

**Influence of root maturity or periodontal involvement on
dental collagen changes following NaOCl irrigation: an ex-
vivo study**

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Abstract

Aims: To refine FTIR protocol for detection of NaOCl-induced dentinal collagen changes using an *ex-vivo* irrigation model, and to apply it to determine the collagen change within 0.5mm of canal or root surfaces, with or without mature roots or periodontal involvement.

Methodology: Extracted human roots were irrigated with control saline (n=3) or 5% NaOCl (n=3) and sectioned into transverse discs for FTIR analyses, 0.5mm from both the canal lumen and root surface, before and after surface-treatment with 17% EDTA. Amide I/phosphate and amide II/phosphate absorbance ratios were compared using [the](#) Wilcoxon sign rank test. Mature roots without periodontal involvement were irrigated with: saline (n=7), 5% NaOCl (n=7), or 5% NaOCl+17% EDTA (n=7); those with periodontal involvement (n=7) or immature roots (n=7) were irrigated with 5% NaOCl. Dentine discs were then prepared for FTIR analyses. The effects of irrigant/root-maturity/periodontal involvement were analysed using linear mixed models.

Results: FTIR analyses of the irrigated samples revealed significant ($P < 0.05$) reduction in collagen bands near the canal lumen after NaOCl irrigation using surface-EDTA treated samples. Irrigation with test solutions resulted in significant ($P < 0.0001$) dentinal collagen changes in the mature roots, whilst those in the immature roots were significantly ($P < 0.05$) greater compared with the mature roots with or without periodontal involvement; but there were no difference between the latter groups.

Conclusion: EDTA surface-treatment of polished dentine surfaces enhanced FTIR detection of NaOCl-induced collagen changes. Both root maturity and irrigation protocol influenced the ability of NaOCl to alter dentinal collagen up to 0.5mm from the canal lumen.

Introduction

The use of sodium hypochlorite and ethylenediaminetetraacetic acid for root canal debridement is known to weaken dentine (Sim *et al.* 2001, Grigoratos *et al.* 2001, Rajasingham *et al.* 2010) through chemical changes in the organic and inorganic phases of the tissue root dentine (O'Driscoll *et al.* 2002, Pascon *et al.* 2012, Ramirez-Bommer *et al.* 2018) and is a deemed an unintentional consequence risk during root canal treatment.

Fourier Transform Infrared (FTIR) spectroscopy has been successfully employed to assess changes caused by NaOCl within dentine (Di Renzo *et al.* 2001a,b, Amarie *et al.* 2012, Tartari *et al.* 2017, Ramirez-Bommer *et al.* 2018). A key effect of NaOCl on dentine is denaturation of dentinal collagen and the organic component of the dentine matrix (Hu *et al.* 2010, Zhang *et al.* 2010a,b, Tartari *et al.* 2016). By monitoring changes in the IR absorption frequencies following NaOCl exposure, it is possible to assess the depth of any changes due to root canal irrigation (Morgan *et al.* 2019).

EDTA chelates calcium ions in hydroxyapatite crystals and facilitates demineralisation of dentine to approximately 20-50 μm in the root canal system (von der Fehr & Östby 1963, Fraser 1974, Verdelis *et al.* 1999). Di Renzo *et al.* (2001b) used photoacoustic FTIRS to examine deproteination by NaOCl and found rapid removal, within 2 mins, of exposed collagen but much slower removal of unexposed (covered by hydroxyapatite) collagen. Ramirez-Bommer *et al.* (2018) estimated the depth of effect in dentine alternately exposed to NaOCl and EDTA using FTIR and found that NaOCl reduced the collagen content of pulverised dentine powder rapidly within the first four minutes of reaction, leading to a plateauing effect, thereafter. Conversely, EDTA continuously reduced the phosphate content of dentine over twenty-four hours and exposed the collagen content in the process. The depth of hypochlorite reaction was 16 ± 13 μm after 10 minutes exposure. The depth of EDTA reaction increased with duration of exposure (19 ± 12 μm by 10 minutes, 27 ± 13 μm by 60 minutes, and 89 ± 43 μm by 24 hours) and also by pre-treatment with NaOCl (62 ± 28 μm by 10 minutes). NaOCl/EDTA/NaOCl alternated treatment resulted in an estimated further 62 ± 28 μm plus 7 ± 4

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3 µm thick collagen-depleted surface compared to the 16±13 µm depletion by initial NaOCl
4 treatment, alone. Di Renzo *et al.* (2001), likewise, found that maleic acid demineralised dentine
5 uniformly below the surface.
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10 Morgan *et al.* (2019) established an FTIR protocol for judging the depth of effect of NaOCl
11 irrigation in teeth. NaOCl was found to degrade the structure of the collagen triple helix within
12 dentine, however, the presence of the mineralised matrix appeared to offer some protection
13 from this degradation. Based on the *ex-vivo* experimental model used, the effect of NaOCl on
14 dentinal collagen extended to at least 0.5 mm into ~~the~~ dentine.
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21 It was hypothesised that the depth of collagen denaturation may be affected by the patency
22 of the dentinal tubules, leading to an irregular boundary, rather than by direct progressive
23 degradation of the intertubular dentine matrix that may result in a more distinct boundary. It
24 was speculated that comparison of the depth of effect on collagen in mature *versus* immature
25 teeth, as well as those periodontally involved (possibly having greater dentinal sclerosis), may
26 help to answer this question. Prior to testing this hypothesis, it was decided to refine the FTIR
27 protocol used by Morgan *et al.* (2019) further, to ~~refine~~ enhance signal detection by surface
28 treatment of the dentine with EDTA to reduce any smear layer left after polishing the sample.
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Materials and Methods

Collection and storage of teeth

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3 Ethical approval was granted for the use of extracted teeth from the UCL Eastman Biobank
4 (Study number: 1301). Intact single-rooted premolars with mature roots (apical constriction <
5 0.7 mm, Andreasen *et al.* 1986) and absence of periodontal disease, teeth with mature apices
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7 0.7 mm, Andreasen *et al.* 1986) and absence of periodontal disease, teeth with mature apices
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9 with extensive loss of periodontal support, and teeth with immature roots (apical constriction
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11 >1.2 mm, Andreasen *et al.* 1986) extracted for orthodontic reasons were collected. They were
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13 stored in 70% ethanol and kept in a refrigerator at 5°C until processing. Those free of caries
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15 and cracks based on inspection under an operating microscope at ×6 magnification (Urban
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17 microscope, DP Medical Systems, Chessington, UK) were selected for experimentation.

18 19 ***Effect of EDTA pre-treatment of sample surfaces on FTIR detection of dentinal collagen*** 20 21 ***changes*** 22

23 24 *Selection and preparation of teeth* 25

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27 Six intact single-rooted premolars with mature roots (apical size < 0.7 mm) were randomly
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29 allocated to saline (control) (n = 3) or NaOCl (n = 3) irrigation groups. The teethpulp chambers
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31 were accessed to allow straight-line access to the canals, their lengths determined by
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33 advancing a file until visible at the apex and the canals prepared to 0.5 mm short of the canal
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35 terminus. A combination of Flexo-files® and rotary ProTaper® instruments (Dentsply-
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37 SironaMaillefer, Ballaigues, Switzerland) were used according to manufacturer's instructions
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39 to a size and taper corresponding to the F3 instrument. Patency filing was used in all teeth
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41 and deionised water as the irrigant between each instrument, with the teeth hand-held and
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43 maintained moist throughout.
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47 Following completion of ~~the~~ canal preparation, the apices were sealed with composite resin
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49 (XRU Herculite®, Kerr Corporation, Orange, CA, USA) and embedded in clear epoxy resin
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51 (Specifix 40®, Struers Ltd., Solihull, UK) using a cylindrical mould. Upon completion of resin
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53 set, the cusp tips were flattened with a diamond bur to provide a reproducible reference point.
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55 The prepared canals were then irrigated with saline (n = 3) or 5% NaOCl (confirmed by
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57 iodometric titration) (n = 3) according to a standardised protocol using a 3 mL Monoject®
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59 syringe (Tyco Healthcare, Gosport, UK) with a 30 gauge Maxi-Probe® needle (Dentsply Rinn,
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3 Elgin, IL, USA). A rubber stop on the needle allowed accurate placement of the tip, 7 mm from
4 the coronal reference. The canals were irrigated with 3 mL of 5% NaOCl or saline, as
5 designated, by emptying the solution at the predetermined level in a delivery time of 1 minute
6 with a push-pull motion of the needle. The canals were then left to soak in the irrigant for a
7 further 4 minutes. This process was repeated four more times. There was a total exposure
8 time of 25 minutes and a volume of 15 mL irrigant. The canals were then washed with 6 mL
9 of deionised water.
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17 Preparation of dentine discs for FTIR analysis

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20 Following irrigation, the tooth roots within the resin cylinders were ~~transversely~~ sectioned
21 ~~transversely~~ into dentine discs at 6 mm and 9 mm below the coronal reference point using a
22 diamond microtome (Leica Model 1600®, Leica, Wetzlar, Germany) to create a single 3 mm
23 thick disc. The disc was then polished on the coronal side with Struers Labopol 5® (Struers
24 Ltd., Solihull, UK) with increasingly fine grade silicon carbide abrasive discs from 500 to 2400
25 grit and diamond polishing paste (Diapro®, Struers Ltd., Solihull, UK). Two lines were scored
26 on the sectioned and polished surfaces with a scalpel blade from the canal lumen towards the
27 cemento-dentinal junction. The two lines were designated A (lingual) and B (buccal). The discs
28 were then ultrasonicated for 30 minutes in deionised water and dried with paper towels (Lotus
29 Hand Towels, Georgia Pacific, Bolton, UK). The lengths of the lines were measured and
30 considered as the thickness of the dentine on the respective sides.
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43 ATR-FTIR analysis of the sectioned and polished surface

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46 ATR-FTIR analysis was carried out using the Perkin Elmer 2000® FTIR spectrometer with an
47 ATR accessory SensIR Technologies DuraScope® (SensIR Technologies, Danbury, CT,
48 USA) following the protocol described ~~by~~ Morgan *et al.* (2019). ATR-FTIR is used to probe
49 surface properties of materials rather than their bulk properties. The penetration depth, or the
50 depth from which the IR signal is generated, is in the range of microns. The DuraScope® is a
51 small diamond ATR accessory fitted with a zinc selenide internal reflectance element (IRE)
52 held at 45° to the incident beam. This ATR accessory gives access to a 38× video-magnified
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3 image of the sample and thus allowed ~~us to carefully~~ positioning of the samples in relation to
4 the IRE. An integrated force readout system allows the user to reproducibly return to the same
5 pressure each time. ATR spectra were recorded with the polished side of the sample in contact
6 with the IRE (contact pressure: 3 bar) and at two points along each engraved line on the
7 sample, the first 0.5 mm from the canal lumen (inner) and the second 0.5 mm from the root
8 surface (outer). These markers were used to accurately place the sample under the IRE with
9 the help of the video-magnified image. The FTIR sampling area around each point had a
10 radius of 0.5 mm. The amount of overlap of the FTIR sampling sites (radius 0.5 mm) was
11 therefore evaluated. If the dentine thickness was less than 2 mm, it was considered that the 2
12 sampling sites overlapped. Spectra were recorded in the range 4000 to 800 cm^{-1} . Twelve
13 scans were obtained for each spectrum with a resolution of 4 cm^{-1} .

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Once all the spectra had been recorded, they were analysed using GRAMS32 AI® (Thermo Scientific, Waltham, MA, USA) software as described ~~by~~ in Morgan *et al.* (2019). The peak maxima absorbance for the amide I (1650 cm^{-1}), amide II (1550 cm^{-1}), and phosphate (1010 cm^{-1}) peaks were recorded from the spectra which were all offset from 0 to 1800 cm^{-1} prior to recording the absorbance.

FTIR analysis of the sectioned and polished surface with 17% EDTA surface treatment

The sectioned and polished surfaces of all samples were then treated with 17% EDTA solution prepared following a standard protocol. One millilitre of EDTA was pipetted onto the polished transverse surface and left for 30 seconds before being washed off with copious amounts of deionised water. The discs were blotted dry with paper towels. The specimens were again subjected to FTIR analysis. The peak maxima absorbance for the amide I (1650 cm^{-1}), amide II (1550 cm^{-1}), and phosphate (1010 cm^{-1}) peaks were recorded as previously described.

Determination of the NaOCl effect on collagen within 0.5 mm of canal wall or root surface depth

Allocation and preparation of samples

Teeth with mature roots and absence of periodontal disease (mature-NP); teeth with mature apices with extensive loss of periodontal support (mature-P) and teeth with immature roots were randomly allocated to one control and four experimental groups (n = 7 each; Table 1).

All pulp chambersteeth (n = 35) were accessed, root canals prepared and embedded in resin as previously described using deionised water as the irrigant between each instrument. Three different irrigation protocols were tested in the mature-NP roots: saline (control group 1); 5 % NaOCl (groups 2); 5 % EDTA + 17 % EDTA (group 5). The 5% NaOCl protocol was also tested in mature-P (group 3) and immature roots (group 4). The irrigation protocols were performed as previously described; for the 5% NaOCl + 17 % EDTA group, the canals were firstly flushed with 5 % NaOCl (6 mL), followed by 17 % EDTA (3 mL); and finally, with 5 % NaOCl (6 mL). In total, the canals were irrigated with 12 mL of NaOCl and 3 mL of 17% EDTA with a total of 25 minutes contact time.

FTIR analysis

Dentine discs were prepared and labelled and the polished surfaces were treated with 1 mL of 17 % EDTA as previously described. The discs were placed in an ultrasonic bath of deionised water for 30 minutes, removed and blotted dry with clean, sterile paper towels. The dentine thickness from the canal lumen to the root surface was measured for each side of each disc as previously described-; the mean dentine thickness for each group was also calculated. Any overlapping of the inner and outer sampling sites and their extent were also determined. The FTIR spectra for all samples were processed and spectral graphs for each group were visually compared as before. The peak maxima absorbance for the amide I (1650 cm^{-1}), amide II (1550 cm^{-1}), and phosphate (1010 cm^{-1}) peaks were recorded as previously described.

Data analysis

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3 The spectra data were processed using Microsoft® Excel® worksheet 2016 (Microsoft
4 Limited, Microsoft Campus, Thames Valley Park, Reading, UK). The data were analysed using
5 STATA 12 (STATA Corporation: College Station, TX, USA).
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10 The amide I/phosphate and amide II/phosphate absorbance ratios for the outer and inner
11 spectra from sides A and B, respectively, were calculated. From this, the relative outer *versus*
12 inner absorbance ratios for amide I/phosphate and amide II/phosphate ratios were calculated
13 for each side of each specimen, respectively.
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19 Kolmogorov-Smirnov and Shapiro-Wilk tests for Normality (IBM SPSS version 21) were used
20 to test the hypothesis that the data did not fulfil the assumption of normal distribution.
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23 *For evaluation of the effect of sample surface treatment with EDTA on FTIR spectra*, the
24 absorbance ratios of amide I/phosphate or amide II/phosphate before and after surface
25 treatment with EDTA, stratified by the saline and NaOCl groups (n=3), were compared using
26 Wilcoxon signed rank test (IBM SPSS version 21).
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32 *For evaluation of the influence of canal irrigant, and root maturity/periodontal involvement*,
33 firstly non-parametric Kruskal-Wallis test (IBM SPSS version 21) was used to compare the
34 *outer* amide I/phosphate or amide II/phosphate absorbance ratios amongst control and
35 experimental groups to confirm NaOCl irrigation had no effects on the outer sample sites.
36 Secondly, four general linear mixed models (STATA 12) were used to analyse: (1) the effects
37 of irrigant (saline, NaOCl, NaOCl+EDTA) on dentine of mature root without periodontal
38 involvement; and (2) the influence of root maturity/periodontal involvement (immature, mature
39 without periodontal involvement, mature with periodontal involvement) on the effect of 5%
40 NaOCl irrigation on root dentine. The relative outer/inner absorbance ratios of amide
41 I/phosphate or amide II/phosphate were used as the dependant variable whilst the dentine
42 thickness, and the clustering effect of the measurements taken from side A and B of the same
43 specimen and tooth were accounted for in the models.
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Results

Effect of EDTA pre-treatment of sample surfaces on FTIR signal detection of dentinal collagen changes

The extent of overlap between outer and inner sampling sites on dentine discs

The thickness of dentine at each side of each disc (n = 6) ranged from 1.9 to 2.5 mm with a mean greater than 2 mm. Only one side of one sample had dentine thickness of less than 2 mm, indicating overlap of the inner and outer FTIR sampling sites for that sample.

Spectral analysis

The spectra for the saline-irrigated samples before and after 17 % EDTA surface treatment demonstrated no obvious difference between the outer and inner aspects (Figure 1a&b). The spectra for the NaOCl-irrigated samples before surface treatment with EDTA (Figure 2a) demonstrated a similar spectral appearance to the saline-irrigated samples with no obvious difference between the outer and inner sampling sites. However, following EDTA surface treatment, the absorbance intensities for the amide I and amide II bands were visibly lower at the inner sampling site compared to the outer sampling site (Figure 2b).

Comparison of absorbance ratios before and after surface EDTA treatment

The mean values of the amide I/phosphate and amide II/phosphate absorbance ratios at the inner and outer sampling sites, before and after surface EDTA treatment, stratified by saline- and NaOCl-irrigated samples are presented in table 2. Wilcoxon sign rank tests revealed no significant difference between the outer *versus* inner: amide I/phosphate ($P = 0.9$, $P = 0.2$, respectively); or the amide II/phosphate ($P = 0.9$, $P = 0.3$, respectively) absorbance ratios for the saline-irrigated samples, regardless of EDTA surface treatment (Table 2).

For the NaOCl-irrigated group, spectra taken before EDTA surface treatment displayed no significant difference between the inner and outer absorbance ratios for amide I/phosphate ($P = 0.5$), and amide II/phosphate ($P = 0.5$) ratios. However, after EDTA surface treatment, the differences between the inner and outer sampling site absorbance ratios for amide

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3 I/phosphate ($P = 0.03$) and amide II/phosphate ($P = 0.03$) were significant at the 5% level
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5 (Table 2).
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7 ***Depth of NaOCl effect on collagen in teeth with or without mature roots or periodontal***
8 ***involvement***
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12 *The extent of overlap between outer and inner FTIR sampling sites on dentine discs*
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14 The mean dentine thickness for each group was greater than 2 mm, ranging from 2.1 mm for
15 the immature roots to 2.6 mm for the mature roots with periodontal involvement; the thickness
16 of individual sides of dentine discs ranged from 1.6 to 3.2 mm. On 10 sides, the dentine
17 thickness was less than 2 mm, indicating overlap between the inner and outer FTIR sampling
18 sites (1 mm in diameter). These occurred as follows: in the mature roots without periodontal
19 involvement, saline-irrigated group (3/14), NaOCl-irrigated group (2/14), NaOCl+EDTA-
20 irrigated group (2/14); and in the immature roots irrigated with NaOCl (3/14). The amount of
21 overlap ranged from 0.1 mm to 0.4 mm. The mature periodontally-involved roots did not have
22 any sampling site overlaps.
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33 *Spectral analysis*
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35 The spectra for the mature roots without periodontal involvement and irrigated with saline (n
36 = 7) had the same appearance on the inner and outer sampling sites (Figure 3a). There was
37 no visible reduction in either the amide I or amide II absorbance band intensities from the outer
38 to the inner aspects.
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43 The spectra for all other groups presented a different picture. The mature roots without
44 periodontal involvement irrigated with NaOCl ($n = 7$) (Figure 3b) or with NaOCl+EDTA ($n = 7$)
45 (Figure 4c) had the same appearance at the *outer* sampling sites as those for the saline group.
46 However, there was a visible reduction in the amide I and amide II absorbance band intensities
47 at the *inner* sampling sites. A similar picture was evident for the mature roots with periodontal
48 involvement irrigated with NaOCl ($n = 7$) (Figure 3d), and immature roots irrigated with NaOCl
49 ($n = 7$) (Figure 3e).
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58 *Comparison of outer absorbance ratios by groups*
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3 The mean *outer* amide I/phosphate and amide II/phosphate ratios stratified by irrigation
4 protocol, root maturity/periodontal involvement are presented in Figure 5. Kruskal-Wallis tests
5 revealed no significant differences in the *outer* amide I/phosphate or amide II/phosphate
6 absorbance ratios amongst various irrigation groups for mature roots without periodontal
7 involvement ($P = 0.1$, $P = 0.2$, respectively), or amongst roots with various maturity/periodontal
8 involvement and irrigated with 5% NaOCl ($P = 0.7$, $P = 0.4$, respectively), respectively.
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18 Comparison of relative outer/inner absorbance ratios by groups

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20 The mean relative outer/inner absorbance ratios for amide I/phosphate and amide
21 II/phosphate by irrigation protocol, root maturity/periodontal involvement are presented in
22 Figure 6.
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26 Linear mixed models 1a&b (Table 3) revealed irrigation with NaOCl or NaOCl+EDTA resulted
27 in significantly ($P < 0.0001$) higher relative outer/inner absorbance ratios for amide I/phosphate
28 and amide II/phosphate, compared with saline (control) irrigation. Use of NaOCl irrigation
29 alone in mature roots without periodontal involvement resulted in significantly lower relative
30 outer/inner absorbance ratios compared with samples irrigated with NaOCl+EDTA (Coeff = -
31 0.3; 95% CI: -0.4, -0.2 for amide I/phosphate; Coeff = -0.4; 95% CI: -0.5, -0.3 for amide
32 II/phosphate) (*results not shown*).
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41 Linear mixed models 2a&b (Table 3) revealed irrigation with NaOCl in mature roots resulted
42 in significantly lower relative outer/inner absorbance ratios for amide I/phosphate or amide
43 II/phosphate absorbance ratios compared with immature roots, regardless of periodontal
44 involvement ($P = 0.001$ for amide I/phosphate, $P < 0.0001$ for amide II/phosphate) or no
45 periodontal involvement ($P = 0.02$ for amide I/phosphate, $P = 0.001$ for amide II/phosphate).
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47 There was, however, no significant difference between mature teeth with or without
48 periodontal involvement, in the relative outer/inner absorbance ratios for amide I/phosphate
49 ($P = 0.2$) or amide II/phosphate ($P = 0.3$) (*results not shown*).
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3 The linear mixed models also ~~revealed~~ showed that the relative outer/inner absorbance ratios
4 increased with the *thickness of dentine*, but this relationship was only significant at the 5%
5 level for amide II/phosphate ($P = 0.02$ in model 1b, & $P = 0.04$ in model 2b).
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9 There was negligible and insignificant variation between the two sampling sides (A&B) within
10 the same root ($P > 0.1$) (Table 3).
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14 15 16 Discussion

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18 Sample tooth selection was predicated upon the notion that mature disease-free roots
19 extracted for orthodontic reasons, would most likely be sourced from patients of similar age,
20 and have minimal amounts of secondary or tertiary dentine with limited deposition of
21 peritubular dentine. In contrast, mature periodontally involved teeth should have a greater
22 likelihood of tertiary and peritubular dentine (Berkowitz *et al.* 2002), whilst, intact immature
23 teeth should exhibit wide dentinal tubules with no secondary or tertiary dentine deposition
24 (Berkowitz *et al.* 2002). Ethanol (70%) was chosen as the storage medium because of its
25 antibacterial properties, coupled with its lack of effect on dentinal collagen or mineral peaks
26 when analysed by FTIR (Strawn *et al.* 1996).
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30 ATR-FTIR is a surface analysing technique capable of sampling only the surface 1-2 μm . It is
31 therefore crucial that any smear created during preparation is ~~completely~~ removed completely
32 before analysis to improve the signal. Morgan *et al.* (2019) had adopted the protocol from
33 Oliveira *et al.* (2002) and Kubinek *et al.* (2007), who found that the smear layer could be
34 removed with a specified polishing technique and ultrasonication in deionised water. In the
35 present study, it proved less effective, as revealed by SEM in pilot studies (not shown). This
36 was consistent with Tani & Finger (2002) who found that polishing dentine samples with 4000
37 grit paper left a smear layer of 1-2 μm , necessitating modification of the protocol. The first part
38 of the study was therefore designed to test a protocol-refinement incorporating surface
39 treatment of the sample with EDTA.
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3 The amide I and amide II bands were selected rather than the amide III and amide A bands
4 as representative of the organic component because the amide A band was too broad and the
5 intensity of the amide III band too low before treatment with NaOCl, making detection of
6 change more difficult. The amide I vibrational mode has been attributed to a combination of a
7 CO stretch, CN stretch, C"CN deformation and other minor contributions. This vibrational
8 mode is directly related to the collagen "backbone" conformation. The amide II vibrational
9 mode has been attributed to a combination of an NH in-plane bend, CN stretch, C"C stretch,
10 as well as other minor contributions. This mode is conformationally-sensitive, providing
11 information on more subtle structural changes in the collagen secondary structure. The
12 phosphate band was used as the constant, and the spectra baseline corrected and normalised
13 to it, based on previous work (Di Renzo *et al.* 2001, O'Driscoll *et al.* 2002, Ramirez-Bommer
14 *et al.* 2018, Morgan *et al.* 2019) that showed it to be unaffected by NaOCl treatment. The
15 amide/phosphate ratios are a recognised parameter for examining the removal of the organic
16 phase (Eliades *et al.* 1997).

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33 Analysis of the spectra from the saline-treated samples before and after surface treatment
34 with 17% EDTA showed no change in the amide I and amide II absorbance peaks between
35 the outer and inner portions of each disc. The recorded spectra bore a resemblance to those
36 reported by Di Renzo *et al.* (2001) and Morgan *et al.* (2019) for untreated dentine on both the
37 outer and inner sampling sites. When the mean absorbance ratios of the amide I/phosphate
38 and amide II/phosphate from the saline-irrigated samples were analysed there was no
39 significant difference between the outer and inner sampling sites before and after surface
40 EDTA treatment. This implied that the 30 second EDTA treatment had a negligible effect on
41 the amide I/phosphate and amide II/phosphate ratios.

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51 The NaOCl-treated samples before surface EDTA treatment were also similar to untreated
52 dentine (Morgan *et al.* 2019) but EDTA surface treatment revealed a clear reduction in the
53 amide I and II band intensities at the inner sampling site compared to the outer sampling site.
54 The spectra for the inner sites were similar to those recorded by Morgan *et al.* (2019) from
55 dentine disc surfaces treated with 5% NaOCl. Statistical analysis of the 5% NaOCl-irrigated
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3 samples revealed no significant difference between the outer and inner sampling sites in the
4 amide I/phosphate and amide II/phosphate absorbance ratios before surface treatment with
5 EDTA. Statistical analysis of the same samples following surface EDTA treatment revealed a
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7 significant difference for the same two mean absorbance ratios.
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11 The reduction of collagen is linked to amino acid degradation and hydrolysis by NaOCl (Estrela
12 *et al.* 2002). The collagen peak reduction confirmed previous findings (Sakae *et al.* 1998, Di
13 Renzo *et al.* 2001, O'Driscoll *et al.* 2001, Ramirez-Bommer *et al.* 2018, Morgan *et al.* 2019).
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15 In this study, surface treatment with EDTA enabled better FTIR detection of the collagen
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17 changes in dentine, consistent with Komabayashi *et al.* (2008).
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21 Visual comparison of the spectral graphs for each of the main experimental groups was
22
23 suggestive of a difference between the mature roots without periodontal involvement irrigated
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25 with saline *versus* all other groups. Spectra for the saline-irrigated group indicated no change
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27 in the amide band intensities between the inner and outer sampling sites. There was a
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29 reduction in the amide absorbance intensities in the collagen component for the inner sampling
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31 sites for all *other* groups (mature root without periodontal involvement irrigated with NaOCl or
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33 NaOCl+EDTA; mature roots with periodontal involvement irrigated with NaOCl; and immature
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35 roots irrigated with NaOCl). The findings were consistent with other reports (Di Renzo *et al.*
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37 2001, Ramirez-Bommer *et al.* 2018, Morgan *et al.* 2019).
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41 The absence of significant differences between any of the groups at the outer sampling sites
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43 implies that NaOCl did not affect the collagen component in the outer 1 mm of the dentine and
44
45 therefore is unlikely to have penetrated this far. This supports Zou *et al.* (2010), who reported
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47 the depth of penetration of NaOCl to be a maximum of 0.3 mm using the bleaching effect of
48
49 NaOCl on crystal violet dye as a tracer. It also supports Morgan *et al.* (2019) who
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51 demonstrated a similar result using ATR-FTIR.
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54 The mean relative amide I/phosphate and amide II/phosphate absorbance ratios for the saline
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56 group were approximately 1, no change. The relative absorbance ratios for the NaOCl and the
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58 NaOCl+EDTA treated groups were greater than 1, indicative of a reduction in collagen at the
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60 inner sampling site. There was a **statistically** significant difference in the mean relative ratio

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3 for NaOCl+EDTA compared to that for NaOCl alone, the latter being lower. This does not
4 indicate greater penetration of the dentine matrix by NaOCl but a greater reduction in the
5 collagen component in the NaOCl & EDTA group, as previously shown in different models
6 (O'Driscoll *et al.* 2002, Ramirez-Bommer *et al.* 2018). Di Renzo *et al.* (2001) noted similar
7 findings when using NaOCl/maleic acid combination and observed a non-uniform penetration
8 into dentine by virtue of destruction of the exposed vulnerable collagen first, followed much
9 more slowly by degradation of collagen protected by mineral deposition (Forien *et al.* 2016).
10 The increased degradation of collagen may also be explained by increased dentine tubule
11 permeability caused by irrigation with both NaOCl & EDTA (Fogel & Pashley 1990), providing
12 a larger surface area of exposure. Removal of the organic phase in mineralised dentine by
13 NaOCl enhances the permeability (Oyarzun *et al.* 2002, Surapipongputr *et al.* 2008) of both
14 peritubular and intertubular dentine to EDTA, which in turn, demineralises the apatite phase
15 and expedites further NaOCl infiltration and collagen destruction (Mai *et al.* 2010). However,
16 this theory contradicts work by Tao *et al.* (1991) and Mello *et al.* (2009) who found that EDTA
17 irrigation only removed the smear layer without increasing dentine permeability.
18 Increased dentine permeability may be due to removal of the smear layer from the
19 instrumented surface (Mader *et al.* 1984), as well as the smear plugs from dentinal tubules
20 (Nygaard-Østby 1957), allowing greater irrigant penetration. It may also be due to the
21 synergistic effect of NaOCl & EDTA leading to dentinal erosion around the canal lumen (Mai
22 *et al.* 2010, Zhang *et al.* 2010). The collagen is protected by hydroxyapatite (Kinney *et al.*
23 2003, Forien *et al.* 2016) and thus the combined effect of NaOCl and EDTA would allow
24 greater destruction of the matrix structure.
25 The statistical analysis of the mean *relative outer versus inner absorbance ratios* for amide
26 I/phosphate and amide II/phosphate for the teeth with mature or immature roots irrigated with
27 5% NaOCl were all greater than 1; indicating that NaOCl had reduced the collagen component
28 at the inner sampling site. Comparison of the periodontally-involved mature roots with the
29 immature roots showed a significant difference in the mean *relative outer versus inner*
30 *absorbance ratios*. This implied a greater reduction in the collagen component in the immature
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3 group than in the periodontally-involved mature roots. A plausible explanation is that immature
4 roots have limited secondary or tertiary dentine (Berkovitz *et al.* 2002) with patent dentinal
5 tubules, allowing greater NaOCl penetration into dentine (Galvan *et al.* 1994). A further reason
6 may be that primary dentine is less mineralised than secondary and tertiary dentine (Berkovitz
7 *et al.* 2002), leaving the collagen component more exposed to hydrolysis and degradation (Di
8 Renzo *et al.* 2001). Additionally, canals in immature teeth require less enlargement and thus
9 generate less smear layer that could hinder NaOCl penetration and exposure of dentinal
10 collagen to the effects of the NaOCl (Mader *et al.* 1984).

11
12 Although not significant, the mean *relative outer versus inner absorbance ratios* of amide
13 I/phosphate and amide II/phosphate for the mature roots with periodontal involvement was
14 less than that for the periodontally involved mature roots. The trend may be explained by the
15 converse observation that periodontally-involved teeth would be expected to have more highly
16 mineralised, thicker and less permeable tertiary dentine (Galvan *et al.* 1994). Additionally, the
17 matureperiodonally involved roots may have required more canal preparation (dentine
18 removal) than those without periodontally-involved roots, potentially because of greater pulpal
19 calcification and tertiary dentine deposition due to periodontal disease (Langeland *et al.* 1974).
20 A greater proportion of the canal wall would, therefore, be expected to have been planed by
21 endodontic instruments in the periodontally-involved teeth, creating more surface smear layer
22 and thus reduced dentine permeability. The lack of significant effect may be explained by the
23 relative lack of patency of both sets of mature canals.

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25 The 3 mm thick root dentine disc was prepared from the root 6 mm apical to the reference
26 point i.e. just apical to the cemento-dentinal junction for all teeth in order to capture the
27 maximum cross-sectional dentine thickness. Therefore, the irrigant was delivered by inserting
28 the needle tip 7 mm apical to the coronal reference point to ensure maximum dentine exposure
29 to irrigant at the sampling point.

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31 The size of the sampling site (0.5 mm) set a threshold limit for boundary detection but the
32 resolution was considered acceptable for the purposes of this study given the previous
33 estimates of depth of penetration of NaOCl into dentine (Zou *et al.* 2010, Ramirez-Bommer *et*

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3 *al.* 2018, Morgan *et al.* 2019). The sampling zone size was a particular limitation if the two
4 sampling sites overlapped when the overall dentine thickness was narrow. Measurement of
5 dentine thickness, however, allowed any potential confounding due to this to be accounted for
6 in the linear mixed models. In fact, dentine thickness was found to have a significant positive
7 influence on the relative absorbance ratio for amide II/phosphate but not for amide
8 I/phosphate. The significant influence of dentine thickness on relative outer/inner amide
9 II/phosphate absorbance ratios could theoretically be attributed to: (1) subtle native
10 differences in collagen structure between the inner and outer sampling sites; or (2) subtle
11 secondary conformational change extending beyond the 0.5 mm sampling zone due to the
12 irrigant. The first explanation may be rejected given the absence of obvious differences
13 between the outer *versus* inner amide II band intensities in specimens irrigated with saline
14 (Table 2), and the negligible (Coefficient = 0.02) and insignificant ($P = 0.6$) influence of dentine
15 thickness amongst the saline-irrigated specimens (*results of further stratified analyses not*
16 *shown*). The second explanation is supported by the fact that dentine thickness had a positive
17 influence amongst the NaOCl-irrigated (thickness range: 1.6-3.2; Coefficient = 0.25; $P = 0.02$)
18 or NaOCl+EDTA-irrigated (thickness range: 1.9-2.6; Coefficient = 0.26; $P = 0.2$) mature roots
19 without periodontal involvement, although such influence was only significant at the 5% level
20 amongst the former group where a greater range of root dentine thickness allowed the effect
21 of NaOCl to be detected statistically (*results of further stratified analyses not shown*). The
22 dentine thickness in the NaOCl-irrigated mature roots with periodontal involvement was
23 greater with no specimens displaying overlapping of the inner and outer sampling sites
24 (thickness range: 2.1-3.2), therefore the negligible and insignificant influence of dentine
25 thickness was expected (Coefficient = 0.05; $P = 0.4$). Similarly, there was a small but
26 insignificant (Coeff = 0.15; $P = 0.7$) effect of dentine thickness amongst the immature roots
27 because of a limited range of thickness (thickness range: 1.7-2.3 mm) within this group.
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29 The depth of effect of irrigants on dentine collagen was likely to be a function primarily of
30 penetration along dentinal tubules but secondarily and over longer time periods as a function
31 of inter-tubular matrix degradation. Any weakening effect of NaOCl and EDTA on dentine (Sim
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3 *et al.* 2001, Grigoratos *et al.* 2001, Ari *et al.* 2004, Slutzky-Goldberg *et al.* 2004, Mareending *et*
4 *al.* 2007, Rajasingham *et al.* 2010), is likely the combined effect of local dentine damage
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6 coupled with alteration of the overall dentine (tooth) geometry (Rajasingham *et al.* 2010,
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8 Zaslansky *et al.* 2016).
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11 At present, a standardised irrigation protocol is recommended for all tooth types, except for
12
13 use of lower concentrations of NaOCl in immature teeth, albeit to preserve the apical papilla
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15 to promote apexogenesis (Cvek *et al.* 1992). The findings of this study suggest that it may be
16
17 prudent to adopt lower concentrations of NaOCl and less aggressive regimens in immature
18
19 teeth to protect remaining dentine and tooth survival (Cvek *et al.* 1992).
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22 23 24 25 **Conclusions**

26
27 A refined protocol for FTIR ~~showed-revealed~~ that irrigation with both 5% NaOCl and 17 %
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29 EDTA resulted in a significantly larger reduction in dentinal collagen than with 5% NaOCl
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31 alone. Root maturity had a significant impact on dentinal collagen reduction, whilst periodontal
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33 involvement did not have a significant impact on dentinal collagen reduction with 5% NaOCl
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35 irrigation. Amide I collagen backbone changes were prevalent mostly within 0.5mm of the
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37 canal lumen but Amide II secondary structure collagen changes were variably evident to a
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39 deeper extent beyond 0.5mm.
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Figure legends

Figure 1. Overlaid spectra for a saline irrigated sample (a) before and (b) after surface treatment with EDTA.

Figure 2. Overlaid spectra for a NaOCl irrigated sample (a) before and (b) after surface treatment with EDTA.

Figure 3. Overlaid spectra for: (a) a saline irrigated sample; (b) a 5% NaOCl irrigated sample; & (c) a 5% NaOCl + 17 % EDTA irrigated sample from mature roots without periodontal involvement, and (d) a periodontally involved mature root sample & (e) an immature root sample after 5% NaOCl irrigation.

Figure 4. Means and standard deviations of outer amide I/phosphate and amide II/phosphate absorbance ratios, stratified by irrigant and root maturity/periodontal involvement.

Figure 5. Means and standard deviations of the relative outer *versus* inner absorbance ratios for amide I/phosphate and amide II/phosphate, stratified by irrigant and root maturity/periodontal involvement.

Table legends

~~**Table 1.** The mean \pm SD dentine thickness (mm) in the control and experimental groups.~~

~~**Table 2.** Comparison of mean absorbance ratios for amide I/phosphate and amide II/phosphate in saline or NaOCl irrigated samples before and after surface EDTA treatment.~~

~~**Table 3.** Linear mixed models incorporating “relative outer/inner absorbance ratios” of amide I/phosphate or amide II/phosphate as the dependent variable and “type of irrigant” or “root maturity” together with “dentine thickness” as the independent variables.~~

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Table 1. The mean±SD dentine thickness (mm) in the control and experimental groups

	Saline (Control) (n = 7)	5% NaOCl (n = 21)	5% NaOCl & 17% EDTA (n = 7)
Mature roots without periodontal involvement (n = 21)	2.3±0.4mm	2.3±0.5mm	2.2±0.2mm
Mature root with periodontal involvement (n = 7)	0	2.6±0.4mm	0
Immature roots (n = 7)	0	2.1±0.2mm	0

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Table 2. Comparison of mean absorbance ratios for amide I/phosphate and amide II/phosphate in saline or NaOCl irrigated samples before and after surface EDTA treatment

		Amide I/phosphate				Amide II/phosphate			
		Before EDTA		After EDTA		Before EDTA		After EDTA	
		Outer	Inner	Outer	Inner	Outer	Inner	Outer	Inner
Saline	Mean	0.306	0.307	0.306	0.307	0.276	0.275	0.252	0.262
	S.D.	0.068	0.065	0.068	0.065	0.085	0.095	0.032	0.020
	*P value	0.9		0.2		0.9		0.3	
NaOCl	Mean	0.307	0.304	0.320	0.245	0.255	0.250	0.252	0.195
	S.D.	0.056	0.054	0.048	0.029	0.053	0.047	0.032	0.020
	*P value	0.5		0.03		0.5		0.03	

*P values for Wilcoxon signed rank tests comparing the outer versus absorbance ratios for amide I or amide II over phosphate

Table 3. Linear mixed models incorporating “relative outer/inner absorbance ratios” of amide I/phosphate or amide II/phosphate as the dependent variable and “type of irrigant” or “root maturity” together with “dentine thickness” as the independent variables.

Independent variables	(a) Amide I/phosphate				(b) Amide II/phosphate			
	Coefficient	95% CI for coefficient	P-value	Z-value	Coefficient	95% CI for coefficient	P-value	Z-value
Model 1 a & b (data from mature roots without periodontal involvement)								
Type of irrigant			*<0.0001				*<0.0001	
<i>Saline (Reference)</i>	0	-	-	-	0	-	-	-
NaOCl	0.45	0.31, 0.60	<0.0001	6.4	0.36	0.25, 0.48	<0.0001	6.1
NaOCl+EDTA	0.75	0.61, 0.89	<0.0001	10.4	0.75	0.63, 0.87	<0.0001	12.6
Dentine thickness (mm)	0.13	-0.02, 0.28	0.1	1.7	0.16	0.03, 0.29	0.02	2.4
Random-effects parameters	Estimate	Standard error			Estimate	Standard error		
Variance for roots	0.002	0.007			5.3E-18	5.9E-17		
Model 2 a & b (data from roots with different root maturity, irrigated with 5% NaOCl)								
Root maturity			*0.008				*0.0002	
<i>Immature root (Reference)</i>	0	-	-	-	-	-	-	-
Mature root	-0.21	-0.40, -0.03	0.02	-2.3	-0.22	-0.36, -0.10	0.001	-3.5
Mature with periodontal involvement	-0.35	-0.56, -0.14	0.001	-3.3	-0.31	-0.45, -0.16	<0.0001	-4.1
Dentine thickness (mm)	0.10	-0.10, 0.31	0.3	1.0	0.16	0.01, 0.30	0.04	2.1
Random-effects parameters	Estimate	Standard error			Estimate	Standard error		
Variance for roots	0.006	0.011			1.0E-19	9.9E-19		

* P values for test of heterogeneity for categorical variable

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For Peer Review

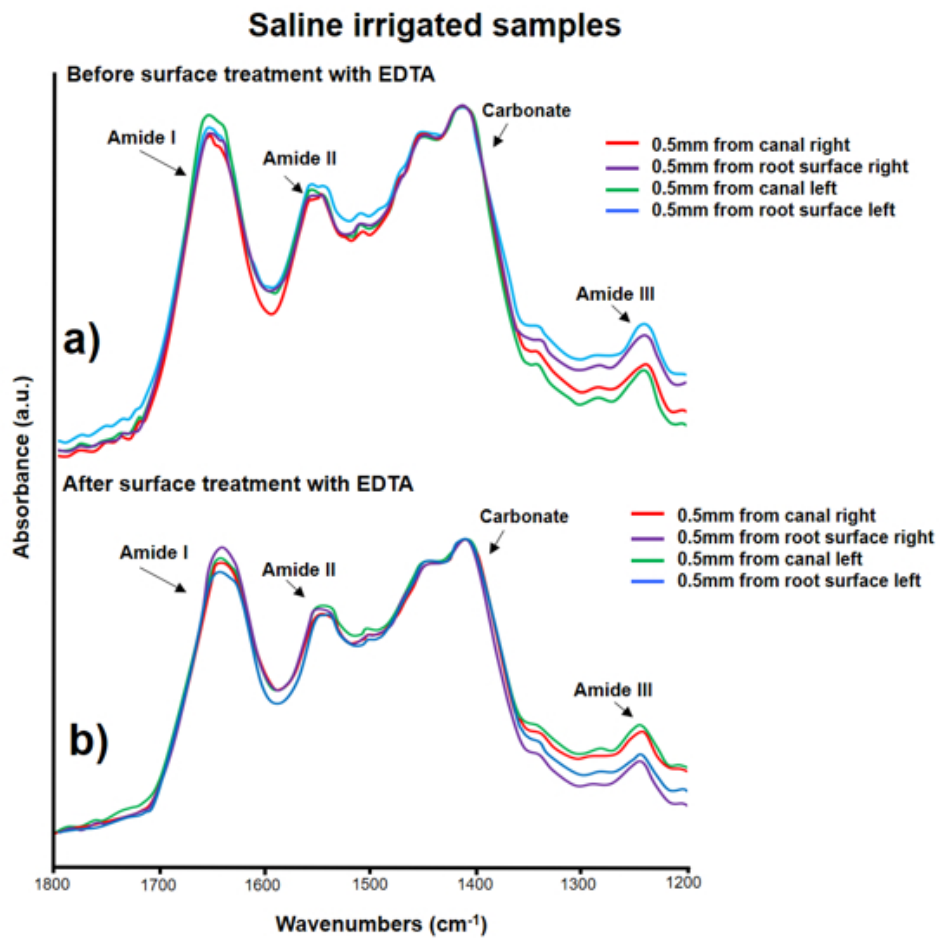


Figure 1. Overlaid spectra for a saline irrigated sample (a) before and (b) after surface treatment with EDTA.

152x148mm (96 x 96 DPI)

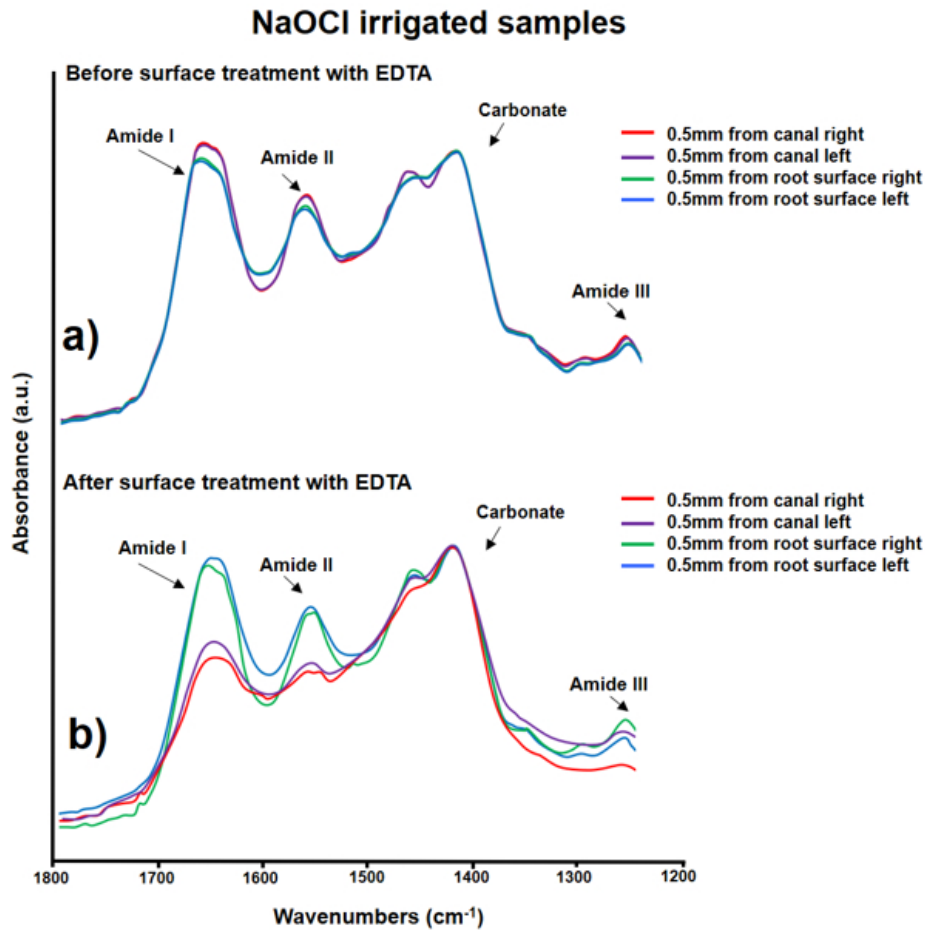


Figure 2. Overlaid spectra for a NaOCl irrigated sample (a) before and (b) after surface treatment with EDTA.

171x164mm (96 x 96 DPI)

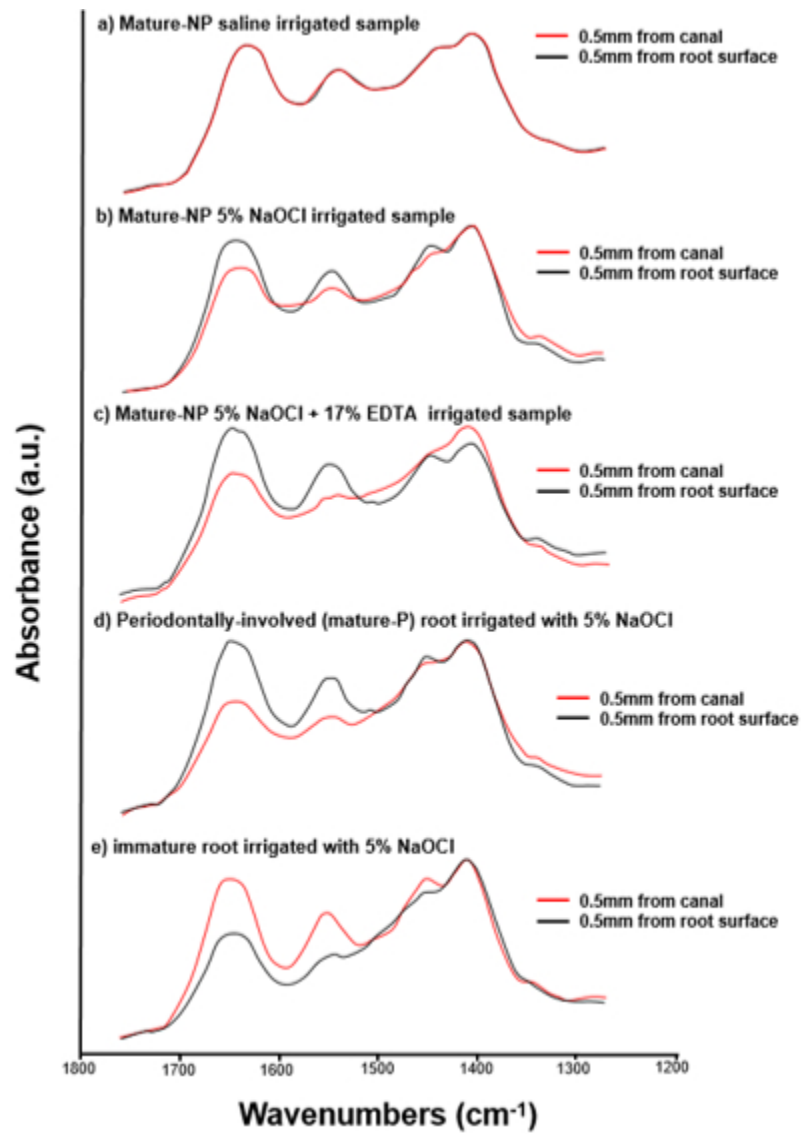


Figure 3. Overlaid spectra for: (a) a saline irrigated sample; (b) a 5% NaOCl irrigated sample; & (c) a 5% NaOCl + 17 % EDTA irrigated sample from mature roots without periodontal involvement, and (d) a periodontally involved mature root sample & (e) an immature root sample after 5% NaOCl irrigation.

118x153mm (96 x 96 DPI)

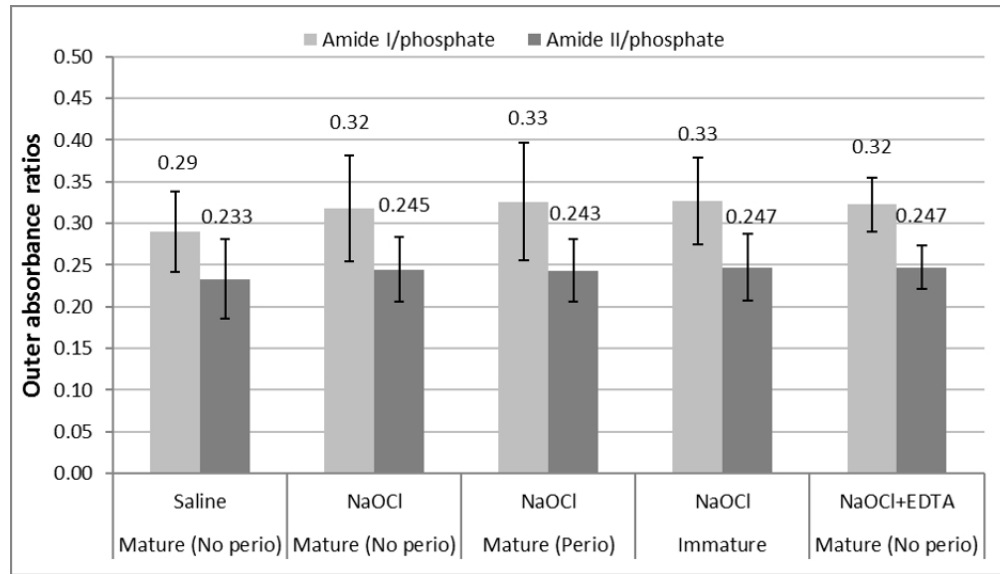


Figure 4. Means and standard deviations of outer amide I/phosphate and amide II/phosphate absorbance ratios, stratified by irrigant and root maturity/periodontal involvement.

159x91mm (149 x 149 DPI)

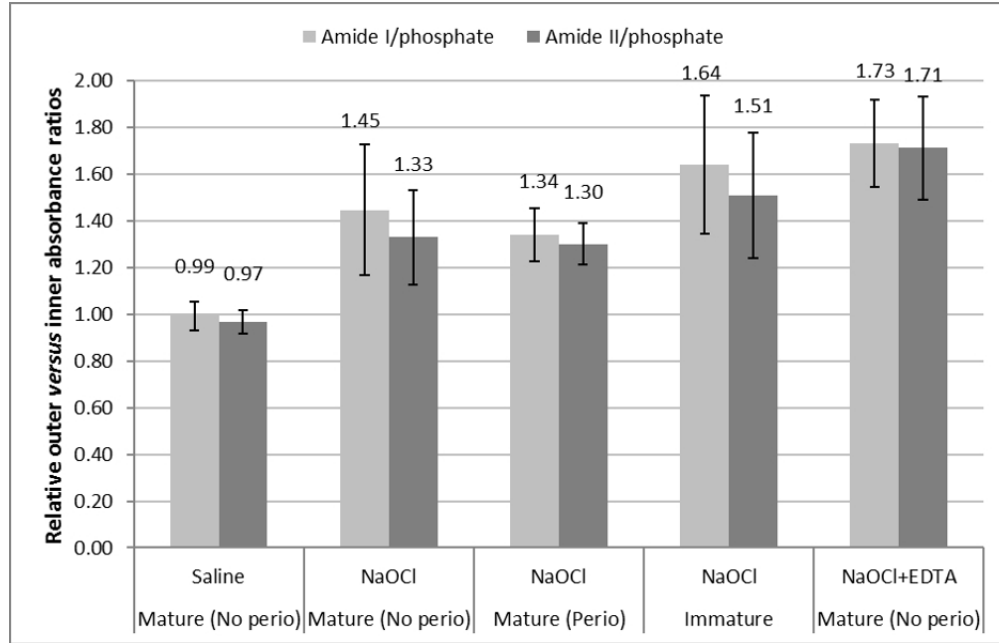


Figure 5. Means and standard deviations of the relative outer versus inner absorbance ratios for amide I/phosphate and amide II/phosphate, stratified by irrigant and root maturity/periodontal involvement.

159x102mm (149 x 149 DPI)