Percentage Exposure of Root Dentin Collagen After Application of Two Irrigation Protocols with Manual or Rotary Instrumentation and Two Methacrylate Resin-based Sealers

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Purpose: To compare the percentage of collagen exposed in dentin root thirds after two irrigation protocols with manual or rotary instrumentation using two methacrylate resin-based sealers.

Materials and Methods: Forty-eight single-root human teeth were prepared with manual (n = 24) or nickel-titanium ProFile rotary (n = 24) instrumentation, using 5% NaOCl between instruments and 5 ml 17% EDTA as final irrigant or 20% citric acid + 2% chlorhexidine (CHX) between instruments and as the final irrigant. RealSeal or EndoREZ were used as filling materials. One 1-mm slice per third was abraded and stained with Masson’s tri-chrome method. Mean exposed collagen values were obtained in four areas from each section (at 60X magnification) and a complete factorial ANOVA was used to analyze the influence of the study variables. Non-parametric Mann-Whitney’s test was used to compare groups. Differences with p < 0.05 were considered significant.

Results: A significantly higher percentage of collagen was exposed in all thirds with the use of the 20% citric acid + 2% CHX protocol with rotary vs manual instrumentation, but percent collagen exposed did not differ as a function of the filling material. After the 5% NaOCl + 17% EDTA protocol, the percentage of collagen exposed did not differ between rotary and manual instrumentation but was higher with the use of RealSeal.

Conclusion: The highest percentage exposure of collagen was with 20% citric acid + 2% CHX using rotary instrumentation, regardless of the filling material.

Keywords: citric acid, chlorhexidine, collagen exposure, EDTA, Masson’s trichrome, methacrylate resin-based sealers.

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Methacrylate resin-based sealers have been developed in an attempt to seal the root canal system more effectively and thereby improve the bonding to radicular dentin. Their utilization requires removal of the smear layer and collagen exposure, because the retention is largely achieved by micromechanical interlocking between the collagen matrix and resin. Ethylenediamine tetraacetic acid (EDTA) has been widely used for this purpose and is recommended by manufacturers as the final irrigant before applying methacrylate resin-based sealers.

It has been reported that utilization of EDTA or other demineralizing agents as a final rinse before the application of a sealer (non-etching or self-etching) produces a thick, partially demineralized area on the dentin surface. The completeness of resin infiltration into the demineral-
imal activities.26 In contrast, CHX and citric acid (CA) are dissolution capability and, to a lesser extent, antimicrobials, and CHX have been reported to reduce the tissue Furthermore, antagonistic interactions among NaOCl, chelators, which result from the demineralizing effect of the acids or chelants removes these elements from collagen on the entire dentin surface of the root canal is essential to obtain the hybrid layer with methacrylate resins. Sodium hypochlorite (NaOCl) is recommended as the main endodontic irrigant because of its ability to dissolve organic matter, but its removal of exposed collagen can compromise hybrid layer formation. Furthermore, antagonistic interactions among NaOCl, chelators, and CHX have been reported to reduce the tissue dissolution capability and, to a lesser extent, antimicrobial activities.26 In contrast, CHX and citric acid (CA) are readily mixed and do not show antagonistic reactions; together they possess a demineralizing capacity similar to that of 17% EDTA.16 Moreover, they expose sufficient collagen for hybridization and protect collagen fibrils that are suboptimally infiltrated by the adhesive agent during hybrid layer formation due to the inhibition of host-derived MMPs by CHX.34,36

Masson’s trichrome staining technique has proven useful to identify exposed collagen. The dye has a high affinity for cationic elements of normally mineralized type I collagen, which it stains green. Etching of dentin with acids or chelants removes these elements from collagen peptide chains, producing a red color. This allows the ready detection of demineralized dentin (red band) and conical formations at the outer extremes of dentinal tubules, which result from the demineralizing effect of the acid on the peritubular dentin.3 We could find no study that has quantified the extent of the partially demineralized layer produced by demineralizing solutions on the dentin surface. Thus, our null hypothesis was that no difference would be found in the percentage of exposed collagen between the application of two irrigation protocols (5% NaOCl between instruments + 17% EDTA as final irrigant; or 20% CA + 2% CHX between instruments and as final irrigant) as a function of the type of biomechanical preparation or filling material. The objectives of the study were to determine the percentage of collagen exposed in the dentin of each root third after application of these two irrigation protocols with manual or mechanical rotary instrumentation, using RealSeal (RS) (SybronEndo; Glendora, CA, USA) or EndoREZ (ER) (Ultradent; South Jordan, UT, USA) methacrylate resin-based sealers.

MATERIALS AND METHODS

We used 48 human maxillary incisors recently extracted for periodontal reasons from anonymous subjects under a protocol approved by the Ethics Committee of the School of Dentistry of Granada University. All teeth had single straight root canals and closed apices, and none showed carious lesions or had received restorative or root canal treatment. After extraction, they were stored in 0.1% thymol solution at 4°C and used within 3 months. They were washed in running water to remove any traces of thymol, and the coronal portion was removed with a water-cooled diamond disk to yield 13-mm-long root segments. The working length was visually determined by subtracting 1 mm from the length of a size 10 K-file (Dentsply Maillefer; Ballaigues, Switzerland) at the apical orifice.

Roots were randomly divided into two groups (n = 24 each): one group underwent manual biochemical preparation with Hedstrom files (Dentsply Maillefer) to the working length of a final file size of 40; the other was prepared by means of a nickel-titanium Profile Rotary Instrument System with an X-Smart motor (Dentsply Maillefer) at 300 rpm, using the files in a crown-down manner to the working length of a final file size of 40 (0.04 taper). Each group of specimens was then randomly divided into two subgroups for one of two irrigation protocols. In protocol 1, 2 ml 5% NaOCl (Panreac Química; Barcelona, Spain) was used for irrigation between instruments for 1 min, and the root canal was then flushed with 5 ml 17% EDTA (MD-Cleanser, Meta Biomed; Cheongju City, Korea), which was left in place for 1 min. In protocol 2, 2 ml of a solution of 20% CA and 2% CHX (Guainama; Valencia, Spain) was used to irrigate between instruments for 1 min, and the root canal was then flushed with 5 ml of the same solution, which was left in place for 1 min. In both subgroups, the irrigation was applied via a 27-gauge Monoject endodontic needle (Sherwood Medical; St Louis, MO, USA) placed as far into the canal as possible. Finally, all specimens were irrigated with 5 ml of distilled water to avoid the prolonged effect of the demineralization solutions. Each irrigation protocol group was then randomly divided into two subgroups (n = 12) according to the root canal sealer used: RS or ER. For the subgroups filled with RS, the root canal was dried with paper points, and the self-etching primer (RS Primer; SybronEndo) was placed in the canal using the applicator supplied in the kit. The excess was then removed with dry paper points and the sealer was directly dispensed from the tip of the automix dual-chamber syringe, following the manufacturer’s instructions. Roots were completely filled with RS sealer, using a single gutta-percha cone technique. Next, the coronal surface of the root filling was light cured with an 800 Spectrum halogen light at 760 mW/cm² (Dentsply; Milford, DE, USA) for 40 s to create an immediate coronal seal, in accordance with manufacturer’s recommendations. For the ER-filled subgroups, the canal was dried with paper points, and the ER was mixed in an Ultra-Mixer (Ultradent) and then dispensed using a narrow-diameter syringe (Skini Syringe, Ultradent).
with a fine-tipped NaviTip cannula (Ultradent). Roots were completely filled with ER sealer using a single gutta-percha cone technique, and the coronal root access was sealed with Cavit (3M ESPE; Seefeld, Germany) to ensure that the setting was not inhibited by the presence of oxygen. They were then stored in a humidifier at 37°C for 24 h for complete sealer setting.

Nine sections were obtained from each root; one from the middle of each third (cervical, middle, and apical) was selected. The medial surface of each slice was attached to a glass holder with the photocuring adhesive Technovit 7210 VLC (Heraeus Kulzer; Wehrheim, Germany), and the slice was then abraded using silicon carbide disks (WS-Flex 18-B, Struers, Denmark) of decreasing grit size (800, 1000, 1200, and 4000) in a polisher (Exakt, Apparatebau Norderstedt, Germany) to a thickness of approximately 10 μm. Slices were then stained with Masson’s trichrome and examined under a light microscope (BX51 Olympus; Tokyo, Japan) at an initial magnification at 4X, followed by the selection of four areas in each section (distal, vestibular, mesial, and lingual) with 12 areas per specimen for study at 60X magnification. Perfect Image V7-7 software (Clara Vision; Verrieres Le Buisson, France) was used to obtain the percentage of red-stained band (corresponding to demineralized dentin) in each field, and the mean value for the four fields was calculated. The reading was done by a single examiner. Intraexaminer reproducibility was evaluated by double determination of the stained areas in 20% of the sample specimens, yielding an intraclass correlation coefficient of 0.977 (p < 0.001). The demineralization depth was also recorded, as were characteristics of the interface with the sealer, and the presence/absence of funnel-shaped formations at the outer extremes of the dentinal tubules.

A complete factorial ANOVA was used to analyze the influence of the study variables on the percentage of exposed collagen. The non-parametric Kruskal-Wallis and Mann-Whitney tests were used to compare among groups. Differences with p < 0.05 were considered significant.

RESULTS

Complete factorial ANOVA with the percentage of exposed collagen as the dependent variable showed that the irrigation protocol (F = 173.374; p < 0.001), obturation material (F = 36.636; p < 0.001), and interaction between them (F = 43.593; p < 0.001) all exerted a significant influence. A first-order interaction was also found between the irrigation protocol and the type of instrumentation. The remaining variables and interactions analyzed did not prove significant.

Table 1 shows the mean (standard deviation [±SD]) percentage of collagen exposure in each group. A significantly higher percentage of collagen was exposed in all thirds (cervical, middle, and apical) with the use of 20% CA + 2% CHX vs 5% NaOCl + 17% EDTA.

When 20% CA + 2% CHX was used, the mean percentage of exposed collagen in the whole sample and apical third was significantly higher with rotary than with manual
When 5% NaOCl + 17% EDTA was used, no significant differences were found between rotary and manual instrumentation in the percentage exposed (ca. 30%), but a 6-fold higher percentage of collagen was exposed with RS than with ER (Table 1).

The percentage of exposed collagen with the 20% CA + 2% CHX vs 5% NaOCl + 17% EDTA irrigation protocol increased 2.8-fold when rotary instrumentation was used and 2.3-fold when manual instrumentation was used.

When ER was used as the filling material, a significant difference was obtained as a function of the irrigation protocol (9x higher with 20% CA + 2% CHX; Table 1), and the percentage collagen exposure was significantly higher with rotary (91.5% ± 10.1%) than with manual (70.8% ± 28.5%) instrumentation (p < 0.05). This percentage was significantly higher (p < 0.05) than was obtained with 5% NaOCl + 17% EDTA using manual (12.8% ± 15.2%) or rotary (4.2% ± 9.3%) instrumentation.

When RS was used as the filling material, significant differences in percentage collagen exposure were obtained as a function of the irrigation protocol (Table 1) with both manual (52.6% ± 32.7% with 5% NaOCl + 17% EDTA vs 75.6% ± 23.4% with 20% CA + 2% CHX; p < 0.05) and rotary instrumentation (57.5% ± 27.6% with 5% NaOCl + 17% EDTA vs 82.7% ± 17.5% with 20% CA + 2% CHX; p < 0.005). No significant differences were found with either irrigation protocol as a function of the instrumentation method.

The percentage collagen exposure was similar between the utilization of ER and RS when the irrigation protocol was 20% CA + 2% CHX. As shown in Table 1, the lowest percentage collagen exposure (<10%) was obtained by using 5% NaOCl + 17% EDTA with ER sealer with either
rotary (4.2% ± 9.3% vs 57.5% ± 27.6% for RS) (p < 0.001) or manual (12.8% ± 15.2% vs 52.6% ± 32.6% for RS) (p < .001) instrumentation.

No difference was observed in the percentage collagen exposure among the root thirds (cervical, middle, and apical) with either irrigation protocol, regardless of the type of biomechanical preparation or obturation material.

Representative light microscopy images of Masson’s trichrome-stained sections of the resin/dentin interface are shown in Fig 1 to 9; the amount of exposed collagen appears as a red line at the interface between root dentin.
and obturation material. Continuous and discontinuous patterns of collagen exposure were observed with both irrigation protocols. With 5% NaOCl + 17% EDTA, discontinuous patterns of exposure predominated in all thirds, and the images show a wide variability in the presence of the red line at the interface, with some showing no line and others showing a red line along most of the interface or a discontinuous pattern. Varied degrees of exposure were observed within the red lines, from partial exposure to exposure of the entire intertubular dentin, and areas differed in the depth of intratubular collagen exposure.

The characteristics of the collagen did not vary as a function of the instrumentation but differed according to the obturation material. A higher frequency of red lines and a greater exposure of the entire intertubular dentin and varying depths of the intratubular collagen were observed with RS than with ER.

Continuous patterns were more frequent in all studied areas and all root thirds with the use of 20% CA + 2% CHX vs 5% NaOCl + 17% EDTA. The former irrigant exposed the entire intertubular dentin and varying depths of the intratubular collagen; typical funnel-shaped formations and only a few areas without collagen exposure could be observed. The depth of collagen exposure was greater with RS than with ER.

With both irrigation protocols, the utilization of RS (vs ER) produced a better interface between obturation material and dentin, with virtually no gaps in areas of exposed or unexposed collagen, and a gray layer corresponding to the adhesive was visible in many areas. When ER was utilized, some gaps could be seen. In general, parts of the canal wall that had not been instrumented usually coincided with non-exposed areas.

DISCUSSION

A significantly higher percentage of collagen was exposed in all root thirds, using two instruments that differ in their action, ProFile rotary system or manual Hedström files, when 20% CA + 2% CHX was used rather than 5% NaOCl + 17% EDTA as an irrigant. This result may be explained by the fact that 20% CA + 2% CHX was used throughout the biomechanical preparation, whereas 17% EDTA was only used as final irrigant for 1 min. With the 5% NaOCl + 17% EDTA irrigation protocol, around 30% of the total examined area was exposed, an inadequate proportion for adhesion, which can probably only be achieved at remaining adhesive interfaces through interaction with the smear layer or penetration of the resin into dentin tubules. The final irrigation with EDTA for only 1 min appears inadequate for removing the smear layer and exposing the collagen in a uniform manner.

One report found that EDTA removed the smear layer and consistently produced a 1- to 1.5-μm-thick area of partially demineralized root dentin at the resin/dentin interface. However, the specimens were placed into an ultrasonic cube with 17% EDTA for 2 min on a flat surface, which is not comparable with the real situation in the root canal, where the interaction of demineralizing agents varies on different areas of the dentin and a constant, uniform collagen exposure cannot be achieved, potentially compromising the success of the root filling. In addition, a certain degree of dentinal sclerosis can be assumed in these teeth, because they were extracted from patients with advanced periodontal disease. The presence of sclerosis may influence the action of the irrigants under study, and it is more frequently observed on mesial and distal than bucco-lingual aspects of the root. We addressed this potential limitation by ex-
amining four areas in each slice (distal, vestibular, mesial, and lingual) from each root third.

In the present study, rotary instrumentation enhanced the demineralizing effect of the irrigants, since the constant revolutions favored their contact with the canal walls. When 20% CA + 2% CHX was used, the collagen was exposed and available for the hybrid layer on almost 90% of the dentin surface with rotary instrumentation and only 73% with manual instrumentation. However, when 5% NaOCl + 17% EDTA was used, the percentage obtained (only around 30%) did not differ as a function of the instrumentation technique, perhaps because 5% NaOCl alone was used between instruments and the EDTA was left to act for only 1 min without agitation.

Low bond-strength values have been reported for methacrylate resin-based sealers, and even gutta-percha has shown significantly higher push-out bond strength in comparison to Epiphany sealer.39 One plausible explanation is that debonding of the sealer may result from polymerization shrinkage stresses along the root dentin/sealer interface.5 These stresses are exacerbated inside the root canal, because the bonding area is large in relation to the volume of filling material, and the canal walls cannot compensate for shrinkage by elastic deformation.33 However, bond strength is only one aspect of the quality of root canal sealing, and it may not correlate with microleakage or gap formation at the cavity margin.30 This lack of correlation may be attributable to the wide variability in the percentage of exposed collagen, which depends on the type of biomechanical preparation, irrigation protocol, duration of the instrumentation, duration of final irrigation, and the obturation material used, among other factors. Furthermore, it has been reported that prolonged exposure to EDTA6,32 and the combined use of hypochlorite and EDTA21 can weaken the tooth and induce cracks, depending on the depth of erosion, the root thickness, and the amount of sclerotic dentin in the root. However, resistance to fracture and root strength depends on the type of biomechanical preparation, instrumentation technique, perhaps because 5% NaOCl alone was used between instruments and the EDTA was left to act for only 1 min without agitation.

When the 5% NaOCl + 17% EDTA protocol was used, a higher percentage of collagen was exposed with RS than with ER sealer. ER does not utilize a dentin bonding system, and the generation of an endodontic seal is dependent on the penetration of the hydrophilic sealer into the dentinal tubules and lateral canals after smear layer removal.19 Leakage and morphological studies showed that the seal was mediocre with the ER system, although long resin tags were observed.2,28 In the present study, more gaps were seen at the interface with ER than with RS. RS utilizes dentin adhesive technology, and its acidic primer penetrates through the smear layer and demineralizes the superficial dentin. This may explain why 5 to 6 times more collagen was exposed with RS than with ER in the present study.

On parts from which the smear layer has not been completely removed, RS primer augments the areas of exposed collagen, although it was reported that RealSeal SE might not be sufficiently aggressive to achieve optimal dentin bonding to root canal walls at sites that cannot be reached by calcium chelating irrigants.20 According to our findings, the use of RS primer produces an increase of 46% in the amount of collagen exposed when 5% NaOCl + 17% EDTA is used but has no effect on the extent of collagen exposure when 20% CA + 2% CHX is the irrigant. Theoretically, the use of a self-etching primer eliminates the possibility of a discrepancy between the depth of dentin demineralization and primer/adhesive penetration, given that the two processes are concurrent.24 However, this behavior may be distinct in the root canal, because the previous application of demineralizing agents during the biomechanical preparation means that the exposed collagen may not be totally infiltrated by self-etching adhesives. The effectiveness of demineralization/infiltration varies among dentin adhesive systems, which helps to explain the wide variability of published results.27 After irrigation with 20% CA + 2% CHX, collagen is exposed on the majority of the surface, and the application of RS, which acts on collagen-exposed areas, increases the depth of exposure in both intertubular and intratubular dentin. It has been reported that prolonged etching times may create a demineralized zone that is too deep for effective resin infiltration,38 resulting in a weaker bond and accelerated degradation.

This may be a major drawback with the utilization of ER because it uses no type of primer/adhesive; therefore, the exposed collagen is only compacted and is more readily degraded because it is not encapsulated by adhesive. Instrumented mineralized intraradicular dentin possesses detectable collagenolytic activity and may adversely affect the longevity of bonded root canal fillings in the same way as conventional bonding for restorations when self-etching adhesives are used,34 accelerating degradation of the bond by the movement of fluid between the hybrid layer and unaffected dentin.30,23 It has also been reported that red-stained areas of completely demineralized tooth sections studied with the trichrome technique correspond to areas with denatured collagen13,37 and intense expression of MMP-2,37 favoring the degradation of collagen not totally encased in adhesive.

NaOCl is the only irrigant with tissue dissolving capacity; therefore, its utilization is essential during early stages of instrumentation, when the smear layer on the canal walls may have a relatively high organic content from necrotic and/or viable pulp tissue in the root canal.7 A mixed technique may be useful when using methacrylate resin-based sealers to ensure that sufficient collagen is exposed to form the hybrid layer, irrigating with NaOCl during the initial phases of biomechanical preparation and with 20% CA + 2% CHX during the final phase. Although CHX is not able to dissolve tissue, it possesses bactericidal activity and the ability to precipitate and coagulate bacterial intracellular constituents.39 Furthermore, its antibacterial action persists in the root canal for 12 weeks after its use.40 CHX is also an MMP inhibitor that can arrest degradation.
of the hybrid layer in vivo.\(^1\) It has been suggested that the positively charged CHX molecules compete for MMPs with adhesive technology,\(^3\) due to the high probability of non-encapsulated areas of exposed collagen. It may be possible to incorporate MMP inhibitors in future methacrylate resin-based sealer systems.

Many questions remain unanswered on the use of methacrylate resin-based sealers in conjunction with adhesive root-filling materials. Further research is warranted to optimize the demineralizing power of irrigants in order to favor the adhesion of methacrylate resin-based sealers and their interaction with MMP. It was recently reported that 17% EDTA inhibits endogenous MMP activity of human dentin within 1 or 2 min,\(^3\) and CHX is a more effective MMP inhibitor than EDTA.\(^3\) However, the interaction of phosphoric acid (PA) with MMPs is controversial. One group found that dentin MMP activity was increased by PA,\(^1\) whereas Mazzoni et al.\(^2\) reported that it was decreased by PA under different experimental conditions. Studies are also required on the deterioration of dentin bonding over time and on the inhibitory effect of CHX on collagen degradation in the root.

The null hypothesis of the study is not supported by the results, because the amount of exposed collagen differed as a function of the irrigation protocol, type of instrumentation, and methacrylate resin-based sealer applied.

**CONCLUSION**

Under the conditions of the present study, the highest percentage of collagen was exposed with 20% CA + 2% CHX as the irrigant and rotary instrumentation, regardless of the filling material used. When 5% NaOCl was used, with 17% EDTA as final irrigant, the type of biomechanical preparation showed no influence, but the percentage of exposed collagen was higher with RS than with ER.

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Clinical relevance: The percentage of exposed collagen depends on the irrigation protocol, type of instrumentation, and methacrylate resin-based sealer used.