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Proof-of-concept study to establish an *in situ* method to determine the nature and depth of collagen changes in dentine using FTIR after sodium hypochlorite irrigation

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Abstract

Aim To establish a method using Fourier Transform Infra-Red spectroscopy (FTIR) to characterise the nature and depth of changes in dentinal collagen following exposure to sodium hypochlorite (NaOCl) during root canal irrigation in an *ex-vivo* model.

Methodology FTIR was used to assess the changes in dentinal collagen when the root canal was exposed to NaOCl. The changes in dentinal collagen caused by NaOCl irrigation of root canals in transverse sections of roots, at 0.5 mm from the canal wall and 0.5 mm from the external root surface were assessed by FTIR. The data were analysed using paired t-test with 5% significance level.

Results FTIR confirmed that NaOCl exposure caused alterations in the chemistry and structure of collagen in dentine. FTIR spectra obtained from dentine surfaces and dentine adjacent to root canals exposed to NaOCl, all consistently showed degradation and conformational change of the collagen structure. FTIR data from the *ex-vivo* model showed that the depth of effect of NaOCl extended to at least 0.5 mm from the canal wall.

Conclusion NaOCl caused changes in dentinal collagen that are measurable by FTIR. In an *ex-vivo* model, the depth of effect into dentine extended at least 0.5 mm from the canal wall.

INTRODUCTION

In the attempt to control resident microbiota during root canal treatment, the canal dentine is unintentionally also subjected to mechanical (instrumentation) and chemical stresses (sodium hypochlorite & ethylenediaminetetraacetic acid). The procedure leads to profound changes in the physical (Niu *et al.* 2002), mechanical (Sim *et al*. 2001, Grigoratos *et al*. 2001, Rajasingham *et al.* 2010), and chemical (O'Driscoll *et al.* 2002, Pascon *et al.* 2012, Ramirez-Bommer *et al*. 2018) properties of root dentine. NaOCl acts predominantly on the organic component of dentine, with no (O'Driscoll *et al.* 2002, Ari *et al*. 2004, Pascon *et al*. 2012, Ramirez-Bommer *et al*. 2018) or some (Sakae *et al*. 1988, Borges *et al*. 2008, Wang *et al.* 2016) effect on the mineral content.

Studies have shown that NaOCl affects the collagen in dentine (Di Renzo *et al*. 2001b, O'Driscoll *et al.* 2002, Ramirez-Bommer *et al*. 2018), leading to a reduction in elastic modulus, microhardness and flexural strength (Sim *et al.* 2001, Slutzky-Goldberg *et al.* 2004) as well as an increase in strain upon loading (Marending *et al.* 2007, Rajasingham *et al.* 2010). Despite the documentation of these changes, the published literature fails to reveal their precise nature, and also how far these effects extend into dentine from the canal wall, *in situ*. A possible reason for this may be the absence of suitable application protocols for available technology to achieve the mapping required.

Moreira *et al*. (2011) explored collagen loss in the surface 200 µm of canal dentine due to NaOCl irrigation as a function of altered birefringence, when viewed in decalcified histological sections produced by staining with 1% Sirius Red in saturated picric acid (picrosirius). Their purpose was to assess the changes in relation to dentine bonding.

Amarie *et al.* (2012) used nano-FTIR to probe the mineral components of marine shells and human teeth at a resolution of 20nm. They found multicomponent phosphate bands in human tooth sections, showing a chemical or structural variation of the apatite nanocrystals.

Ramirez-Bommer *et al.* (2018) used FTIR to *estimate* the depth of effect in graded dentine powder, alternately exposed to NaOCl and EDTA, previously shown by Di Renzo *et al.* (2001b) using photoacoustic FTIRS. Ramirez-Bommer *et al.* (2018) estimated the depth of reaction assuming that the bulk fraction of reacted collagen (1 minus ratio of collagen over phosphate peak height) could be equated with the volume fraction of a fully collagen-depleted shell of thickness around a spherical particle of original radius *without actual mapping* of the dentine sample. According to Ramirez-Bommer *et al.* (2018), 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 μm thick collagen-depleted surface compared to the 16±13 μm depletion by initial NaOCl treatment, alone.

The studies exploring the effect of chemical irrigants on the mechanical properties of dentine were conclusive in showing their negative effect on tooth resilience, elastic modulus, and flexural strength (Sim *et al*. 2001, Grigoratos *et al*. 2001, Rajasingham *et al.* 2010, Zhang *et al.* 2010a,b). These changes are almost certainly due to the altered chemical composition of dentine, although to date this has not yet been investigated, *in situ*. The nature, extent and depth of the chemical change, *in situ*, in an *ex-vivo* model are the focus of the present study.

The chemical composition and structure of dentine has been studied using many techniques, including microscopy (Marshall *et al.* 1997), immunohistochemistry (Hall & Embery 1997), spectroscopy (di Renzo *et al.* 2001a,b), X-ray microanalysis (Kinney *et al.* 2001, Marten *et al*. 2010), atomic force microscopy (Marshall *et al*. 1993, Habelitz *et al.* 2002), inductively coupled plasma atomic emission spectrometry (ICP-AES) (Ari & Erdemir 2005) and nano-

FTIR mapping (Amarie *et al.* 2012). Of these, 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 may be possible to assess the depth of any changes due to root canal irrigation. The aim of this study was to establish a protocol to investigate the depth of effect of NaOCl on collagen in dentine using FTIR in an *ex-vivo* model. The null hypothesis for the study was that there was no significant difference at the 5% level, between the mean FTIR absorbance ratios of amide I, amide II or amide III to phosphate, taken at 0.5 mm from the canal boundary compared to those taken at 0.5 mm from the root boundary, after canal irrigation with 5% NaOCl.

Materials and Methods

Effect of NaOCl on collagen scaffolds

In order to evaluate the impact of NaOCl directly on collagen model samples, five Type I collagen gels were made up following a standard protocol. Acid solubilised rat-tail collagen (First Link, Birmingham, UK) was neutralised using GIBCO® 10X Minimum Essential Medium (MEM) (Invitrogen, Renfrew, UK) and dropwise addition of aqueous NaOH on ice. The neutralised collagen was poured into a 2mL casting reservoir, allowed to set at room temperature (20°C) for 30 minutes and then placed in 10% phosphate buffered saline for storage prior to analysis. A commercially available stock solution of NaOCl (Merck Chemicals, Nottingham, UK) was diluted with water to a concentration of 5%; this was verified by iodometric titration. The gels were removed from the storage solution, cut in half, stretched out onto a glass microscope slide, and rinsed with deionised water. One of the

resulting pieces was exposed to 10 µL of 5% NaOCl. The NaOCl solution was dropped onto the left hand corner of the gel from a Gilson pipette and left for 20 minutes. Further pieces were taken and the process repeated using the same volume and concentration of NaOCl, but with varying exposure time intervals of 1, 5, 10 and 20 minutes. One gel was left untreated to act as a control. van Gieson's stain (a mixture of picric acid and acid fuschin, (Sigma Aldrich, Poole, UK) was added to each of the gels for five minutes, washed off with deionised water and photographed. Changes in the appearance of the gels were assessed visually, prior to repeating the experiment a further two times.

Effect of NaOCl on dentine

Preparation of dentine samples to assess the effect of NaOCl

Ethical approval was granted for the use of extracted human permanent teeth from the UCL Eastman Biobank (Study number: 1301). Five non-carious, intact, and crack-free human premolar teeth were obtained and stored in a solution of 70% ethanol in deionised water. They were embedded in clear epoxy resin (Specifix 40®, Struers Ltd., Solihull, UK) using a cylindrical mould. One resin cylinder was randomly selected for this study and sectioned transversely using a diamond microtome (Leica Model 1600®, Leica, Wetzlar, Germany) to create a 3 mm thick, 35 mm diameter disc with a transverse cross-section of the coronal third of the tooth root embedded in it. The discs were polished using a Struers Labopol 5® (Struers Ltd.) with increasingly fine grade silicon carbide abrasive discs up to 2400 µm grit (Struers Ltd.) and finished by polishing with diamond polishing paste (Diapro®, Struers Ltd.). The discs were ultrasonicated for 30 mins in deionised water. After this time, the discs were removed and dried with tissue paper.

Exposure of dentine to NaOCl and analysis by FTIR

One half of the transverse section was covered with masking tape (3M, Bracknell, UK). Two millilitres of 5% NaOCl (concentration verified by iodometric titration) was placed using a Gilson pipette (Gilson Inc., Middleton, WI, USA) onto the unmasked half, ensuring all the dentine was covered, and was left to stand for 20 minutes. The NaOCl was washed off with deionised water, the disc dried with paper tissues and the masking tape removed. The discs were then analysed by FTIR using the PE 2000® FTIR spectrometer with a SensIR Technologies Durascope® (Smiths Detection, Watford, UK). The dentine sample was placed in the sample compartment. The Durascope was fitted with a zinc selenide internal reflectance element (IRE) held at 45° to the incident beam. Spectra were recorded with the polished side of the sample in contact with the IRE, and constant force between the IRE and sample monitored by the graded scale (500 μm diameter target) of the Durascope®. The Durascope® provides a 38× video-magnified image of the sample, seen through the diamond ATR element. This enabled the area of the sample to be scanned and fitted within the calibrated area of the Durascope®. Thus, each spectrum recorded was the result of averaging the infrared absorption over 11 mm diameter area of the sample. Spectra were recorded in the range 4000 to 800 cm^{-1}. Twelve scans were obtained for each spectrum with a resolution of 4 cm^{-1}. Prior to any of the samples being analysed, the background FTIR spectrum was measured in the absence of the test sample. Spectra were recorded at six separate randomly selected locations on each half (masked and unmasked) of the disc.

Effect of NaOCl canal irrigation on dentine

Preparation of root samples to assess the effect of NaOCl at 0.5 mm from the canal boundary

Fourteen teeth were randomly selected, assessed to ensure they were intact, caries and crack-free and then endodontic access gained; the root canals were prepared with rotary

Protaper Universal® files (Dentsply Sirona Endodontics, Ballaigues, Switzerland) using the manufacturer's protocol, to a taper and apical size corresponding to instrument size F3. Deionised water was used to irrigate between files. The teeth were then embedded upright in the Specifix 40® resin, with the coronal 5 mm of the crowns above and out of the resin. Once the resin had set, the cusp tips were ground flat with a diamond bur to give a reproducible reference point for the extent of irrigation.

Irrigation of root canal samples with NaOCl and analysis by FTIR

The root canal samples were irrigated with 15 mL of 5% NaOCl (n = 12) or deionised water (n = 2) using a Monoject® syringe (Tyco Healthcare, Gosport, UK) and a 30 gauge Maxi-Probe® needle (Dentsply Rinn, Elgin, IL, USA). A rubber stop was placed on the needle to allow accurate placement of the tip at 7 mm from the reference point; the canal was irrigated by emptying the syringe at this level. Twelve of the fourteen samples were irrigated five times at 5 min intervals each with 3 mL of 5% NaOCl. After the last measure of NaOCl had had a dwell time of five minutes, the canal was washed out with 6 mL of deionised water. The other two samples were prepared as controls in the same way but irrigated with deionised water rather than NaOCl. The resin block was then sectioned using a horizontal diamond microtome (Leica Model 1600®, Leica, Wetzlar, Germany) at 6 mm from the reference point, and then a further 3 mm below that, giving a 3 mm thick disc of the coronal third of the root; incorporating the location at which the needle tip deposited the irrigant. Once the discs had been polished using a Stuers labo Pol-5 with 2400 grit paper and diamond polishing paste (Diapro, Struers Ltd.), they were ultrasonicated for 10 min in Ultra-High Quality water produced in the laboratory. Following this, indentations were made with a diamond inscriber 0.5 mm from the edge of the canal and 0.5 mm from the outer edge of the sample, on both halves of the disc whilst examining them under a dental operating microscope (Global Surgical, St Louis, MO, USA) as presented in Figure 1. The halves of

the disc were then designated left and right to facilitate the location of the discs in the spectrometer. Spectra were recorded using the PE 2000® FTIR at points immediately above the four marks on each dentine sample with 0.5 mm radius sampling area, and the spectra were compared and the data analysed as described below.

FTIR data analysis

The spectra obtained from FTIR analysis of the dentine samples were exported into Grams32 AI® spectroscopic software (Thermo Scientific, Waltham, MA, USA). The spectra were overlaid for direct visual comparison, then peak maxima were calculated for the bands of interest in the spectra, which were the amide A (peak heights: 3600 cm^{-1} and 3200 cm^{-1}), I (peak height: 1650 cm^{-1}), II (peak height: 1550 cm^{-1}), III (peak height: 1230 cm^{-1}) bands (Paschalis *et al.* 2001). These data were then processed in Microsoft Excel® (Microsoft Inc., Redmond, WA, USA). All FTIR spectra were offset to 0 at 1800 cm^{-1} to allow comparisons between samples of different intensities.

For the dentine sample with half of the surface directly exposed to the NaOCl, the spectra of the native dentine and of the dentine exposed to NaOCl in all 12 positions (6 from the masked and 6 from the exposed halves) was recorded and initially compared descriptively. The absorbance of the three carbonyl types of amide I band at 1690 cm^{-1}, 1660 cm^{-1} and 1630 cm^{-1} of the native dentine and of the dentine exposed to NaOCl in all 12 positions (6 from the masked and 6 from the exposed side) was recorded and compared.

For the dentine samples harvested from the roots after canal irrigation, the changes in the amide bands were quantified by compairing their relative intensities against the stable mineral component, phosphate peak, of the spectra. Briefly, the absorbances of the amide I, II and III bands and the phosphate peak at 1010 cm^{-1} were recorded from the spectra and their ratios calculated. The ratios of amide I / phosphate, the amide II / phosphate, and amide III / phosphate, taken at 0.5 mm from the canal boundary *versus* those taken at 0.5

mm from the root boundary, were compared using paired t-test. For each sample, the mean value of the data obtained from the left and right sides was calculated and included for analysed.

RESULTS

Effect of NaOCl on collagen

The control collagen gel (n = 1) not exposed to NaOCl revealed complete red staining of its surface when van Gieson's stain was added, as expected (Figure 2). The gels (n = 4) that had been exposed to NaOCl all had varying degrees of degradation, which correlated with the duration of exposure. Although, only a droplet of 5% NaOCl was added to the top-left hand side of the collagen gel, the impact of exposing the gel to NaOCl increased over time. From the 1 min exposure time, a significant change in the appearance of the gel was noticed with almost a third of its surface having been denatured. As the exposure time increased, so did the proportion of the gel affected, ending with complete degradation after 20 min (complete loss of staining and gel structure). A key observation in these experiments was the diffusion of NaOCL throughout the gel, despite its deposition just at the top-left corner of the gel; in the case of the 20 min exposure, the entire gel (~3 ×3 cm^2) was affected.

The effect of NaOCl on dentine structure was assessed by monitoring changes in the FTIR spectrum of the dentine. Figure 3 shows a typical spectrum of native dentine with each of the characteristic absorption bands (amide, carbonate and phosphate) labelled. Overlaying the native spectrum over the spectrum of NaOCl exposed dentine, showed that the intensity of all four amide bands reduced following NaOCl exposure, whereas the mineral peaks of carbonate and phosphate remained stable. The repeatability of the response was demonstrated by 6 replicates before and after exposure (data not shown). Table 1 shows a relative increase in absorbance at 1630 cm^{-1}, compared to that at 1660 cm^{-1}, indicating greater contribution from the carbonyl groups at 1630 cm^{-1} after NaOCl exposure.

Effect of NaOCl on dentine collagen at 0.5 mm from canal boundary

The FTIR spectra for the control discs (n=2) with canals irrigated with deionised water (figure 4) were consistent with those for native dentine (Figure 3), at all four points on these samples.

The FTIR spectra for the experimental discs (n=12) with canals exposed to NaOCl, consistently showed a different picture at the 4 points (Figure 5); those obtained from 0.5 mm from the outer boundary of the root had the same appearance as those for native dentine, whereas those obtained from 0.5 mm from the canal boundary had the same appearance as those for dentine exposed to NaOCl.

Figure 6 depicts the means and 95% confidence intervals of the ratios of the absorbance of each of the amide bands to the phosphate peak for each of the 12 samples, stratified by the canal- *versus* outer-edge sampling site. Paired t-tests revealed significant difference in the absorbance ratio for amide I (P = 0.0005), amide II (P = 0.001) and amide III (P = 0.02) to phosphate, between the spectra taken at the canal- *versus* outer-edge, respectively.

DISCUSSION

The exposure of 5% NaOCl to a control collagen gel was an important step in understanding the potential impact of the chemical on the structure and native aspect of collagen. In this approach, only one of the corners of the gel was exposed to a small droplet of the solution and yet after 20 min, complete degradation of the gel was recorded as Van Gieson's staining was unable to show any red area, suggestive of native collagen structure. This degradation was progressive over time and space. It is the picric acid component of the Van Gieson staining that is particularly pertinent in this approach as the picric acid molecule binds to collagen along its fibrillar long-axis. Any changes in the quaternary structure of the collagen would result in prevention of this interaction and the failure of the sample to stain. During

these experiments, the gels were also air-dried on a glass slide, yet NaOCl diffused throughout the gel. This is an important parameter to consider but further studies would have to be performed to fully characterise the diffusion potential of NaOCl in such an environment.

The *ex-vivo* irrigation root canal model aimed to mimic the clinical irrigation scenario. It may be argued that NaOCl should be used during the mechanical enlargement of the canal as well to be properly representative. However, the adoption of this strategy would preclude proper control of the total volume, duration of exposure, and precise location of the apical extent of irrigant needle tip during this phase due to variations in the initial canal dimensions. It was therefore decided to restrict exposure of the canal to NaOCl only after canal enlargement was complete, confining canal preparation irrigation to deionised water to enable better standardisation. It may be argued that since the adoption of the paradigm that the mechanical preparation of the canal simply serves as access to the canal system, the key irrigation step is the dynamic phase after canal preparation geared towards delivery of the irrigant to all canal surfaces including those not planed by the instruments. The position at which the irrigant was deposited was standardised so that the harvested root disc (3mm in height) incorporated this site, 1mm below the coronal surface and 2mm above the apical surface. The root dentine thickness was approximately 2.3 mm, whilst the FTIR sampling site had a radius of 0.5 mm, therefore only one data point could be taken from the canal boundary without overlapping with the surface boundary data point. Further studies are planned using probes with higher resolution to enable detailed mapping of the effect of NaOCl.

The FTIR spectrum obtained from the analysis of dentine contained several peaks that could be used to monitor changes in chemical composition (Di Renzo *et al*. 2001b, Hu *et al*. 2010, Zhang *et al*. 2010, Tartari *et al*. 2016). A broad peak was observed in the spectrum between 3600 and 3200 cm^{-1} present because of N-H stretching vibrations. This is described as the amide A band. The main structural repeating unit in collagen is the peptide bond, which exists between the amino acids. This amide bond contributes significantly to the FTIR

spectrum by virtue of stretching and bending vibrations of the carbonyl bond (C=O) at 1650 cm^{-1} (known as the amide I band), the N-H and C-N bonds at 1550 cm^{-1} (the amide II band) and at 1230 cm^{-1} (the amide III band). Each of these amide bands was observed in the dentine FTIR spectrum. In addition, peaks corresponding to the mineral component of the matrix were also observed; these were found at 1410 and 870cm^{-1} due to carbonate groups, and at 1100 cm^{-1}, due to phosphate groups (Di Renzo *et al*. 2001a).

Loss of or denaturation of collagen from dentine treated with NaOCl was confirmed (Di Sakae *et al*. 1988, Di Renzo *et al*. 2001b, O'Driscoll *et al*. 2002, Tartari *et al.* 2016) through a decrease in the intensity of the absorbance peaks assigned to collagen (Figure 3, Table 1) (amide A, I, II and III bands), compared to the phosphate peak. The effect of NaOCl on the mineral content of dentine has been reported with conflicting results. Several studies (Sakae *et al*. 1988, Barbosa *et al*. 1994, Tomazic *et al*. 1993, Tsuda *et al*. 1996) found a decrease in the carbonate content of dentine following treatment with NaOCl, whereas others (Di Renzo *et al*. 2001a, O'Driscoll *et al*. 2002) reported that both the carbonate and phosphate peaks, as measured by FTIR, remained constant. Given that the 'shoulder' of the amide II peak (Figure 3) showed absorption at a co-incident wavenumber to one of those associated with the carbonate peak, then a loss of collagen from the dentine may also be expected to result in a decrease in absorbance at this frequency, leading to confusion in interpretation of the spectra. For this reason, the phosphate peak was selected as the comparison reference (Hu *et al*. 2010, Tartari *et al*. 2016).

The amide I / phosphate absorbance ratio and the amide II / phosphate absorbance ratio decreased following exposure to NaOCl, confirming the widely reported finding of loss of dentinal collagen. The effect was most apparent with the amide I band, whereas the amide II band formed a 'shoulder' on the carbonate peak, potentially allowing some convolution of its peak, whilst the intensity of the amide III band was initially much lower. The band used to judge the effect of NaOCl was not always clearly depicted in previous studies, whilst Tartari *et al.* (2016), seem to exclusively use the Amide III band.

Reduction in the collagen content of the dentine has been linked to hydrolysis and amino acid degradation by NaOCl (Estrela *et al*. 2002), which occurs following chloramination of the amine functionality, resulting in the formation of organic chloramines. Breakdown of the collagen structure may result in smaller, more soluble products that are dissolved in the irrigant and removed from the dentine surface. Another mechanism for loss of organic content is amino acid neutralisation, where peptide linkages are broken and the resulting free carboxylic acid group combines with the Na^+ of the NaOCl to form a soluble sodium salt.

Not all of the bands associated with collagen were removed during exposure to NaOCl, implying its continued integrity and detectable presence, as previously reported by Di Renzo *et al*. (2001b) after NaOCl-exposure for 48 hours. They proposed that this was because of collagen being encapsulated by hydroxyapatite, which remained unaffected by NaOCl.

The amide I band in the FTIR spectrum of dentine is a relatively broad peak because of the conformationally distinct carbonyl moieties that produce the peak. At 1690cm^{-1} the contribution to the signal is derived from carbonyl groups held within the collagen fibrils that are not available for hydrogen bonding. In the collagen triple helix, the carbonyl group of an amino acid (usually proline) is held in close proximity to the N-H group of the glycine amino acid. The intra-molecular hydrogen-bond that forms between them helps to hold the triple helix together. The weakly hydrogen-bonded carbonyl groups give an absorbance at 1660 cm^{-1}. A third contribution to the amide I band at 1630 cm^{-1} arises from the carbonyl groups of the triple helix of the collagen which are held in a position pointing out of the triple helix. These carbonyl groups can form relatively strong inter-molecular hydrogen bonds that provide the contribution at the lower frequency. As a consequence of the different contributions from the three types of carbonyl groups present, the amide I band derived from the collagen is particularly sensitive to changes in its conformation (Lazarev *et al*. 1985, Vyavahare *et al.* 1998).

It has been reported that denaturation of collagen (following heat treatment rather than exposure to NaOCl) results in a relative increase in the amide I component at 1630cm^{-1} and a relative decrease in the amide I component at 1670 cm^{-1} (George & Veis 1991). This is consistent with the breaking of the intra-molecular hydrogen bonds in the triple helix, which releases the carbonyl groups, making them available to form stronger inter-molecular hydrogen bonds. If NaOCl breaks open the triple helix, the same effect may be expected to be observed.

The change in the contour of the amide I band in the FTIR spectrum of a pure collagen gel exposed to NaOCl was assessed in pilot studies and showed that just considering the band maximum, no real difference was evident; the maximum for the untreated collagen gel occurred at 1658 cm^{-1}, whilst that exposed to NaOCl for 30 seconds occurred at 1656 cm^{-1}. The band maxima for the exposed and unexposed dentine were also similar. If denaturation of collagen is judged simply by measuring the frequencies of the band maxima, the broad peak width may cause confusion. If however, the ratio of the responses at 1660 and 1630 cm^{-1} are compared then an increase in the contribution due to the 1630 cm^{-1} carbonyl stretching frequency may be apparent. The ratio 1630/1660 for the unexposed collagen gel sample was 0.70, whilst that for the collagen gel exposed to NaOCl for 30 seconds was 0.91, suggesting a greater contribution to the spectrum from carbonyl groups that are not held within the triple helix. Exposing the collagen gel to NaOCl for a further 30 seconds gave a spectrum with a 1630/1660 ratio of 0.99, highlighting an even greater contribution from the carbonyl group(s) at 1630 cm^{-1}. The rate of change decreased over the second 30 second period suggesting that the majority of the triple helix had degraded in the first 30 second exposure.

Examination of the dentine FTIR spectra did not reveal the changes as readily. Calculation of the ratio of the absorbances at 1630/1660 revealed a small increase in the relative contribution of the 1630 cm^{-1} carbonyl stretch. Table 1 shows a relative increase in absorbance at 1630 cm^{-1}, compared to that at 1660 cm^{-1}, consistent with the breaking of the

intra-molecular hydrogen-bonds of the collagen and the unwinding of the triple helix. In doing so, the collagen would be more susceptible to chemical attack by NaOCl and subsequent dissolution of the smaller, more soluble breakdown products. A 20 minute exposure of the dentine discs was selected because this was considered more clinically relevant. During this time, it is proposed that the triple helix should have broken open and the dissolution of the collagen in the NaOCl be complete. Therefore the loss of the carbonyl contribution at 1630cm^{-1} would have 'caught up' with the loss at the 1660 cm^{-1}, making the observed difference smaller.

Changes in 1660 to 1690 cm^{-1} ratio have been linked to crosslinking of collagen in bone. It may be expected that the cross-linked form of collagen would be more stable than the free triple helix, and as such, the rate of its loss from the dentine would be slower. The ratio of the absorbance at 1690 cm^{-1} to that at 1660 cm^{-1} was recorded (Table 1), and the data support this hypothesis with an increase in the relative contribution of the component at 1690 cm^{-1} following exposure to the NaOCl (Paschalis *et al*. 2001).

Having established the FTIR spectral changes brought about by NaOCl exposure of pure collagen and that within dentine, they were adopted as outcome measures for changes within dentine a root canal irrigation *ex-vivo* human tooth model. The trends for FTIR changes seen on exposure of dentine to NaOCl, were also observed in 10 of the 12 experiments where the FTIR spectra were taken 0.5 mm *from the edge of the irrigated canal*. In all cases, the spectra taken at 0.5 mm *from the outer edge of the root* were consistent with that of native dentine. The purpose of this model was to ensure that the tests would be conducted on either affected or unaffected dentine to obtain clear results; partial exposure could potentially confuse the picture. The overall inference drawn from the study as designed, was that the model worked and that NaOCl could be seen to exert a clear effect to a depth of at least 0.5 mm from the canal wall. This does not exclude the possibility of effect deeper into dentine but the probe used in this study and the design precluded this test. There is no published literature on the depth of collagen degradation brought about by

NaOCl exposure of dentine *in situ*; nevertheless, the finding of this study is consistent with Slutzky-Goldberg *et al.* (2004), who reported a significant reduction in dentine microhardness at 0.5 mm depth following exposure to 6% NaOCl. It would be desirable to have greater accuracy in the measurement described here, a feat that may be possible with a different ATR platform.

Exposure of dentine to NaOCl has been reported to alter its mechanical properties (Sim *et al.* 2001, Grigoratos *et al.* 2001, Ari *et al.* 2004, Fuentes *et al.* 2004, Lee *et al.* 2004, Slutzky-Goldberg *et al.* 2004, Marending *et al.* 2007) and increase in tooth surface strain (Rajasingham *et al.* 2010). These findings have the potential to cause the dentine in root-treated teeth, exposed to NaOCl, to be weakened and more disposed to fracture. The removal, or the structural alteration of collagen, which is the major building block in the matrix of mineralised tissues, has a significant effect on the physical characteristics of the tissue (Ottani *et al.* 2001). It has been suggested that the cross-linking of collagen in root-filled teeth differs from that in vital teeth (Rivera *et al.* 1988) and, although convincing experimental evidence of this is lacking, conformational changes in collagen structure have been shown to have a detrimental effect on the physical characteristics of dentine (Balooch *et al.* 2008). The presence of collagen and its particular arrangement within the dentine matrix contributes considerably to the mechanical strength of the dentine, and ultimately the tooth. All of the mechanical and physical changes described in the literature which result from exposing dentine to NaOCl can, in the main, be ascribed to a loss of collagen from the dentine matrix. Although studies (O'Driscoll *et al.* 2001, Pascon *et al*. 2012, Ramirez-Bommer *et al*. 2018) have shown that demineralising agents do not affect collagen *per se*, they fail to consider how disruption of the matrix may affect the fibrillar structure of collagen and potentially leave it more exposed to damage from NaOCl (Di Renzo *et al*. 2001, Habelitz *et al*. 2002, Tartari *et al.* 2017). A number of studies have demonstrated that the synergistic effect of NaOCl and ethylenediaminetetraacetic acid (EDTA) leads to a greater change in dentine than either agent in isolation (O'Driscoll *et al.* 2002, Sayin *et al.* 2007, Ramirez-

Bommer *et al.* 2018). Additionally, it has been proposed that the nano-structural calcium-phosphate phases in devitalised teeth may differ in size and hence ability to resist forces (Zelic *et al*. 2014). It is important therefore, from an endodontic perspective, to understand how NaOCl affects collagen within the native dentine matrix, without the confounding effects of demineralisation, and then to consider how this effect may be enhanced when combined with EDTA exposure. If this can be studied using irrigants *in situ*, it may be possible to design an irrigation protocol that achieves the biological objectives of root canal treatment, whilst minimising the damage to the tooth structure.

CONCLUSIONS

An *ex-vivo* experimental model suitable for studying composition and ultra-structural changes within dentine *in situ*, has been developed. Based on the FTIR findings, NaOCl was found to degrade the structure of the collagen triple helix within dentine, however, the presence of the mineralised matrix appeared to offer some protection from this degradation. Based on the *ex-vivo* experimental model used, the effect of NaOCl on dentinal collagen extended at least 0.5 mm into the dentine.

Conflict of Interest statement

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

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Figure legends

Figure 1 Embedded dentine sample with indentations 0.5mm from the canal and sample edges

Figure 2 Effect of NaOCl on collagen scaffold demonstrated using Van Gieson staining

Figure 3 The FTIR spectra of native dentine and dentine exposed to NaOCl (spectra normalised to the phosphate peak)

Figure 4 FTIR spectra taken at four labelled points on dentine surrounding the canal after irrigation with deionised water

Figure 5 FTIR spectra taken at four labelled points on dentine surrounding the canal after irrigation with 5% NaOCl

Figure 6 Means and standard deviations of the absorbance ratios of amide I, amide II, and amide III to phosphate, at 0.5 mm from the canal or the outer edge of the root (n = 12)

Table 1 Comparing the relative contribution of the three carbonyl types to the amide I band

Sample	Absorbance at 1690cm^{-1}	Absorbance at 1660cm^{-1}	Absorbance at 1630cm^{-1}	Ratio 1690 / 1660	Ratio 1630 / 1660
Exposed (position 1)	0.0449	0.1011	0.1118	0.44	1.11
Exposed (position 2)	0.0461	0.1045	0.116	0.44	1.11
Exposed (position 3)	0.0376	0.0869	0.0964	0.43	1.11
Exposed (position 4)	0.0438	0.0998	0.1104	0.44	1.11
Exposed (position 5)	0.0437	0.1014	0.1109	0.43	1.09
Exposed (position 6)	0.0434	0.0992	0.1078	0.44	1.09
Average exposed				0.44	1.10
Native/masked (position 1)	0.0059	0.0146	0.0154	0.40	1.05
Native/masked (position 2)	0.0429	0.108	0.1136	0.40	1.05
Native/masked	0.0175	0.0418	0.0435	0.42	1.04

(position 3)					
Native/masked (position 4)	0.0571	0.1489	0.1554	0.38	1.04
Native/masked (position 5)	0.0659	0.1735	0.181	0.38	1.04
Native/masked (position 6)	0.0337	0.0947	0.0983	0.36	1.04
Average native				0.39	1.05
Relative increase in absorbance at 1690 compared to 1660 following exposure				1.12	
Relative increase in absorbance at 1630 compared to 1660 following exposure					1.05

Figure 1. Embedded dentine sample with indentations 0.5mm from the canal and sample edges

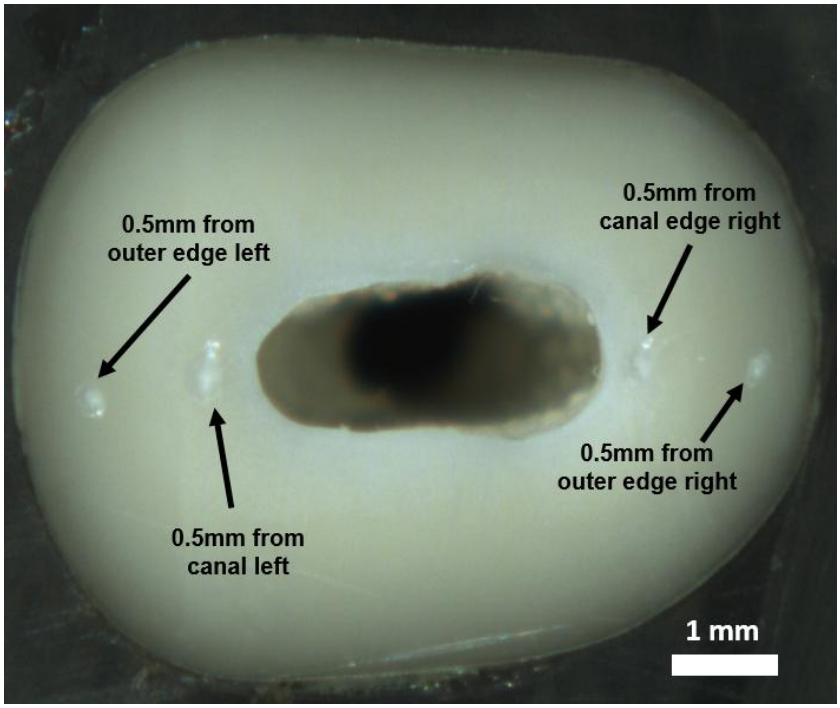


Figure 2. Effect of NaOCl on collagen scaffold demonstrated using Van Gieson staining

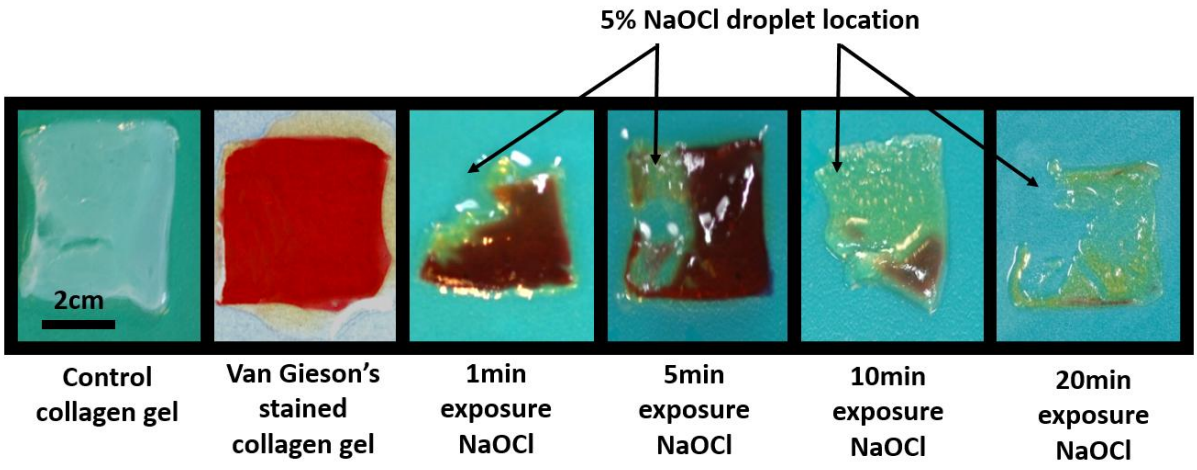


Figure 3. Comparing the FTIR spectra of native dentine and dentine exposed to NaOCl (spectra normalised to the phosphate peak)

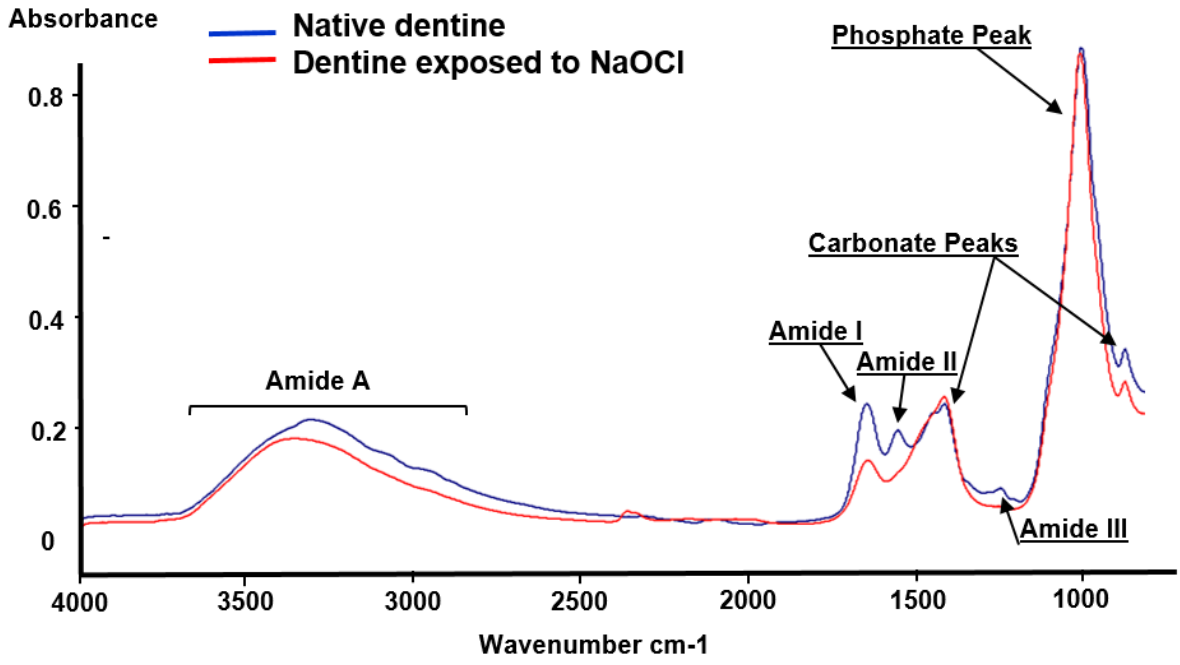


Figure 4. FTIR spectra taken at four labelled points on dentine surrounding the canal after irrigation with deionised water

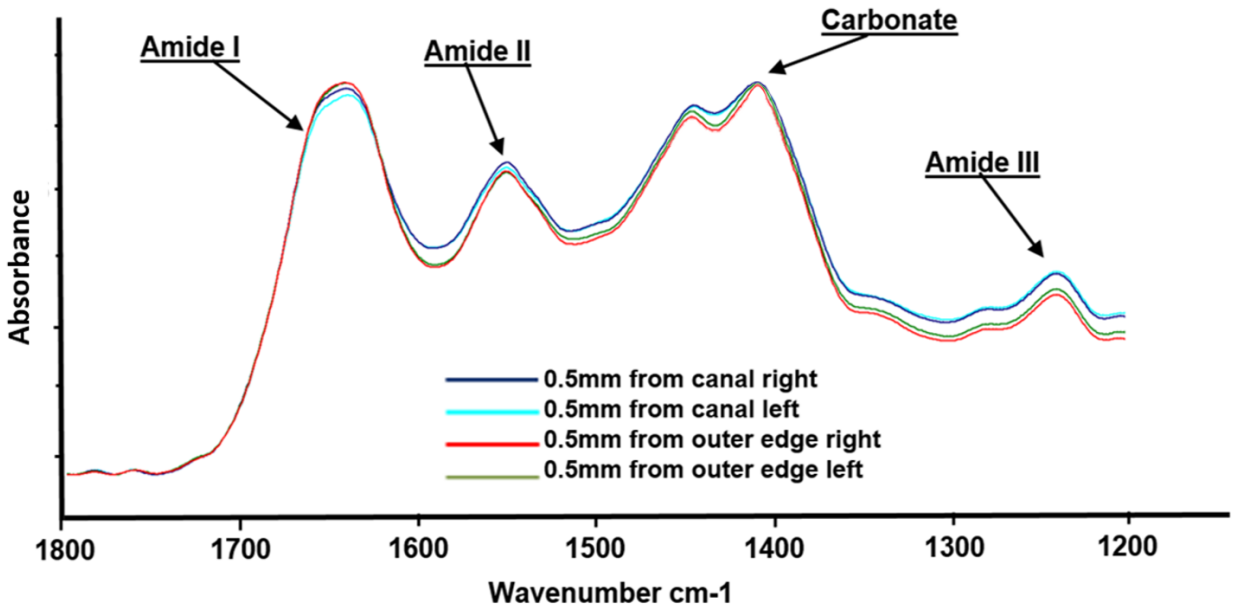
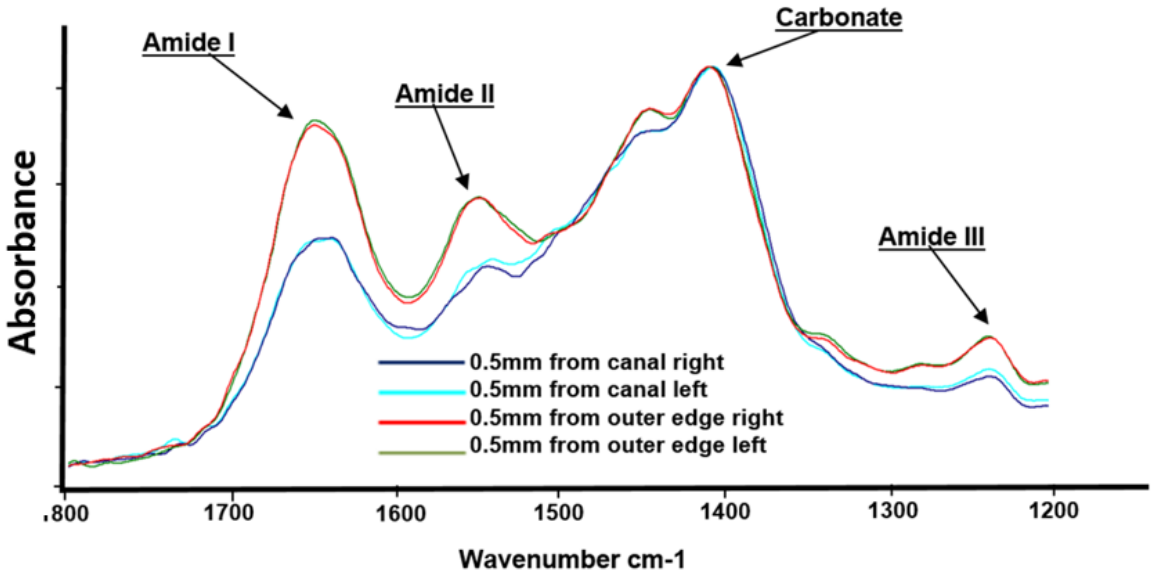


Figure 5. FTIR spectra taken at four labelled points on dentine surrounding the canal after irrigation with 5% NaOCl



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