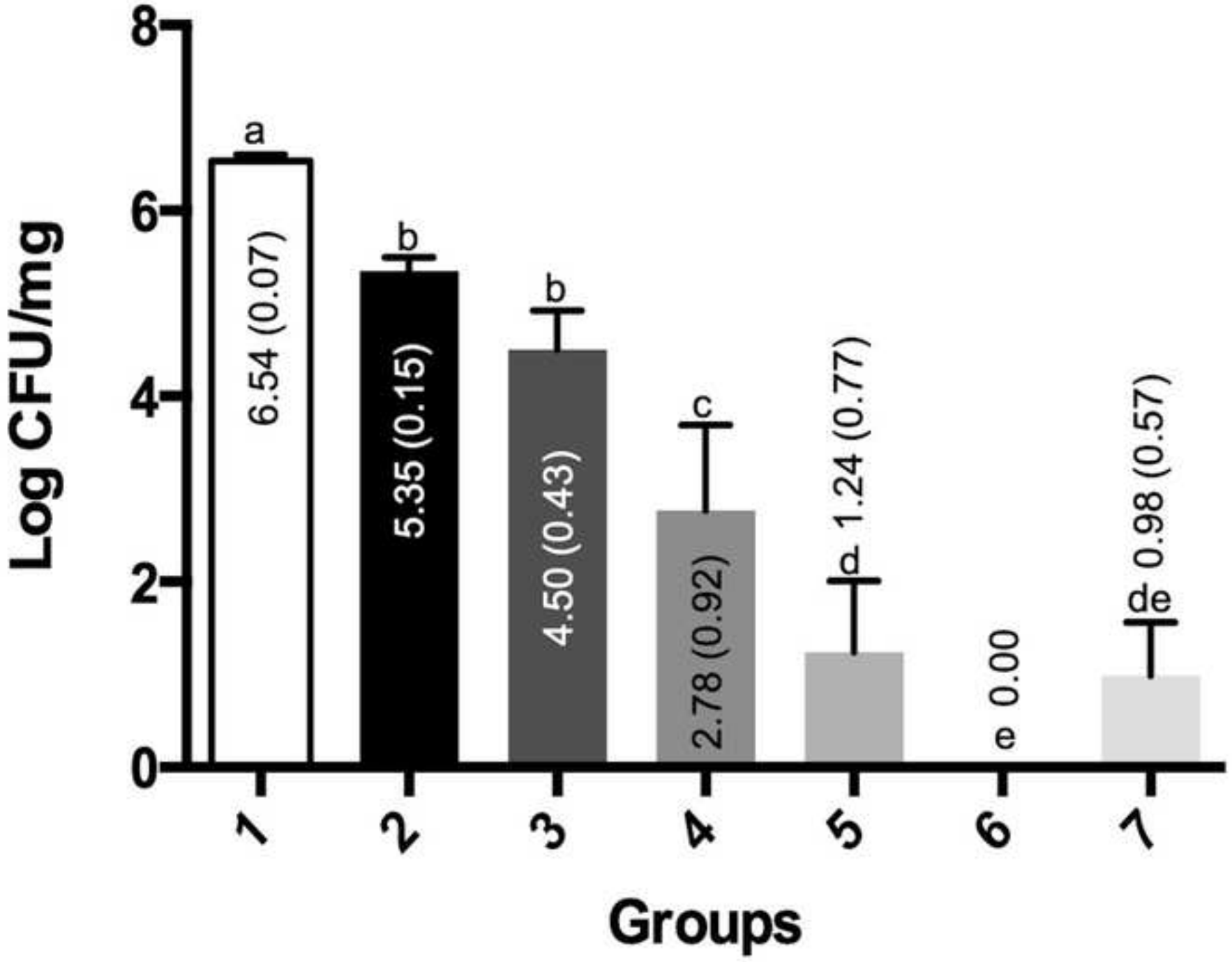

Figure-3

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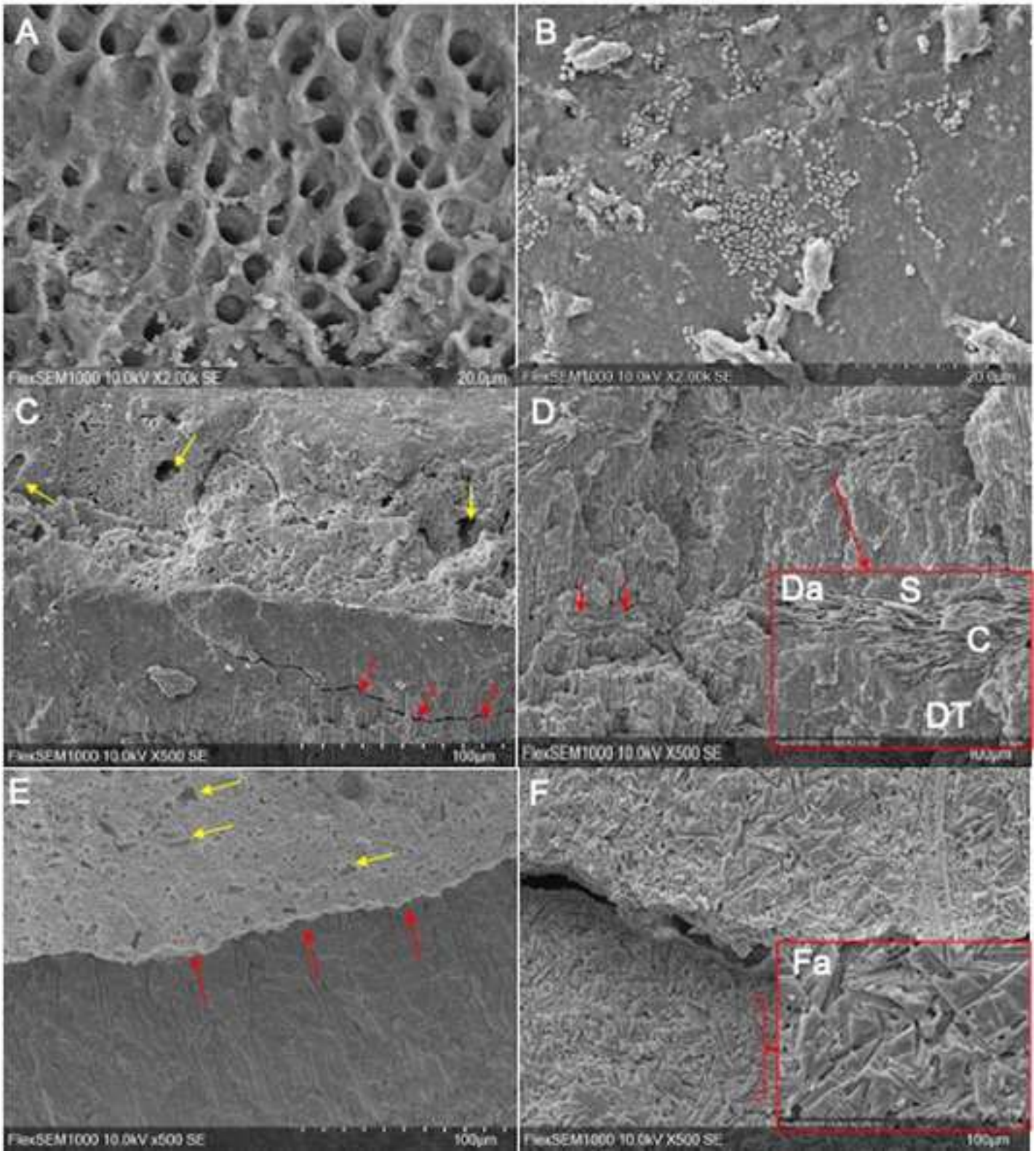

Table 1: Experimental groups for fracture resistance test

Group (n=8)	2.5% NaOCl	17% EDTA (3 min)	CS-HA solution (5 min)	GP+TCS	GP+TCS/CS-HA
1	x	x	x	x	x
2	✓	✓	x	x	x
3	✓	✓	✓	x	x
4	✓	✓	x	✓	x
5	✓	✓	x	x	✓
6	✓	✓	✓	✓	x
7	✓	✓	✓	x	✓

Table 2: Experimental groups for antibacterial test

Group (n=5)	2.5% NaOCl	17% EDTA (3 min)	CS-HA solution (5 min)	GP+TCS	GP+TCS/CS-HA
1	x	x	x	x	x
2	✓	✓	x	x	x
3	✓	x	✓	x	x
4	✓	✓	✓	x	x
5	✓	✓	x	✓	x
6	✓	✓	✓	✓	x
7	✓	✓	✓	x	✓

Table 3. Fracture resistance in Newton after root canal dentin conditioning with CS-HA and filling TCS or TCS/CS-HA.

Group	Mean (*SD*)	Min-Max	95% CI
1	946.07 (212.02) [ac]	635.1 - 1134.03	768.81- 1123.32
2	444.34 (61.29) [b]	360.48 – 543.43	393.10 – 495.58
3	928.28 (199.59) [ad]	734.39 - 1292.77	761.42 - 1095.15
4	735.69 (215.21) [a]	405.45 – 969.15	555.77 – 915.62
5	939.30 (159.92) [ad]	762.26 – 1148.04	805.60 – 1072.99
6	1116.94 (171.04) [cd]	903.76 – 1362.86	973.94 – 1259.93
7	1134.06 (238.00) [cd]	915.74 – 1516.05	935.08 – 1333.03

Faculty of Dentistry
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Endodontics
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August 4, 2021

Professor Kenneth M. Hargreaves DDS, PhD
Editor, Journal of Endodontics
Dept. of Endodontics, UTHSCSA
7703 Floyd Curl Drive, San Antonio, TX 78229-3900

Subject: Submission of an original research manuscript

Dear Dr. Hargreaves,

I am herewith submitting an original article titled "The impact of dentin conditioning and sealer modification with chitosan-hydroxyapatite nanocomplexes on the antibacterial and mechanical characteristics of root dentin", towards consideration for publication in the *Journal of Endodontics*.

The primary purpose of this study was to characterize the effectiveness of dentin-conditioning with bio-mineralizable chitosan-hydroxyapatite precursor (CS-HA) nanocomplexes alone or associated with tricalcium silicate sealer (TCS/CS-HA) on the mechanical property and antibiofilm efficacy in root dentin. The findings from this experimental investigation highlighted that dentin substrate modification with CS-HA enhanced the mechanical integrity of root dentin leading to higher resistance to fracture, while decreasing the residual bacterial burden. TCS/CS-HA potentiated the nano-physical characteristics of TCS. Given the originality of the study, and its relevance, we presume it will be appropriate for publication in the *Journal of Endodontics*.

All the authors have read the manuscript and approved the submission. This is an original manuscript that has not been previously published either in totality or in part, including the illustrations, and that it is not under consideration for publication elsewhere. In consideration of the editors of the *Journal of Endodontics* taking action in reviewing and editing this submission, the author(s) undersigned hereby transfer, assign or otherwise convey all copyright ownership to the AAE in the event that such work is published in that Journal.

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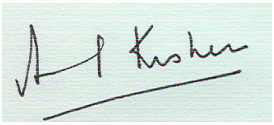
Yours sincerely,



Anil KISHEN
(On behalf of all authors)
Professor of Endodontics
Graduate Coordinator, Graduate Education
Principal Investigator, Dental Research Institute

Reply to the Associate Editor's (AE) comment

The authors would like to thank the AE for the feedback. We have considered the comment and have now included supplemental information with additional information on the synthesis, solubility test and fracture resistance model for the JOE readership. Thank you.

Supplemental data

Synthesis of CS-HA precursor nanocomplexes:

CS-HA nanocomplex was synthesized according to a previously published methodology (Chen, *et al*. 2015. ref.[12]). Please realize the use of a different nomenclature to name carboxymethyl-chitosan / hydroxyapatite precursors nanocomplexes in the present study (CS-HA) as compared to Chen's paper, where CMC/ACP initials were used. The final synthetized nanocomplexes are the same in both studies.

Solubility test:

Stainless steel ring moulds (*n*=10/group, 10 mm internal diameter, 1mm thickness) were filled with TCS or TCS/CS-HA and allowed to set for 7 days at 37°C and 100% humidity. The solubility test was performed according to a previously published methodology (Elyassi, *et al*. 2019. ref.[19]). Nonetheless, in the present study, simulated body fluid (SBF) was used instead of distilled water (DW) and the samples were allowed to dry for 1 hour at room temperature followed by vacuum desiccator overnight. Two glass beakers (A and B) for each material were used, which were placed in an oven at 90°C, removed after reaching the temperature and weighted at room temperature. Then, 2 specimens per beaker (A) were immersed in 60 ml of SBF and stored in an incubator at 37°C for 24 hr. After that period, the content of A was poured into B using funnel/filter paper. Beaker A was washed with 5 ml of SBF, which was also placed in beaker B. The discs were weighted after 1 hour of drying at room temperature followed by vacuum desiccator overnight. However, beaker B was placed into an oven at 90°C for 24 h to allow evaporation and then weighted at room temperature. The whole procedure was repeated after 1 and 4 weeks. The leachate (amount of sealer dissolved from the specimen) was calculated by recording the difference between the original mass of B and its final mass. Solubility was calculated by expressing the mass of the leachate as a percentage of the original combined mass of the specimens.

Fracture resistant test (setup):

The samples were prepared according to the different combinations of dentin conditioning/obturation protocols (Table 1). The filled specimens were embedded in self-curing resin (SR-Ivolen, Ivoclar-Vivadent, Lichtenstein). Note in green, a 0.2 mm silicone barrier (AquasilLV, Dentsply DeTrey GmbH, Germany) surrounding the roots to mimic the periodontal ligament (PDL). The samples were submitted to a compressive force with a 6.3 mm ball-indenter, along the long axis of the root at the crosshead speed of 1 mm/min until fracture (Instron, Canton, MA) (Fig: 1S). A 6.3 mm ball-indenter was chosen in order to contact with at least 85-90% of the obturation-dentin interface. The resistance to fracture was equal to the maximum compressive load recorded.

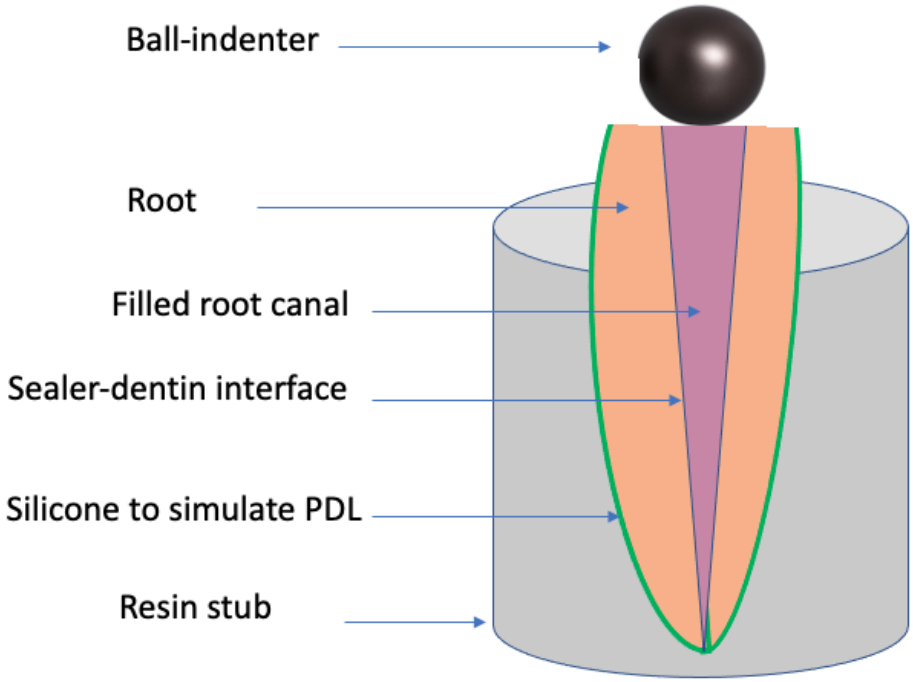


Figure 1S: Schematic diagram showing the experimental setup during fracture resistance study