The effect of NaOCl and heat treatment on static and dynamic mechanical properties and chemical changes of dentine

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Abstract

Objectives: To determine the effect of heat on flexural strength (FS), maximum strain (MS), storage modulus (SM), tan delta (TD) and chemical changes through micro-Raman spectroscopy of dentine exposed to 2.5% NaOCl or saline.

Methodology: Dentine bars were randomly allocated to 8 test groups. Half (groups 2,4,6,8) were treated with NaOCl for 20 mins; the rest (groups 1,3,5,7) remained in saline. FS/MS were measured in groups 1-4 (n=15) (3/4 were also heated to 200°C & re-hydrated in saline). Micro-Raman spectroscopy was performed on bars from groups 1–4. SM/TD were measured in 5-8: in 5/6 (n=10), repeated after heating (200°C), then following re-hydration; in 7/8 (n=3) after heating to 25−185°C.

Results: Increase in MS on heat and FS/MS on heat+NaOCl was not significant (P>0.05). SM increased (P=0.06) after heat treatment but reduced to initial state after rehydration (P=0.03). TD did not change (P=0.4) after heat (200°C) treatment but rehydration increased it compared with pre-treatment state (P=0.001). For dentine bars pre-treated with NaOCl, SM did not change (P=0.6) after heat (200°C) treatment or rehydration but TD significantly increased (P=0.02) upon re-hydration compared with pre- (P=0.007), or post- (P=0.03) heat-treatment states. SM and TD varied between 25−185°C with no consistent trend amongst the NaOCl pre-treated bars. Micro-Raman only detected chemical changes following NaOCl treatment in the mineral phase.

Conclusions: Exposure of dentine bars to heat and NaOCl produced only moderate changes to quasi-static but marked changes to viscoelastic properties, which may be explained by chemical alterations.

1. Introduction

The combination of mineral, organic and water components determine the mechanical behaviour of mineralised tissues (Gul-E-Noor et al., 2015; Unal and Akkus, 2015). Intra-collagen fibrillar mineralisation provides elastic behaviour, whilst extra-fibrillar granular mineralisation, reinforced by inter-penetrating collagen fibrils, provides the viscous element when saturated with water (Kinney et al., 2003). Dentine dehydration may consolidate the granular mineral matrix by shrinkage of the interpenetrating collagen, thereby increasing Young's modulus.

Water in dentine resides in "bound" or "free" compartments; the latter within dentinal tubules (75%) and former (25%) within mineral and collagen phases (Gul-E-Noor et al., 2015; Unal and Akkus, 2015). Free water in tubules may contribute to resistance to occlusal forces by the work required to displace it upon compression (Granke et al., 2015; A. Kishen and Asundi, 2005; A. Kishen and Vedantam, 2007; Pashley, 1990), whilst bound-water contributes toughness, visco-elasticity and strength. Water molecules influence mechanical properties through formation of hydrogen bonds with various structural components in mineral as well as collagen (Gul-E-Noor et al., 2015). Free-water can be removed by heating dentine to 100 °C (Anil Kishen, 2006), whilst the bound water may be lost at temperatures above 200 °C (Helfer et al., 1972; Anil Kishen, 2006).

The rigours of caries, tooth surface loss, wear and tear, age and loss of pulp vitality lead to a greater susceptibility of teeth to fracture in later life. This appears to be mediated by a combination of factors, including unfavourable changes in the architecture of the tooth due to dentine loss (Hansen and Asmussen, 1993; Mondelli et al., 1980; Reeh et al., 1989; Zelic et al., 2015), the mineral content of dentine (Nalla et al., 2003; Zelic et al., 2014), collagen cross-linking in dentin (Miura et al., 2014; Montoya et al., 2018; Yan et al., 2017) and pulpal proprioception (Bleicher, 2014; Byers and Cornel, 2018; Randow and Glantz, 1986).

Root canal treatment may potentially further impose physical weakening of teeth (Gulabivala et al., 2005; Anil Kishen, 2006) through changes in the properties of dentine (Driscoll et al., 2002; Grigoratos et al., 2001; Rajasingham et al., 2010; Sim et al., 2001) caused by sodium hypochlorite (NaOCl), EDTA or calcium hydroxide. NaOCl may unfavourably alter flexural strength and elastic modulus (Sim et al., 2001) of dentine and increase tooth surface strain (Rajasingham et al., 2010). The changes have been attributed to depletion and alteration of dentinal collagen by NaOCl (Driscoll et al., 2002; Morgan et al., 2018; Ramirez-Bommer et al., 2018). Despite these observations, NaOCl remains the first choice root canal disinfectant and has been adopted at concentrations ranging between 0.5% to >5.0% (Dutner et al., 2012; Gulabivala et al., 2005; Koch et al., 2009). The most popular "compromise-concentration" between antimicrobial or dissolution efficacy and potential harm to soft and hard tissues is 2.5% (Hommez et al., 2003), assuming a dose-dependent effect. The limitation of harm to dentine is also reliant upon the relatively superficial extent of effect on dentine (Morgan et al., 2018).

In addition, however, heat applied to dentine during or after root canal treatment, either deliberately (to soften gutta-percha) or inadvertently (during removal of gutta-percha, separated instruments or post-preparation) may through temperature increases of 200 °C cause damage to hydroxyapatite or collagen and dehydrate dentine (Lin et al., 2010). Dehydration makes dentine brittle (Hayashi et al., 2012; Huang et al., 1992; Jameson et al., 1993; A. Kishen and Asundi, 2005), although this change is reversible upon re-hydration (Hayashi et al., 2012; Hayashi et al., 2010; Jameson et al., 1993). Hence the clinical impact of moisture loss may be transient because dentine may re-hydrate from surrounding fluids (Linden, 1968). Furthermore, dentine kept moist, is little affected in strength, when heated to 100 °C (Rasmussen and Patchin, 1984).

The effect of heat on dentine may be mediated by altered collagen properties. Collagen, a key component of dentine, consisting of 3 helical polypeptide chains bound together by hydrogen and covalent bonds into a super-helix (Wang et al., 2002) undergoes various

temperature-dependent and time-dependent changes (Suwa et al., 2016). Collagen structure is reportedly altered to different degrees at different temperatures (20-200 °C) and levels of hydration and physical confinement (Armstrong et al., 2008; Gevorkian et al., 2013; Hayashi et al., 2012; Miles and Ghelashvili, 1999; Suwa et al., 2016; Vangsness et al., 1997; Wang et al., 2002). Collagen has been reported to denature at 200 °C (Yamashita et al., 2002).

It was hypothesised that heating dentine to 200 °C may denature or alter its constituent collagen and mineral content, as well as cause water loss, thus affecting its physical properties. Furthermore, NaOCl pre-treatment of dentine, could also theoretically deplete collagen, and this may either reduce the impact of heat compared to untreated dentine or exert an accumulative effect. The aim of this study was to investigate the effect of heat on dentine pre-treated with either 2.5% sodium hypochlorite or 0.9% saline; by assessing changes in its physical and chemical properties.

2. Material and Methods

2.1 Sample preparation and allocation to groups

Ethical approval was granted for the use of extracted teeth from the UCL Eastman Biobank (Study number: 1301). Eighty-six standardized plano-parallel dentine bars of dimensions (2 mm depth × 0.6 mm height × ≥11 mm length) were cut exclusively from the roots of eighty-four extracted human teeth (25 molars, 49 premolars, 4 incisors and 8 canines stored in 4% formal-saline at room temperature (Jameson et al., 1993)) using a diamond saw (Exakt, GmbH, Nordestedt, Germany) under a coolant water spray. The dentine bars were examined under magnification (×3.2) (Global Surgical™ Microscope, St. Louis, USA) and discarded if they included cracks or parts of the root canal; the tubule direction was parallel to loading direction. The dentine bars were also stored in 4% formal-saline (Normasol, SSL, Oldham, UK) and randomly allocated to 8 test groups (Table 1). The randomization was stratified; ensuring that bars from each tooth type were distributed approximately evenly amongst the groups.

2.2 Effect of NaOCl and heat treatment on flexural strength (Groups 1-4; n = 15 each)

Samples in groups 1 to 4 were exposed to 40 mL of 0.9% saline (Groups 1 & 3) or 2.5% sodium hypochlorite solution (Teepol Bleach®, Teepol products, Surrey, UK) (Groups 2 & 4), respectively, for 20 minutes. The concentration of sodium hypochlorite was verified by iodometric titration. Dentine bars in groups 1 & 2 were then stored in 0.9% saline solution without being subjected to heat treatment, whilst those in groups 3 & 4 were held for 10 minutes at 200 ºC in a laboratory chamber furnace (Lenton, Hope Valley, UK), allowed to cool and replaced in 0.9% saline solution.

After exposure, the dentine bars were subjected to a 3-point bend-test using an Instron loading machine (Instron®, High Wycombe, UK), with tubule orientation parallel to the loading, to measure the flexural strength (MPa) and maximum strain (%) of the dentine bars

at fracture (Grigoratos *et al.* 2001). The cross-head diameter was 2.20 mm and the speed 1 mm/min.

2.3 Effect of NaOCl and heat treatment (at 200 °C) on Storage Modulus and Tan Delta (Groups 5 & 6; n = 10 each)

The dentine bars in groups 5 & 6 were immersed in 40 mL of 0.9% saline or 2.5% NaOCl, respectively, for 20 minutes (Table 1). Following removal from their respective solutions, each dentine bar was left to partially air-dry, at room temperature for 10 minutes, whilst maintained on an electronic balance (Analytic balance, Denver instrument Company, Norfolk, UK). The rate of weight loss after 10 minutes (due to water loss) was found to be low and stable after this time. The final weight of the dentine bar was recorded.

Each bar was subsequently subjected to dynamic mechanical analysis (Ferry, 1980) to determine SM and TD (test 1); and then subjected to heat treatment at 200 °C for 10 minutes in a furnace (as previously described), allowed to cool and stored in a dry airtight storage container, and DMA repeated (test 2). The dentine bars were subsequently re-hydrated in 0.9% saline for 24 hours and the DMA repeated once again (test 3).

2.4 Effect of NaOCl and heat treatment on Tan Delta and Storage Modulus under conditions of dynamic temperature increase (Groups 7 & 8; n = 3 each)

Dentine bars in group 7 & 8 were immersed in 40 mL of 0.9% saline or exposed to 2.5% NaOCl, respectively, for 20 minutes, left to partially air-dry, at room temperature for 10 minutes as previously described. Each dentine bar was then subjected to DMA under a continuous temperature scan at a starting ambient temperature of 18 °C, increasing at a rate of 5 °C per minute and rising to a maximum temperature of 185 °C.

2.5 Micro-Raman spectroscopy

Three dentine bars randomly selected from groups 1, 2, 3 and 4 were retained for micro-Raman analysis (LabRam 300, Horiba Jobin-Yvon, France). The samples were placed at a

focus of a 50x microscope and three random point spectra were acquired at a resolution of ~ 6 cm^{-1}, over the spectral region of 850 to 1750 cm^{-1}, with an accumulation time of 20 s and 3 acquisitions for each point. The resulting average spectra of each sample was gathered, processed using post processing tools (Labspec 5 software, Horiba Jobin-Yvon, Palaiseau, France) and peak intensities of the mineral phase and organic phase were averaged and the ratio between the two (mineral/organic) was calculated.

2.6 Data analysis

The data were analysed using the STATA® statistics/data analysis software version 13 (StataCorp, College Station, TX, USA). All inferential analysis was carried out at a significance level of 5%.

General Linear Models were used to analyse the effects of 2.5% NaOCl and heat treatment on the flexural strength (MPa) and maximum strain (%) of the dentine bars in groups 1 to 4.

Repeated measures ANOVA with pair-wise comparisons of marginal linear predictions were performed to compare values of tan delta and storage modulus (GPa) of dentine bars in groups 5 and 6, prior to heat treatment, after heat treatment and following re-hydration.

The storage modulus and tan delta values of dentine bars in groups 7 and 8 were plotted against the temperature and analysed descriptively.

The micro-Raman data were analysed by a two-way factorial ANOVA to compare the effect of the surface treatment (NaOCl) and heat treatment on the mineral phase (960 cm^{-1}), organic phase (1667 cm^{-1}) and their ratio (Mineral/Collagen). All inferential analysis was carried out at a significance level of 5%.

3. Results

3.1 Effect of NaOCl and heat treatment on flexural strength and maximum strain at fracture (Groups 1-4; n = 15 each group)

The mean values of flexural strength (MPa) and maximum strain (%) at fracture for groups 1 to 4 are presented in Table 2. General linear model analysis revealed that chemical (NaOCl or saline) (P=0.8, P=0.2) or heat (room temperature or 200 °C) (P=0.7, P=0.07) treatment individually had no statistically significant effect on the flexural strength or maximum strain at fracture of the dentine bars at the 5% level, respectively.

3.2 Effect of NaOCl and heat treatment (at 200 °C) on Storage Modulus and Tan Delta (Groups 5 & 6; n = 10 each)

Data for the effect of heat and re-hydration on storage modulus and tan delta of dentine bars, with or without NaOCl pre-treatment, are presented in Table 3.

3.2.1 Group 5 (Saline)

Two dentine bars fractured during testing, reducing the sample size to 8. The repeated ANOVA revealed that the heat treatment and subsequent rehydration did not have a significant effect on storage modulus (GPa) (P=0.06), but a significant effect on tan delta (P=0.0002) at the 5% level. *Post hoc* pairwise comparison of marginal linear predictions revealed an increase in storage modulus after the heat treatment (P=0.06) with a significant subsequent reduction after rehydration (P=0.03) returning to the pre-treatment state (P=0.7). There was no significant change in tan delta after heat treatment (P=0.4) but the subsequent rehydration resulted in a highly significant increase in tan delta compared with the pre-treatment state (P=0.001) or after heat treatment (P< 0.0001).

3.2.2 Group 6 (2.5% NaOCl)

Amongst the dentine specimens pre-treated with NaOCl, subsequent heat treatment and rehydration had no significant effect on storage modulus (P=0.6) but significant effects on tan delta (P=0.02) (Table 3). *Post hoc* pairwise comparisons of marginal linear predictions

revealed there were no significant changes in the tan delta after heat treatment (P=0.5); however, the subsequent re-hydration resulted in significant increase in tan delta compared with the pre- (P= 0.007), or post- (P=0.03) heat-treatment states.

3.3 Effect of NaOCl and heat treatment on Tan Delta and Storage Modulus under conditions of dynamic temperature increase (Groups 7 & 8; n = 3 each)

3.3.1 Group 7 (Saline)

The changes in storage modulus and tan delta with temperature amongst the three bars followed a similar trend. The plots of storage modulus and tan delta against temperature of a representative dentine bar are presented in Figure 1. It shows a change in storage modulus and tan delta with temperature increase. The storage modulus initially fell in value from 25-60 °C and then increased linearly in value, reaching a plateau around 143–170 °C, followed by a further drop. In contrast, tan delta values showed an inverse trend with values increasing up to temperatures of 80–100 °C, after which they fell at around 118 °C, followed by an increase to values above the baseline. The two plots intersected at around 100 °C for all three bars.

3.3.2 Group 8 (2.5% NaOCl)

The changes in storage modulus and tan delta with temperature amongst the three dentine bars pre-treated with NaOCl did not follow similar trends and hence are all presented (Figures 2 a-c).

The storage modulus of dentine bars 1 & 2 increased with temperature but with fluctuation in dentine bar 1 and plateauing at 120 °C in dentine bar 2. The storage modulus of dentine bar 3 fluctuated throughout, with a sharp peak at 40 °C and a smooth peak at 110 °C.

Tan delta values showed unpredictable variation in pattern that was different to that observed in dentine bars not exposed to NaOCl. In dentine bar 1, there was a small decrease in tan delta between 25-60 °C, and the values fluctuated thereafter until it

increased steeply after 160 °C. In dentine bar 2, there was small fluctuation in the tan delta values with peaks at around 65 °C and 115 °C. In dentine bar 3, the tan delta values increased with a peak at 70 °C and then decreased thereafter. Regardless of the variations, tan delta values increased above the baseline values when 185 °C was reached.

3.4 Micro-Raman spectroscopy

The Raman spectra of the selected dentine bars from groups 1–4 are shown in Figure 3. The region below 1100 cm^1 in dentine spectra represent phosphate (960 cm^{-1}) and carbonate groups (1072 cm^{-1}). The organic phase, representing collagen includes (amide I 1667 cm^{-1}, amide III 1243 cm^{-1} and CH_2 deformation at 1457 cm^{-1}). Saline with no heat gave the baseline spectra.

Application of NaOCl resulted in depletion of the 1667 cm^{-1} peak and increase in the 1072 cm^{-1} peak (Table 4). The minor changes reflected in the mean differences between groups considering the mineral and organic phases alone were not statistically significant (P>0.05). However, the interaction between the two factors (surface treatment vs. heat treatment) was statistically significant (P=0.04) when the hydroxyapatite (HA) peak was taken into account: Heat treatment resulted in depletion of HA peak in samples exposed to saline but increase in HA peak amongst samples exposed to NaOCl. The only statistically significant difference (P=0.04) lay in the carbonate peak, which increased following the application of NaOCl.

4. Discussion

The reported Young's modulus values for dentine vary enormously, the likely true estimated value is said to lie between 18-20 GPa (Kinney et al., 2003). The variations are attributed to specimen preparation and flaws (Ryou et al., 2011), experimental design, stress relaxation, and storage conditions (Kinney et al., 2003). Dentine bar preparation and their dimensions could affect their mechanical properties (Grigoratos et al., 2001; Sim et al., 2001) but the consistency in values *within* previous and current study suggests this was relatively well controlled. Any limited size discrepancy between samples was accounted for by individual measurement of samples.

Dentine properties may also differ by location within the source teeth; Ryou et al., (2011) reported significantly lower elastic modulus, strength and energy to fracture in dentine harvested near the pulp than that harvested superficially. Bars of larger dimensions would therefore exhibit heterogeneous properties or property gradients. Additionally, intertubular dentine heated to 140 °C has been shown to be affected more than peritubular dentine (Hayashi et al., 2012), suggesting an influence mediated to a greater extent by changes to collagen.

Dentine is moderately anisotropic by virtue of its tubular structure and in a perpendicular direction due to its intertubular collagen matrix (Kinney et al., 2003), particularly in the context of heated dentine (140 °C) (Hayashi et al., 2008). The tubular structure does not cause anisotropy relative to its elastic properties (Kinney et al., 1999) but tensile strength was deemed lowest when aligned with the tubules (Hayashi et al., 2008; Ryou et al., 2011). The DMA and Instron tests in this study would be deemed to have been performed in the plane of maximum strength - parallel to the dentinal tubules (Forien et al., 2015; Kinney et al., 2003; Anil Kishen, 2006; Nalla et al., 2003; Zaslansky et al., 2016). Hayashi *et al*. (2008) found that flexural and microtensile strength in specimens with tubules oriented parallel to the load were 2-2.4× higher after heating to 140 °C for 1 hour but heating to 200 °C reduced

the flexural (178 MPa at 140 °C; 145 MPa at 200 °C) and microtensile (195 MPa at 140 °C; 167 MPa at 200 °C) strength.

The present study revealed that chemical or heat treatment individually had no significant effect on flexural strength or maximum strain at fracture of the dentine bars at the 5% level. This would seem consistent with Hayashi *et al*. (2008). The interactive effects of chemical and heat treatments on both flexural strength (P=0.06) and maximum strain at fracture (P=0.08), were not significant at the 5% level, suggesting minimal accumulative effect and possibility of mediation via changes to both collagen and hydroxyapatite.

The outcome of FS related to NaOCl treatment contrasts with the findings of Grigoratos et al., (2001) and Sim et al., (2001), in the level of statistical certainty. In Grigoratos et al., (2001), exposure of dentine bars (11.7×1.0×1.0mm) to 3% NaOCl significantly reduced flexural strength (from $8.7×10^7$ Pa [for saline] to $3.7×10^7$ Pa). Whilst in Sim et al., (2001), exposure of dentine bars (11.7×0.8×0.8mm) to 0.5% NaOCl did not reduce flexural strength, whereas exposure to 5% NaOCl did ($17×10^7$ Pa vs $10.3×10^7$ Pa). Dentine tubule orientation to the applied force was the same as in the present study. The differences in outcomes may reflect different sample parameters or test conditions; possibly a dose-dependent effect, 2.5% lying between the virtual absence of effect at 0.5% (Sim et al., 2001) and manifest effect at 3.0% (Grigoratos et al., 2001). Nevertheless, the direction of effect is consistent and explainable by collagen denaturation at the dentine surface (Ramirez-Bommer et al., 2018). Strength and elasticity are largely contributed to by the mineral structure, which should be minimally affected by NaOCl, except possibly at the surface.

The Raman spectra in this study (Figure 3) showed slight changes in the intensity of peaks, reflecting alteration in the chemistry of the dentine substrate. The mineral/collagen ratio (ratio between the 960 cm^{-1} peak for hydroxyapatite and 1667 cm^{-1} for amide I) was increased in samples subjected to 2.5% NaOCl alone and followed by heat treatment, revealing the organic phase of the substrate shows initial depletion, however, these changes

were not statistically significant compared to the controls. These observations are consistent with the mechanical findings and in accordance with Pascon et al., (2012), since in their study pretreatment with 5.25% NaOCl (a higher concentration) did not alter, significantly, the organic content of permanent dentine as confirmed by micro-Raman analysis. The collagen is surrounded and protected by the mineral phase, this may explain why no marked changes were evident (Miles and Ghelashvili, 1999). The increase in the carbonate peak (CO_3 at 1072 cm^{-1}) in this study, however, was statistically significant and is consistent with the formation of carbonate, known to occur due to treatment with NaOCl as previously reported (Tsuda et al., 1996). As carbonate content increases, there is a resulting decrease in crystallinity which in turn is known to affect mechanical properties (Xu et al., 2012). However, in the current study, no significant change in FS was observed after exposure to 2.5% NaOCl, regardless of heat treatment, suggesting the chemical changes that did occur were mainly a surface effect (Morgan et al., 2018; Ramirez-Bommer et al., 2018).

The effect of heat on the quasi-static test outcomes on dentine reveals a range of effects, depending upon the source of the sample, test method and property under test (Lin et al., 2010).

The present findings contrast with those of Watts et al., (1987) who showed when testing dentine loaded *perpendicular* to tubules that Young's modulus, proportional limit, compressive strength and resilience all linearly *decreased* with increasing temperature (0-80 °C). The toughness decreased from 0 °C to 37 °C and then remained constant up to 80 °C. Whilst, Rees et al., (1994) also using DMA, found an *increase* in Young's modulus with temperature (27 °C – 37 °C); the authors postulating that the effect may be due to dessication. The effect on flexural strength and maximum strain at fracture in the present study may be attributed to some initial denaturation of collagen and in the case of effect of heat, in addition the loss of bound water from the inter-tubular matrix and may also be due to changes in the mineral composition, with the increase of carbonate.

In the present study, when dentine specimens were pre-treated with NaOCl, heat treatment and rehydration had no significant effect on storage modulus (P=0.6) but significant effects on tan delta (P=0.02). There were no changes in tan delta after heat treatment (P=0.5) but there was a significant increase in tan delta upon rehydration compared with the pre- (P=0.007), or post- (P=0.03) heat-treatment states.

The results of groups 7 and 8 confirmed the outcomes in groups 5 and 6 but conveyed interesting insight into variations leading up to the final outcomes evident in the latter groups. They showed variation in the storage modulus and tan delta values with changing ambient temperature. The patterns of change in group 7 compared with group 8 were starkly different. In the saline-exposed specimens of group 7, the data for the 3 bars consistently showed a decrease in storage modulus between 25-50 °C, followed by a subsequent rise between 50-140 °C. In the same temperature ranges, tan delta showed a mirror image increase and then decrease. Between the temperatures of 100-145 °C, the trend was reversed with storage modulus increasing, whilst tan delta continued to decrease upto 115 °C and thereafter slowly increasing. Between 145-185 °C, storage modulus decreased, whilst tan delta continued its increase to converge with storage modulus at 185 °C. It was speculated that the dentinal collagen was becoming more flexible with the rise in temperature, thus accounting for the fall in storage modulus as the temperature rose to 60 °C. However above 60 °C, collagen shrinkage (Vangsness et al., 1997) may have caused stiffening until at 145 °C, collagen dehydration probably limited further increase and led to a decline.

The behaviour of dentine bars pre-treated with sodium hypochlorite (Group 8) was starkly different and showed variation in pattern and actual values amongst the 3 bars. The inverse relationship between storage modulus and tan delta (in Group 7) was less evident. Storage modulus showed an increase in two bars and a slight decrease in one. Tan delta showed variations that were similar in pattern in bars 2 and 3 but different in the first. The variations

may be accounted for by differences in collagen depletion between the 3 bars, which in turn may also reflect anatomical differences in the dentine.

Given the close conjunction of collagen and apatite in dentine, there are likely to be mechanical interactions between these two components with chemical or thermal alterations playing a part in any altered physical properties of the composite. The effect of heat on natural composites containing organic and inorganic components has been studied in various tissue types. Heating a native collagen fibril reduced its Young's modulus at temperatures between 20-58 °C, due to partial denaturation of triple helices; but it then remained constant between 58-75 °C because of stabilization by inter-molecular interactions when hydrated. This stabilization phase was associated with absorption of water under heating, which was deemed to be due to restructuring of the triple helices (Gevorkian et al., 2013). The thermal stability of collagen depends on resistance to unfolding as a result of heat (Armstrong et al., 2008). The unfolding occurs first by separation of triple helices into individual helices due to disruption of hydrogen bonds between the polypeptide chains. Individual helices may then unfold into random coils due to disruption of intra-helical hydrogen bonds. As water is lost, the collagen fibrils are said to approximate, allowing collagen peptides to form new intrinsic associations due to hydrogen bonds in addition to covalent intermolecular cross-links critical to the stability of the triple helices. The new hydrogen bonds stiffen and strengthen the collagen matrix making it more stable to elevations in temperature in the absence of water. When rehydrated, there is a rapid loss in stiffness, indicating that the changes are reversible and due to weak non-covalent forces. In addition, hydration is enhanced by unfolded collagen in the presence of water (McGee et al., 2012). Thermal denaturation of collagen may occur to different levels at 65 °C and 150 °C under hydration but continue at 220 °C, 305 °C, and 345 °C when dry (Bozec and Odlyha, 2011). Denaturation of collagen fibrils is dependent on water hydration, temperature and duration for which it is held at that temperature. One minute at temperatures *higher than* 80 °C in water may achieve collagen fibril denaturation, whilst fibrils in water held at

temperatures of 80 °C for *only* 1 min achieved partial denaturation, complete denaturation taking 90 mins. In dry conditions, fibrils maintain their native structure even after heating to 120 °C for 90 mins, although partial denaturation could not be prevented (Suwa et al., 2016).

Heating collagen in tendons caused it to shrink by 10% and reduced its load to failure by 33% (Vangsness et al., 1997). When confined within bone, heat-induced unwinding of collagen molecules could alter bone stiffness; the same effect but to a lesser degree could be induced by enzyme denaturation of collagen (Wang et al., 2002). Both types of collagen denaturation caused similar changes in strength and work to fracture in bone.

When collagen is confined within the mineralized structure of dentine, the collagen in its natural state is thought to be under tension, whilst the attached intrafibrillar apatite nanoparticles are in compression (Forien et al., 2015; Zaslansky et al., 2016). Furthermore, as explained above, confinement of the collagen in a mineral matrix may stabilize it to thermal denaturation by the so-called "polymer-in-a-box" phenomenon (Miles and Ghelashvili, 1999). Taking these two phenomena into account, heating dentine to 200 °C may still have a denaturing effect on the collagen throughout the bulk of the specimen, possibly drawing the mineral together and causing stiffening. Rehydration may soften the collagen components and reduce the stiffness, explaining the findings on the quasi-static properties in this study.

The effect of NaOCl on surface degradation of collagen would be to alter the state of balance of compressive and tensile forces within dentine to the depth of its chemical effect. This may have a marginal effect on flexural strength and increase the maximum strain before fracture as the material in the outer boundaries would be less tightly interwoven by the collagen mesh.

Similarly, the observations for storage modulus with saline pre-treatment contrast with NaOCl treatment and may be explained by the effect of NaOCl on the collagen component. Presumably, degradation of the collagen component deprives the structure of the build-up in

stresses created by shrinkage in the saline treatment group. The effect on tan delta is as expected given that partially degraded and denatured collagen would still attract hydration (McGee et al., 2012) and increased viscous behaviour.

Yamashita et al., (2002) assessed the viscoelasticity of bone heated to 100 °C or 200 °C with DMA; half the specimens were rehydrated after heat treatment, whilst the other half were tested dry. Significant collagen denaturation only occurred when bone was heated to 200 °C, irrespective of hydration; the percentage of denatured collagen did not show a significant effect on tan delta but higher tan δ values were observed in wet samples compared to dry. They concluded that hydration condition rather than denatured collagen affected tan delta, whilst storage modulus was not strongly affected either by percentage of denatured collagen or by water content. In this respect, the present study differed because a significant increase in storage modulus (P=0.06) was found upon heating to 200 °C that was restored upon rehydration (P=0.03), returning to the pre-treatment state (P=0.7). Simultaneously, there was no change in tan delta after heat treatment (P=0.4) but subsequent rehydration significantly increased tan delta in comparison to the pre-treatment state (P=0.001) or after heat treatment (P<0.0001). The differences could be explained at least partly by the differences in degree of confinement of the collagen fibres in the two tissue types. The effect of heat may be to drive out the free and bound water from the dentine leading to shrinkage of both the collagen and apatite nanoparticles, causing a "tightening" and consolidation of the structure to make it stiffer and elastic. Restoration of the hydrated status would then return the mineral and organic components to their normal structure and hence physical properties (Forien et al., 2016). Denatured collagen may draw greater water saturation resulting in higher viscous tendency (McGee et al., 2012).

In summary, heat has an effect on the chemical and mechanical properties and physical behaviour of dentine, the extent of which may be dependent on the type of dentine and the relative exposure of the bulk material to heat and/or NaOCl. Clinically, this implies that caution should be exercised in the extent of exposure to heat and on the application of

forces, particularly whilst dentine is in a dehydrated or semi-hydrated state immediately after heat application.

5. Conclusions

Within the limitations of this study it may be concluded that exposure of dentine bars to heat and NaOCl, independently and accumulatively, produced moderate changes in quasi-static mechanical properties but more obvious and marked changes in viscoelastic properties. These may be explainable by chemical changes in collagen.

List of tables

List of figures

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Table 1 – The test groups, chemical, heat treatments and physical tests

Group	Chemical treatment	Heat treatment	Sample size	Test	Outcome measure
Group 1	0.9% Saline	None	15	Instron	Flexural strength
Group 2	2.5% NaOCl	None	15	Instron	Flexural strength
Group 3	0.9% Saline	200ºC for 10 mins	15	Instron	Flexural strength
Group 4	2.5% NaOCl	200ºC for 10 mins	15	Instron	Flexural strength
Group 5	0.9% Saline	Test 1: Air-drying for 10 mins Test 2: 200ºC for 10 mins Test 3: Rehydration in 0.9% saline	10	DMA	Tan delta Storage modulus
Group 6	2.5% NaOCl	Test 1: Air-drying for 10 mins Test 2: 200ºC for 10 mins Test 3: Rehydration in 0.9% saline	10	DMA	Tan delta Storage modulus
Group 7	0.9% Saline	18 ºC – 185 ºC	3	DMA	Tan delta Storage modulus
Group 8	2.5% NaOCl	18 ºC – 185 ºC	3	DMA	Tan delta Storage modulus

Table 2 – Mean (±SD) flexural strength and maximum strain of dentine bars in groups 1-4 and the effects of sodium hypochlorite, and heat treatment

Groups	N	Mean (±SD) flexural strength (MPa)		Mean (±SD) maximum strain at fracture (%)	
Group 1 (Saline – no heat)	15	0.30±0.30		1.64±0.60	
Group 2 (NaOCl – no heat)	15	0.18±0.04		1.02±0.41	
Group 3 (Saline – 200ºC)	15	0.22±0.06		1.66±0.76	
Group 4 (NaOCl – 200ºC)	14	0.31±0.34		1.79±1.25	
General Linear Models					
Independent variables	**df**	**F**	**P value**	**F**	**P value**
Heat (200ºC vs room temperature)	1	0.17	0.6	3.54	0.07
NaOCl (NaOCl vs Saline)	1	0.08	0.8	1.36	0.2
Heat*NaOCl	1	3.59	0.06	3.21	0.08

Table 3 – Mean (±SD) storage modulus and tan delta of dentine bars in groups 5 & 6, before heat treatment, after heat treatment and following rehydration in the saline (NB. Two bars in the saline group fractured during testing).

Group	Mean storage modulus (GPa)				Mean tan delta			
	Test 1 No heat	Test 2 200ºC for 10 minutes	Test 3 Rehydration	Repeated ANOVA	Test 1 No heat	Test 2 200ºC for 10 minutes	Test 3 Rehydration	Repeated ANOVA
Group 5: Saline (n = 8)	14.3±4.1	15.9±4.3	14.0±14.0	P=0.06	0.056±0.059	0.054±0.005	0.068±0.011	P=0.0002
Pairwise comparisons of marginal linear predictions		Test 2 vs 1 (P=0.06) Test 3 vs 1 (P=0.7) Test 3 vs 2 (P=0.03)				Test 2 vs 1 (P=0.4) Test 3 vs 1 (P=0.001) Test 3 vs 2 (P< 0.0001)		
Group 6: NaOCl (n = 10)	16.3±7.3	16.3±13.5	14.9±8.3	P=0.6	0.059±0.007	0.061±0.012	0.068±0.013	P=0.02
Pairwise comparisons of marginal linear predictions		Test 2 vs 1 (P=1) Test 3 vs 1 (P=0.4) Test 3 vs 2 (P=0.4)				Test 2 vs 1 (P=0.5) Test 3 vs 1 (P=0.007) Test 3 vs 2 (P=0.03)		

Table 4 – Table with micro-Raman intensity means (±SD) for groups 1 – 4 and the effects of heat and NaOCl treatment on the apatite, carbonate, collagen peak intensity or ratio of mineral/collagen intensity.

Groups	Dependent variables			
	Apatite (960 cm^{-1})	Carbonate (1072 cm^{-1})	Collagen (1667 cm^{-1})	Mineral/Collagen Ratio
Group 1 (Saline – no heat)	1.52 ± 0.17	0.23 ± 0.004	0.15 ± 0.02	10.3 ± 1.99
Group 2 (NaOCl – no heat)	1.40 ± 0.17	0.26 ± 0.02	0.13 ± 0.02	11.1 ± 2.43
Group 3 (Saline – 200 ºC)	1.32 ± 0.15	0.23 ± 0.02	0.14 ± 0.003	10.0 ± 0.05
Group 4 (NaOCl – 200 ºC)	1.63 ± 0.08	0.26 ± 0.01	0.13 ± 0.02	12.6 ± 1.87

Two-way factorial ANOVA

Independent variables	P value	P value	P value	P value
Heat (200 ºC *vs* room temperature)	0.8	0.9	0.7	0.6
NaOCl (NaOCl *vs* Saline)	0.3	0.04	0.1	0.1
Heat*NaOCl	0.04	0.9	0.5	0.4

*P values for Two-way factorial ANOVA for the effect each independent variable on the respective dependent variable in the same column

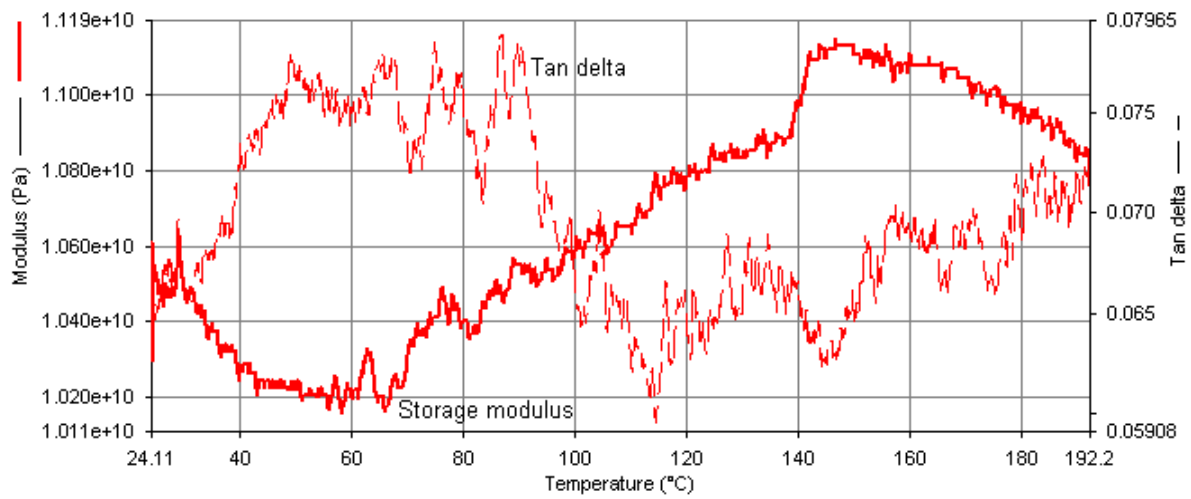**Figure 1 –** Changes in storage modulus and tan delta with temperature increase; saline group.

Figure 2 – Changes in storage modulus and tan delta with temperature increase; sodium hypochlorite group.
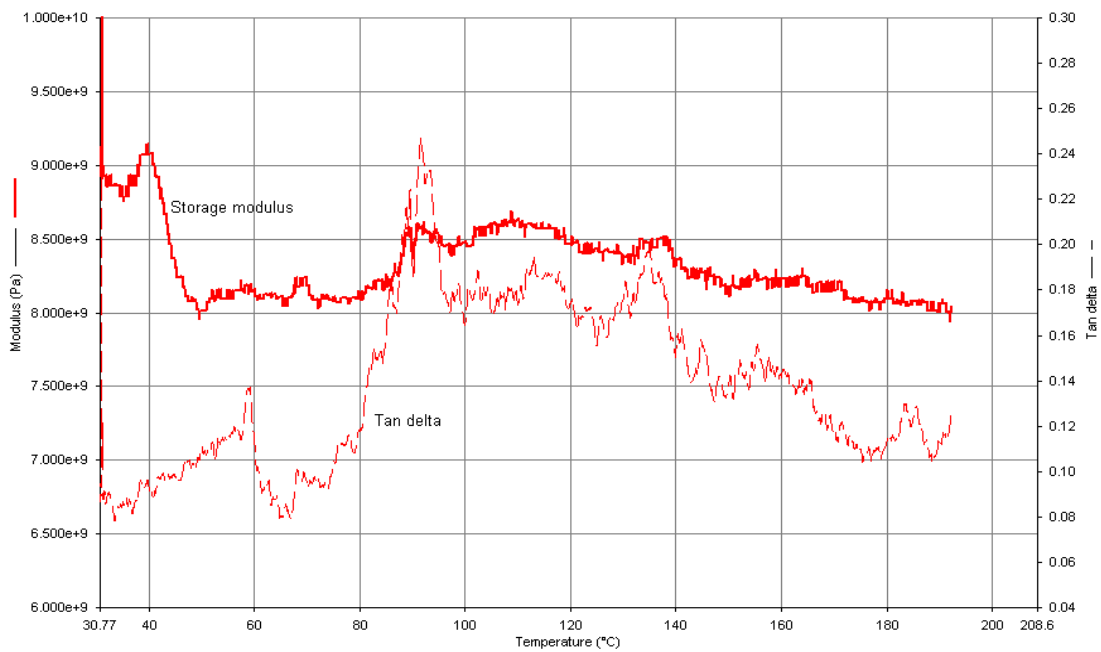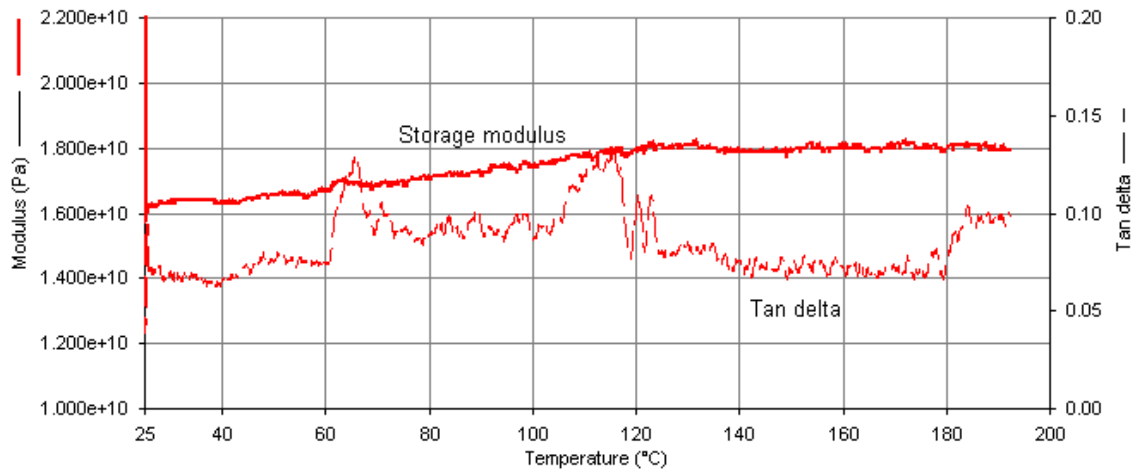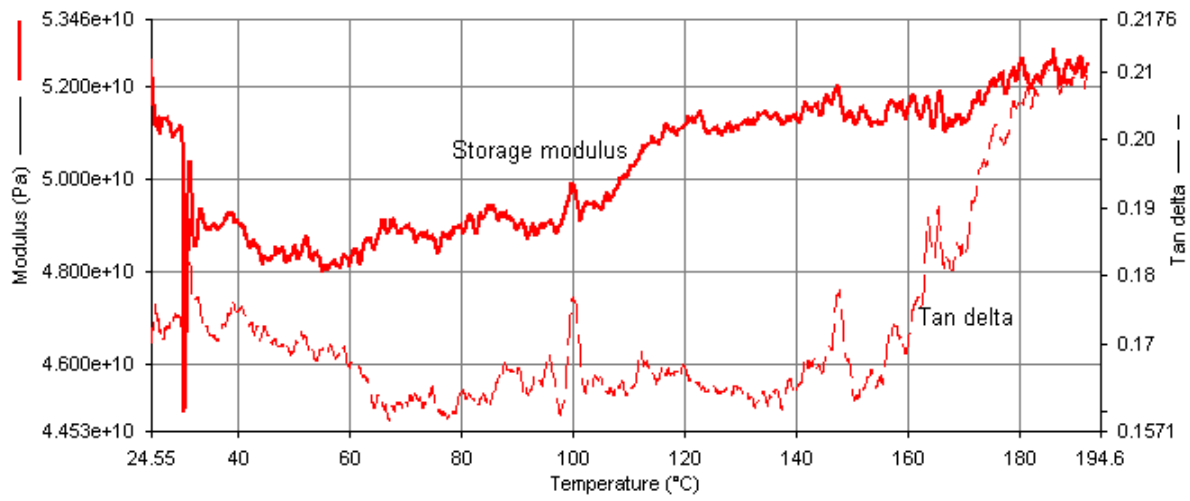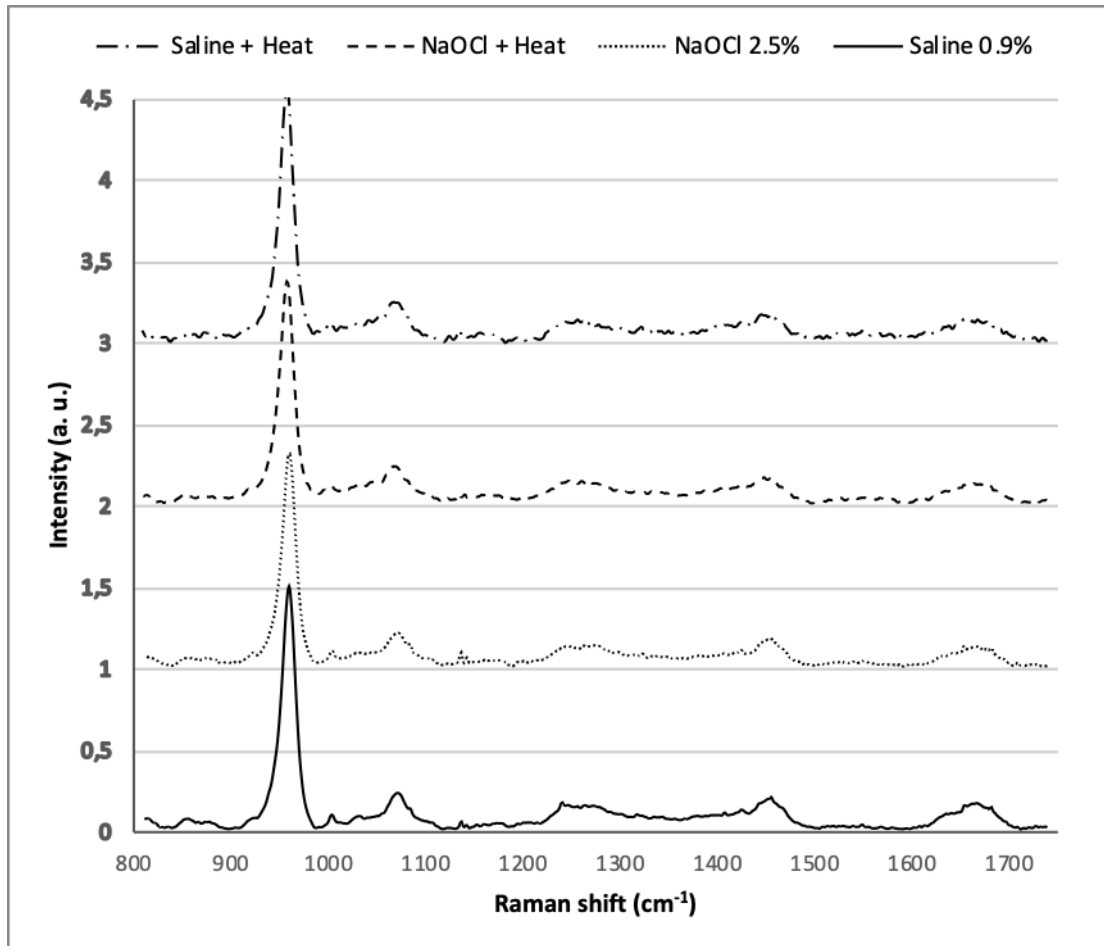
Figure 3 – Micro-Raman spectra of dentine from groups 1- 4 (800-1750 cm^{-1} region).

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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