The influence of different irrigation regimes on the cleanliness and physical properties of the root canal walls

By

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Abstract

This thesis describes a series of investigations into the effects of root canal irrigants on dental root dentine.

Imaging of human, ovine and bovine root dentine revealed no significant differences in tubule density or diameter. Canal volumes were estimated to be significantly different between human and bovine, but not between human and ovine teeth. EDAX identified significant differences in Ca/P ratios between ovine and bovine, and between ovine and human dentine at points up to 300µm from the root canal lumen. The Ca/P ratio of bovine dentine was significantly lower than human at the canal lumen only.

Atomic Force Microscopy (AFM) revealed human and bovine root dentine to be significantly stiffer than ovine dentine at all depths from the canal lumen; human root dentine was stiffer than bovine dentine up to 300µm from the lumen. Animal teeth were concluded to be imperfect replacements for human teeth in endodontic research.

Serial polishing down to 0.05µm aluminium oxide was refined for the gentle removal of laboratory-generated smear layers from sectioned dentine specimens, allowing analysis of subtle surface and sub-surface changes following exposure to root canal irrigants, and AFM analysis of smooth, flat dentine surfaces.

A new 5-point scale was developed for scoring root canal cleanliness. NaOCl (5% & 10%) removed pulpal debris and predentine from canal walls after 5 minutes exposure at room temperature, with no evidence of chemical etching (score 4). NaOCl (2.5%) also resulted in a score of 4 in the coronal third, although in middle and apical thirds it was less effective (score 3).

AFM analysis revealed no change in surface or sub-surface dentine stiffness after exposure to NaOCl, (5% & 10%, 5 minutes). Significant changes in dentine stiffness after exposure to 17% EDTA and 6% citric acid for 1 minute, were not increased after 2 minutes exposure.

Author’s Declaration

I declare that the work in this thesis is my own original work. I also state that it has never been submitted for any other degree course at Newcastle University or any other Institute, except where indicated.

Supervisor's Certificate

This is to certify that the entitled thesis **"**The influence of different irrigation regimes on the cleanliness and physical properties of the root canal walls**"** has been prepared under my supervision at the school of Dental Sciences/Newcastle University for the degree of PhD in Conservative Dentistry.

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Dedication

To all my family, my parents, my sister and my brother Ammar Al-Khafaji,

I owe you all my utmost respect and thanks for your support and encouragement.

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Table of contents

Chapter 1: Introduction 1

Chapter 2: Literature review 3

2.1 Purpose of root canal treatment 3

2.2 Challenges of contained pulp canal infections 4

2.2.1 The nature of pulp space infection 4

2.2.2 Complexities of the pulp canal space 6

2.3 Dentine as the tissue on which treatment is performed 8

2.3.1 Structure and chemical composition of dentine 14

2.3.2 Nanostructure of dentine 15

2.4 Role, limitations and risks of mechanical instrumentation in root canal treatment 18

2.5 Need for irrigants to complement instrumentation 20

2.5.1 Overview of common irrigant solutions 20

2.5.2 Alternative irrigant agents 35

2.5.3 Irrigant combinations 37

2.5.4 The delivery and activation of irrigant solutions 40

2.6 Important properties of dentine 45

2.6.1 Chemical properties 45

2.6.2 Mechanical properties of dentine 45

2.7 Devices for analysing dentine 49

2.7.1 SEM 49

2.7.2 EDAX 50

2.7.3 ESEM 52

2.7.4 TEM 52

2.7.5 CLSM 53

2.7.6 Microhardness tester 54

2.7.7 Nanoindenter 55
2.7.8 SPM and AFM 56
2.8 Evidence for damage to dentine 57
2.8.1 Evidence for damage to dentine by root canal treatment procedures 57
2.8.2 Evidence for chemical damage to dentine by irrigant solutions 58
2.9 Methods of scoring the cleanliness of root canal 60
2.10 Suitability of animal dentine and dental roots for endodontic research 64
2.10.1 Volume of root canal 65
2.10.2 Density and diameter of dentinal tubules 65
2.10.3 Chemical and physical composition of human and animal teeth 67
2.10.4 Sterilisation and storage procedures 68
2.10.5 Some experimental situations of using animal teeth 69
2.11 Unanswered questions emerging from this literature review 69
Chapter 3: The validation of animal teeth as a model for human teeth 71
3.1.1 Introduction 71
3.2 Diameter and density of dentinal tubules 73
3.2.1 Aim 73
3.2.2 Materials and methods 73
3.2.3 Results 74
3.2.4 Discussion 81
3.2.5 Conclusion 83
3.3 Estimation of root canal volume 83
3.3.1 Aims 83
3.3.2 Materials and methods 83
3.3.3 Results 89
3.3.4 Discussion 91
3.3.5 Conclusion 93

3.4 Analyses of Ca/P ratio of root dentine by EDAX 93
3.4.1 Aims 93
3.4.2 Materials and methods 93
3.4.3 Results 95
3.4.4 Discussion 99
3.4.5 Conclusion 101
3.5 Analysis of dentine stiffness by Atomic Force Microscopy (AFM) 101
3.5.1 Aim 101
3.5.2 Materials and methods 101
3.5.3 Results 103
3.5.4 Discussion 108
3.5.5 Conclusion 109
Chapter 4: The removal of saw-generated smear layer from dentine 110
4.1.1 Introduction 110
4.2 Mechanical brushing and ultrasonication 111
4.2.1 Aim 111
4.2.2 Materials and methods 111
4.2.3 Results 114
4.2.4 Discussion 119
4.2.5 Conclusions 123
4.3 Macro and micro polishing 123
4.3.1 Aim 123
4.3.2 Materials and methods 123
4.3.3 Results 124
4.3.4 Discussion 127
4.3.5 Conclusion 127
4.4 Micro polishing 128

4.4.1 Aim....128
4.4.2 Materials and methods....128
4.4.3 Results....129
4.4.4 Discussion....134
4.4.5 Conclusion....134
Chapter 5: Removal of pulp debris and predentine from the walls of root canals....135
5.1.1 Introduction....135
5.2 Continuous irrigation to remove pulp debris and predentine from ovine root canal and its effect on the Ca/P ratios of root dentine....137
5.2.1 Materials and method....137
5.2.2 Results....142
5.2.3 Discussion....157
5.2.4 Conclusion....162
5.3 The substitution of ultrasonic agitation of NaOCl irrigant with other methods 162
5.3.1 Materials and methods....163
5.3.2 Results....165
5.3.3 Discussion....170
5.3.4 Conclusions....171
5.4 Debris removal from human root canals....172
5.4.1 Materials and Methods....172
5.4.2 Results....175
5.4.3 Discussion....182
5.4.4 Conclusion....183
Chapter 6: AFM analysis of the effect of irrigants on human root dentine....184
6.1.1 Introduction....184
6.2 Effect of NaOCl on human root dentine surfaces....185
6.2.1 Aim....185

6.2.2 Materials and methods 185
6.2.3 Results 187
6.2.4 Discussion 194
6.2.5 Conclusions 197
6.3 The effect of citric acid on human root dentine surface 197
6.3.1 Aim 197
6.3.2 Materials and methods 198
6.3.3 Results 201
6.3.4 Discussion 206
6.3.5 Conclusions 208
6.4 Effect of EDTA on human root dentine surface 208
6.4.1 Aim 208
6.4.2 Materials and methods 208
6.4.3 Results 209
6.4.4 Discussion 215
6.4.5 Conclusions 217
Chapter 7: General discussion 218
7.1 The suitability of ovine and bovine teeth as a model for human teeth 218
7.1.1 Estimation of the root canal volume 220
7.1.2 Analyses of the Ca/P ratios of root dentine 221
7.1.3 Analysis of dentine stiffness 222
7.1.4 Summary 223
7.2 The preparation of root dentine specimens to analyse the effects of irrigants 223
7.3 The effect of different irrigants on the root dentine 224
7.3.1 Selection of the correct type of needle to deliver the irrigant 224
7.3.2 Selection of irrigant 225
7.3.3 Method of application of the irrigant 226

7.3.4 Efficiency of the irrigant and its effect on root canal walls 227
7.3.5 The effect of other irrigants on dentine 227
Chapter 8: Conclusions and recommendations 230
8.1 Conclusions 230
8.2 Recommendations for future work 230
Chapter 9: References 232
Chapter 10: Appendices 290
10.1 Appendix A 290
10.2 Appendix B 293
10.3 Appendix C 296
10.4 Appendix D 297

List of figures

Figure 2.1. Illustration of dentinal tubules (A) which are narrower and more widely spaced at o the dentinoenamel junction compared to those in deeper areas (B). Dentinal tubules in superficial (C) and deep (D) root dentine are also narrower and more sparse than their counterparts in the coronal areas (Roberson *et al.*, 2006).....10

Figure 2.2. Photographic image by transmission electron microscope shows age-related root dentinal sclerosis starting from the apex of the root and progressing coronally. It shows the transition between transparent apical root dentine (dark) and opaque dentine (white) (Nalbandian *et al.*, 1960). 10

Figure 2.3. Photographic image by transmission electron microscope (Nalbandian *et al.*, 1960), and photographic image by SEM shows globular dentine, highly mineralized peritubular dentine contrasts with intertubular dentine which represents the bulk of dentine volume. 11

Figure 2.4. AFM images of human dentine collagen fibrils in which surface imaging was taken with a mixed-mode of the tapping and phase mode. There is a difference in the diameter of collage fibrils between image a (in liquid) and image b (in air) (Habelitz *et al.*, 2002a).....17

Figure 2.5. AFM images of human dentine collagen fibrils taken in liquid. (a) Tapping mode image: gap and overlap zones of adjacent fibrils interlock. (b) Phase mode image reveals the presence of mineral particles attached to fibrils (Habelitz *et al.*, 2002a). 17

Figure 2.6. The saponification reaction of NaOCl and fatty acids which results in fatty acid salts (detergent) and glycerol (alcohol) (Kandaswamy and Venkateshbabu, 2010). 23

Figure 2.7 The reaction of amino acid neutralisation by NaOCl and the formation of salt and water (Kandaswamy and Venkateshbabu, 2010). 23

Figure 2.8 Chloramination process during the reaction of NaOCl with amino acids NH which results in chloramine and water (Kandaswamy and Venkateshbabu, 2010).....24

Figure 2.9. The chemical structure of EDTA.....28

Figure 2.10. Metal ion (calcium) in dental hard tissue is sequestered by EDTA to form stable complexes of calcium edetate (CaEDTA) (Qian *et al.*, 2011).....28

Figure 2.11. Structure of citric acid (Chemical Education Digital Library Staff, 2010).32

Figure 2.12. The dissolution of hydroxyapatite by acid. 32

Figure 2.13. Structure of chlorhexidine digluconate (Basrani and Haapasalo, 2012). ... 35

Figure 2.14. The mechanism of action of chlorhexidine (CHX) on bacteria (Kandaswamy and Venkateshbabu, 2010). 35

Figure 2.15. A simplified diagram showing the production of x-ray radiation by collision of incident electrons with those of the inner shells of an atom. 51

Figure 3.1. SEM image (2000x magnification) to show the structure of root canal dentine walls in the cervical third of ovine teeth in a native, non chemically-treated state. 76

Figure 3.2. SEM image (2000x magnification) to show the structure of root canal dentine walls in the cervical third of bovine teeth in a native, non chemically-treated state. 76

Figure 3.3. SEM image (2000x magnification) to show the structure of root canal dentine walls in the cervical third of human teeth, in a native, non chemically-treated state. 77

Figure 3.4. Median dentinal tubule density in ovine, bovine and human root canals. The difference found is not significant ($p<0.05$, Kruskal-Wallis test). The error bars are the upper and lower quartiles for each group. 79

Figure 3.5. Median dentinal tubule diameter (µm) in ovine, bovine and human root canals. The difference is not significant ($p<0.05$, Kruskal-Wallis test). The error bars are the upper and lower quartiles for each group. 80

Figure 3.6. Radiographic images show the bucco-lingual dimensions of root canal of 3 species, a. ovine, b. bovine and c. human teeth, respectively. They show an imaginary outline of the root canals. All are at the same magnification. 85

Figure 3.7. Photographic image of a horizontal section from the cervical third of the root of an ovine tooth with a plastic scale, the ruler depicted is to indicate scale and was not used for the measurements. 87

Figure 3.8. Photographic image of a horizontal section from the cervical third of the root of a bovine tooth with a plastic scale, the ruler depicted is to indicate scale and was not used for the measurements. 87

Figure 3.9. Photographic image of a horizontal section from the cervical third of the root of a human tooth with a plastic scale, the ruler depicted is to indicate scale and was not used for the measurements. 88

Figure 3.10. Mean volume of ovine, bovine and human root canal spaces measured using distilled water. The difference was significant ($p<0.05$, One way ANOVA). The error bars are the standard deviation values for each group. The number of samples was five for each group, and each sample was measured 5 times. 89

Figure 3.11. Mean assumed volume of ovine, bovine and human root canal spaces measured digitally using ImageJ software. The difference was significant ($p<0.05$, One way ANOVA). The error bars are the standard deviation values for each group. 90

Figure 3.12. Typical SEM photograph showing locations at which EDAX measurements were taken at intervals from the canal lumen (x), 0µm, 100µm, 200µm, 300µm and 1000µm. Note: the black curved-line is a crack resulting from drying of the sample within the vacuum chamber of the EDAX machine. 95

Figure 3.13. Typical EDAX spectra of ovine cervical root dentine. 97

Figure 3.14. Typical EDAX spectra of human cervical root dentine. 97

Figure 3.15. Median Ca/P ratios of horizontal cervical dentine sections of ovine, bovine and human dentine at different depths from the root canal lumen. The error bars are the upper and lower quartiles for each group. 98

Figure 3.16. AFM image to show the structure of root canal dentine walls in the cervical third of ovine teeth. 104

Figure 3.17. Typical force curve of root canal dentine walls in the cervical third of ovine teeth. 104

Figure 3.18. AFM image to show the structure of root canal dentine walls in the cervical third of bovine teeth. 105

Figure 3.19. Typical force curve of root canal dentine walls in the cervical third of bovine teeth. 105

Figure 3.20. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth. 106

Figure 3.21. Typical force curve of root canal dentine walls in the cervical third of human teeth. 106

Figure 3.22. Median dentine stiffness (nN/µm) of horizontal cervical dentine sections of ovine, bovine and human dentine at different depths from the root canal lumen. The error bars are the upper and lower quartiles for each group. 107

Figure 4.1. This figure shows horizontal root section..... 114

Figure 4.2. SEM image (500x magnification) to show Hülsmann score 5 in Group 1 (control group). 116

Figure 4.3. SEM image (500x magnification) to show Hülsmann score 4, in Group 2, treated by ultrasonication in distilled water for 15 minutes. 116

Figure 4.4. SEM image (500x magnification) to show Hülsmann score 3, in Group 3, treated by brushing in distilled water for 15 minutes. 117

Figure 4.5. SEM image (500x magnification) to show Hülsmann score 2, in Group 7, treated ultrasonication in distilled water for 30 minutes. 117

Figure 4.6. SEM image (500x magnification) to show Hülsmann score 1, in Group 4, treated by brushing and ultrasonication in distilled water. 118

Figure 4.7. Median smear layer scores (Hülsmann *et al*'s. 1997) on the surface of horizontal dentine slices of groups 1-8. The difference was significant between the non-treated group and all of the other groups, except group 2 ($p<0.05$, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the maximum and minimum scores for each group. 118

Figure 4.8. SEM image (500x magnification) to show image from pilot study with 6% citric acid treatment, showing smear layer removal but with dentine erosion (Hülsmann score 1). 122

Figure 4.9. SEM image (500x magnification) to show image from pilot study with ultrasonication with 5% SDS without mechanical brushing (Hülsmann score 4). 122

Figure 4.10. SEM image (500x magnification) to show the control group (Group 1) with complete smear layer (Hülsmann score 5). 125

Figure 4.11. SEM image (500x magnification) to show a representative Group 2 specimen after polishing with sand papers (600, 800, 1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1). 125

Figure 4.12. Median score of smear layer persistence on the surface of horizontal dentine slices of groups 1 and 2. The difference was significant ($p<0.05$, Kruskal-Wallis

test). Smear layer scores used the Hülsmann *et al*'s. (1997) scale. The error bars are the maximum and minimum scores for each group. 126

Figure 4.13. SEM image (500x magnification) to show the control group (Group 1) with complete smear layer (Hülsmann score 5). 130

Figure 4.14. SEM image (500x magnification) to show representative Group 2 specimen after polishing with only aluminium oxide (Hülsmann score 1). 130

Figure 4.15. SEM image (500x magnification) to show representative Group 3 specimen after polishing with sand papers (1200µm), and then with aluminium oxide (Hülsmann score 1). 131

Figure 4.16. SEM image (500x magnification) to show representative Group 4 specimen after polishing with sand papers (1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1). 131

Figure 4.17. SEM image (500x magnification) to show representative Group 5 specimen after polishing with sand papers (800, 1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1). 132

Figure 4.18. SEM image (500x magnification) to show representative Group 6 specimen after polishing with sand papers (600, 800, 1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1). 132

Figure 4.19. Median smear layer scores (Hülsmann *et al.*, 1997) for control (1) and experimental (2-6) Groups, following micro polishing. The difference between Group 1 and Groups 2-6 was significant ($p<0.05$, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the maximum and minimum scores for each group. 133

Figure 5.1. Cylindrical mould with a tooth mounted in soft red wax to hold it in position and create an apically closed system for irrigation. 140

Figure 5.2. SEM image (500x magnification) of the ovine root canal wall in the cervical third to show debris and predentine Score 1. 143

Figure 5.3. SEM image (500x magnification) of the root canal wall in the cervical third after dynamic continuous replenishment with 5% NaOCl at 25˚C for 5 minutes (Group 6), with a debris and predentine score of 3. 143

Figure 5.4. SEM image (500x magnification) of the root canal wall in the cervical third after dynamic continuous replenishment with 5% NaOCl at 25˚C for 10 minutes (Group 7), with debris and predentine score of 4. 144

Figure 5.5. SEM image (500x magnification) of the root canal wall in the cervical third after dynamic continuous replenishment with 10% NaOCl at 25˚C for 5 minutes (Group 9), with debris and predentine score of 4. Damage to the canal walls was noted, probably from contact with the ultrasonic file. 144

Figure 5.6. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 25˚C for 0, 5 and 10 minutes. The difference between all experimental groups and the controls was significant ($p<0.05$, chi square test). 145

Figure 5.7. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 25˚C for 0, 5 and 10 minutes. The difference between all of the experimental groups and the controls was significant ($p<0.05$, chi square test)..... 145

Figure 5.8. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 25˚C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test). 146

Figure 5.9. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled for water 0, 5 and 10 minutes and irrigation with 5% NaOCl at 60˚C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test). 146

Figure 5.10. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 60˚C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test). 147

Figure 5.11. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0 minutes, 5 minutes and 10 minutes and irrigation with 5% NaOCl at 60˚C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test). 147

Figure 5.12. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 25°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).....148

Figure 5.13. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 25°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).....148

Figure 5.14. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 25°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).....149

Figure 5.15. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).....149

Figure 5.16. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).....150

Figure 5.17. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).....150

Figure 5.18. Chi square analysis to identify significant differences in pulp debris and predentine scores for ovine root canals in their cervical, middle and apical thirds after no-treatment and irrigation with distilled water and irrigation with 5% NaOCl at 25°C,

5% NaOCl at 60°C, 10% NaOCl at 25°C, 10% NaOCl at 60°C, for 0 minutes, 5 minutes and 10 minutes respectively. NaOCl treatments were significantly more effective than untreated control and water treatments at all levels (cervical, middle and apical thirds) ($p<0.05$, chi square test). 151

Figure 5.19. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no treatment and irrigation with distilled water for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group. 155

Figure 5.20. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 5% NaOCl at 25°C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group. 155

Figure 5.21. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 5% NaOCl at 60°C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group. 156

Figure 5.22. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 10% NaOCl at 25°C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group. 156

Figure 5.23. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 10% NaOCl at 60°C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group. 157

Figure 5.24. SEM image (500x magnification) of an ovine root canal wall in the cervical third without treatment (Group 1, untreated control), showing full coverage with debris and predentine (score 1). 166

Figure 5.25. SEM image (500x magnification) of the ovine root canal wall in the cervical third from Group 2 to show debris and predentine score of 2. 166

Figure 5.26. SEM image (500x magnification) of the ovine root canal wall at the cervical third from Group 3 to show debris and predentine score of 4. 167

Figure 5.27. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no-treatment, static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25°C for 5 minutes. The difference was significant between the treated Groups 2, 3 and 4 and the non-treated Group 1. In addition, the difference was significant between the treatment groups 2, 3 and 4 ($p<0.05$, chi square test). 167

Figure 5.28. Pulp debris and predentine scores from the middle thirds of ovine root canals after no-treatment, static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25°C for 5 minutes. The difference was significant between the treated groups 2, 3 and 4 and the non-treated group 1. The difference was not significant between the treatment groups 2, 3 and 4 ($p<0.05$, chi square test). 168

Figure 5.29. Pulp debris and predentine scores from the apical thirds of ovine root canals after no-treatment, static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25°C for 5 minutes. The difference was significant between the treated groups 2, 3 and 4 and the non-treated group 1. In addition, the difference was significant between the treatment groups 2, 3 and 4 ($p<0.05$, chi square test). 168

Figure 5.30. Chi square test to investigate significant differences in debris and predentine scores in the cervical, middle and apical thirds of ovine roots after static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25°C for 5 minutes. The difference was significant between the treated groups 2, 3 and 4 and the non-treated group 1. At the cervical third, the difference between groups 2 and 3 was not significant, between groups 2 and 4 was significant, and between groups 3 and 4 was significant. At the middle third, the difference between groups 2 and 3, groups 2 and 4 and groups 3 and 4 was not significant. At the apical third, the difference between groups 2 and 3, between groups 2 and 4 and between groups 3 and 4 was significant ($p<0.05$, chi square test). 169

Figure 5.31. The agreement between the first and the second examiners, which shows the reliability of the first examiner (Kappa score) for pulp debris and predentine scoring of SEM images of human root canal after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25°C for 5 minutes. 177

Figure 5.32. The agreement between the first and the third examiners shows the reliability of the first examiner (Kappa score) for pulp debris and predentine scoring of SEM images of human root canal after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes. 177

Figure 5.33. SEM image (500x magnification) of the human root canal wall in the cervical third of an untreated control specimen (Group 1), with a debris and predentine score of 1. 178

Figure 5.34. SEM image (500x magnification) of the human root canal wall in the middle third of a specimen from Group 3 (NaOCl 2.5%), with a debris and predentine score of 3. 178

Figure 5.35. SEM image (500x magnification) of the human root canal wall in the cervical third of a specimen from Group 4 (5% NaOCl), with debris and predentine score 4. 179

Figure 5.36. Pulp debris and predentine scores for the cervical thirds of human root canals after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes. The difference was significant between the experimental groups 3, 4 and 5 and groups 1 and 2 ($p<0.05$, chi square test). 179

Figure 5.37. Pulp debris and predentine scores for the middle thirds of human root canals after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes. The difference was significant between the experimental groups 3, 4 and 5 and groups 1 and 2. ($p<0.05$, chi square test). 180

Figure 5.38. Pulp debris and predentine scores for the apical thirds of human root canals after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes. The difference was significant between the experimental groups 3, 4 and 5 and groups 1 and 2 ($p<0.05$, chi square test). 180

Figure 5.39. Chi square test to identify significant differences in debris and predentine scores for human root canals in their cervical, middle and apical thirds after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes. The difference was significant between all groups at the cervical, middle and apical thirds. While, the difference between groups treated with 2.5% and 5% NaOCl , between groups treated with 2.5% and 10% NaOCl, and between groups treated with 5% and 10% NaOCl was not significant at the cervical, middle and apical thirds ($p<0.05$, chi square test). 181

Figure 6.1. AFM image to show the structure of untreated root canal dentine walls (Group 1) in the cervical third of non-treated human teeth, at 100µm depth from the root canal lumen. 188

Figure 6.2.Typical force curve from horizontal section of root dentine in the cervical third of non-treated human teeth (Group 1). 188

Figure 6.3. AFM image from horizontal section of root dentine to show the structure of root dentine in the cervical third of human teeth, at 200µm depth from the root canal lumen, after 5 minutes treatment with normal saline (Group 2). 189

Figure 6.4.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, at 100µm depth from the root canal lumen, after 5 minutes treatment with normal saline (Group 2). 189

Figure 6.5. AFM image from horizontal section of root dentine to show the structure of root dentine in the cervical third of human teeth, at 100µm depth from the root canal lumen, after 5 minutes treatment with 2.5% NaOCl (Group 3). 190

Figure 6.6.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, at 200µm depth from the root canal lumen, after 5 minutes treatment with 2.5% NaOCl (Group 3). 190

Figure 6.7. AFM image from horizontal section of root dentine to show the structure of root dentine in the cervical third of human teeth, at 100µm depth from the root canal lumen, after 5 minutes treatment with 5% NaOCl (Group 4). 191

Figure 6.8.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, after 5 minutes treatment with 5% NaOCl (Group 4). 191

Figure 6.9. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, at 200µm depth from the root canal lumen, after 5 minutes treatment with 10% NaOCl (Group 5). 192

Figure 6.10.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, after 5 minutes treatment with 10% NaOCl (Group 5). 192

Figure 6.11. Median dentine stiffness at different depths from the lumen of horizontal cervical human root dentine 0µm, 100µm, 200µm, 300µm and 1000µm, after no treatment or root canal irrigation with normal saline and different concentrations of sodium hypochlorite, 2.5%, 5% and 10% (Groups 1-5 respectively). The differences

were not significant (p>0.05, Kruskal-Wallis test). The error bars are the upper and lower quartiles for each group.....193

Figure 6.12. Shows a specimen within the AFM liquid cell, with a white square on the surface which represents the area in which the 9 points of surface analysis were positioned.....200

Figure 6.13. AFM image to show the structure of root canal dentine walls in the cervical third of non-treated human teeth (Group 1).....202

Figure 6.14. Typical force curve of root canal dentine walls in the cervical third of non-treated human teeth (Group 1).....202

Figure 6.15. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 6% citric acid (Group 2).....203

Figure 6.16.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 6% citric acid (Group 2).....203

Figure 6.17. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 6% citric acid (Group 3).....204

Figure 6.18.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 6% citric acid (Group 3).....204

Figure 6.19. Median dentine stiffness (nN/µm) of human cervical root canal wall, with no treatment, 1 and 2 minute treatments with 6% citric acid. The difference was significant between the control group and the 1 minute treatment, and between the control group and the 2 minute treatment (p<0.05), but between the 1 minute and 2 minute treatments, the difference was not significant (p>0.05, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the upper and lower quartiles.....205

Figure 6.20. AFM image to show the structure of root canal dentine walls in the cervical third of non-treated human teeth (Group 1).....211

Figure 6.21.Typical force curve of root canal dentine walls in the cervical third of non-treated human teeth (Group 1).....211

Figure 6.22. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 17% EDTA (Group 2).....212

Figure 6.23.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 17% EDTA (Group 2).....212

Figure 6.24. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 17% EDTA (Group 3). 213

Figure 6.25.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 17% EDTA (Group 3). 213

Figure 6.26. Median dentine stiffness (nN/µm) of human cervical root canal wall, after no treatment, 1 and 2 minute treatments with 17% EDTA. The difference was significant between the control group and the 1 minute treatment, and between the control group and the 2 minute treatment ($p<0.05$), but between the 1 minute and 2 minute treatments, the difference was not significant ($p>0.05$, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the upper and lower quartiles. 214

List of tables

Table 2.1. Typical constituents of human dentine (Nanci, 2008). 15

Table 3.1. Normality test of the data of dentinal tubule density (combined cervical, middle and apical thirds) in ovine, bovine and human root canals ($p<0.05$). 78

Table 3.2. Normality test of the data of variance dentinal tubule diameter (combined cervical, middle and apical thirds) in ovine, bovine and human root canals ($p<0.05$). .. 78

Table 3.3. Median (upper and lower quartiles) of the dentinal tubule density in ovine, bovine and human root canals. The difference found was not significant ($p<0.05$. Kruskal-Wallis test). 79

Table 3.4. Median (upper and lower quartiles) of the dentinal tubule diameter (µm) in ovine, bovine and human root canals. The difference was not significant ($p<0.05$. Kruskal-Wallis test). 80

Table 3.5. Mean volume and standard deviation of ovine, bovine and human root canal spaces measured using distilled water. 89

Table 3.6. Mean estimated volume and standard deviation of ovine, bovine and human root canal spaces measured digitally using ImageJ software. 90

Table 3.7. Median (upper and lower quartiles) of the Ca/P ratios of ovine, bovine and human teeth at, 0µm, 100µm, 200µm, 300µm and 1000µm from root canal lumen. 98

Table 3.8. Median (upper and lower quartiles) of the dentine stiffness of horizontal cervical dentine sections of ovine, bovine and human teeth at, 0µm, 100µm, 200µm, 300µm and 1000µm from root canal lumen. 107

Table 4.1. A summary of pilot studies to remove saw-generated smear layer from horizontal tooth sections. Smear layer scores were based on the Hülsmann *et al.* (1997) scale. 120

Table 4.2. It is a continuation of the previous table. It shows a summary of pilot studies to remove saw-generated smear layer from horizontal tooth sections. Smear layer scores were based on the Hülsmann *et al*'s. (1997) scale. 121

Table 5.1. Summary of treatment regimes for ovine roots. 141

Table 5.2. Median (upper and lower quartiles) of the Ca/P ratio of the cervical third of root dentine in control and experimental groups at different depths, and the significant (a) and non-significant (b) differences ($p<0.05$), between the non-treated control (Group 1) and the other Groups (2-16). 153

Table 5.3. It is a continuation of the previous table. It shows median (upper and lower quartiles) of the Ca/P ratio of root dentine at different depths and the significance (a) and non-significance (b) differences ($p<0.05$), between the non-treated group (group 1) and the other groups 2-16. 154

Table 5.4. Summary of treatment regimens of ovine roots. 165

Table 5.5. Summary of treatment regimes for human roots. 174

Table 6.1. Summary of treatment regimens of human roots. 186

Table 6.2. Median (upper and lower quartiles) dentine stiffness of horizontal cervical dentine sections of human teeth at, 0µm, 100µm, 200µm, 300µm and 1000µm from root canal lumen, after root canal irrigation with normal saline and different concentrations of sodium hypochlorite, 2.5%, 5% and 10% (Groups 1-5 respectively). 193

Table 6.3. Median (upper and lower quartiles) dentine stiffness of human cervical root canal walls, with no treatment, and after 1 and 2 minute treatments with 6% citric acid. 205

Table 6.4. Median (upper and lower quartiles) dentine stiffness cervical human root canal wall, after no treatment, 1 and 2 minute treatments with 17% EDTA. 214

Chapter 1: Introduction

Endodontology is the field of dentistry that studies the form, function and health of the dental pulp and the periradicular tissues that surround the root(s) of teeth. Central to its remit are injuries and diseases of the pulp and periradicular tissues and their prevention and treatment (European Society of Endodontology, 2006).

Endodontic treatment is undertaken to retain the function of teeth with damaged pulps and can be provided for teeth with vital and non-vital pulp tissues. Aspects of successful treatment include:

1. Canal preparation to remove pulp tissue, create space for irrigant exchange and medicament delivery, and shape for the dense and controlled compaction of filling materials (Peters, 2004).

2. Instrumentation and medication to reduce the microbial load of the canal space to as low a level as possible (Distel *et al.*, 2002).

These processes involve the application of mechanical enlarging tools and medicaments to dentine surfaces, which may be damaged as a result. A balance must therefore be found between sufficient instrumentation and chemical treatment to win success, and excessive treatment, with the risk of tooth failure for iatrogenic reasons.

The focus of endodontic treatment has long been on maximising shaping and cleaning outcomes, but emerging insights on the fragility and potential failure modalities of root canal treated teeth are beginning to re-focus attention on the possible deleterious effects of treatment and ways of protecting teeth from failure. This thesis will explore some of these issues, considering models for the investigation of dentine damage, aspects of sample preparation for analysis, and *in vitro* experiments on the effects of endodontic irrigants on root dentine.

General factors could influence the outcome of non-surgical root canal treatment such as, steroid therapy, thyroxin therapy and diabetic disease, pre-operative factors such as pain sinus, periodontal probing depth, fractured instrument inside canal, and the fate of foreign material. There are also intra-operative factors such as root canal blockage, root

canal patency, extrusion of root canal filling out of the root apex, tooth perforation and post-operative factors such as terminal tooth, proximal contacts, cast post and core and type of the coronal restoration (Ng *et al.*, 2011) which may have some bearing on outcome.

Failure of root filled teeth is low, with 97% remaining in the oral cavity 8 years after treatment. A key determinant of failure was the absence of a cuspal coverage restoration after treatment (Tickle *et al.*, 2008).

During root canal treatment, the biomechanical properties of dentine can be adversely affected by mechanical instrumentation (Pantvisai and Messer, 1995), and irrigation with agents such as NaOCl, EDTA and citric acid (Slutzky-Goldberg *et al.*, 2002; Tartari *et al.*, 2013).

This thesis will focus on the suitability and preparation of specimens, and the effects of various irrigant agents on the properties of mammalian dentine.

Chapter 2: Literature review

This chapter will provide background and justification for the studies included in this thesis, by reviewing literature on the purpose and challenges of root canal treatment, the nature of dentine as the tissue subject to endodontic procedures, the role, limitations and risks of root canal irrigant solutions, and methods of assessing their effects.

2.1 Purpose of root canal treatment

Root canal treatment is an element of routine dental practice, which aims to preserve teeth in a healthy and functional condition by removing diseased pulp tissue, managing internal infection and preventing its recurrence. Specific indications include:

1. Teeth with irreversibly damaged or necrotic pulps, with or without clinical and/or radiographic signs of apical periodontitis (European Society of Endodontology, 2006).
2. Teeth with no clear evidence of pulp disease, where use of the pulp space is required for tooth restoration (e.g. post space preparation), where the condition of the pulp may be in doubt prior to complex restoration, or where the pulp may be severely compromised by restorative procedures (e.g.: preparation of overdenture abutments, tooth hemisection, crown preparation on mis-aligned teeth) (European Society of Endodontology, 2006).

The management of microbial infection is a central theme in both categories of treatment. In teeth with irreversibly damaged or necrotic pulps, the pulp space is generally infected, and treatment is focussed on eliminating both microbial infection and the substrate on which it may thrive (Sjögren *et al.*, 1991; Takahashi, 1998; Basmadjian-Charles *et al.*, 2002; Siqueira *et al.*, 2002). Treatment should balance the need to eliminate microorganisms and organic matter from the pulp space, with the imperative to minimise harm to the host and to the dental hard tissues that must subsequently be restored and provide function.

2.2 **Challenges of contained pulp canal infections**

2.2.1 The nature of pulp space infection

Dental pulp necrosis and the formation of a periapical lesion usually result from microbial infection from the oral cavity (Siqueira Jr, 2002). The most common source of this infection is dental caries, which results in microbial invasion of the previously sterile pulp space, and subsequent necrosis of pulp tissues (Langeland, 1987). Pulps devitalised by non-infectious means, such as trauma, no longer have the potential to defend themselves and are highly susceptible to infection (Langeland, 1987). Infections under such circumstances are opportunistic, and supported by nutrients derived from pulp tissue remnants and exudate from the periodontium (Sundqvist, 1994; Nair, 2004). The invasion of microorganisms into the root canal space is through dentinal tubules or by direct pulp exposure to the mouth, and follows the breakdown of enamel and cementum, which in health protect dentine and thus the pulp from microbial invasion (Sundqvist and Figdor, 2003).

When protective barriers break down, many microorganisms from the oral cavity theoretically have the same opportunity to invade root canal space, but certain species have been found in infected root canals (Kantz and Henry, 1974; Wittgow and Sabiston, 1975; Sundqvist, 1994). This reflects the specialised and demanding environment of the root canal with its low oxygen tension, restricted space and nutritional opportunities (Thilo *et al.*, 1986; Costerton *et al.*, 1994; Costerton *et al.*, 1999; Potera, 1999), and the restrictions imposed by microorganisms that may have invaded earlier (Molven *et al.*, 1991; Sundqvist and Figdor, 2003).

It has become apparent that microbial communities exist wherever they can in complex biofilm communities (Costerton *et al.*, 1987; Nair, 1987; Costerton *et al.*, 1994; Johnson *et al.*, 2002; de Paz, 2007). The walls of root canal systems appear to be no different from other surfaces in this regard, playing host to complex arrays of microorganisms in biofilm formation (Nair, 1987; Svensäter and Bergenholtz, 2004). In biofilms, single or multiple microbial species arrange themselves in mutually supportive networks, adhering to surfaces, protecting themselves with extracellular matrices and presenting quite different challenges compared to planktonic microorganisms (Costerton *et al.*,

1994; Costerton, 1999). Biofilms are less susceptible to antimicrobial agents than their planktonic counterparts, which have traditionally been used *in vitro* to test antimicrobial agents (Wilson, 1996; Potera, 1999). This difference may reflect a number of biofilm features including their relatively impervious extracellular matrix, ability to remain dormant in a non-dividing state, diffusion gradients, transport mechanisms, inter-cellular signalling and a variety of other mechanisms within formations of many cells-thickness (Costerton *et al.*, 1987; Wilson, 1996; Gilbert *et al.*, 1997; Shani *et al.*, 2000; Johnson *et al.*, 2002; Larsen, 2002).

Our historical understanding of root canal infections has largely focussed on planktonic microorganisms, which by contrast are free-floating microorganisms intermingled with necrotic pulp tissues and suspended in tissue fluids. Microorganisms in such arrangements may have been considered straightforward to eliminate by tissue extirpation, canal instrumentation and irrigation, and much microbiological research has adopted this model, investigating the killing of microorganisms in simple plate or broth cultures (Torabinejad *et al.*, 2003c; Williams *et al.*, 2006; Arias-Moliz *et al.*, 2009). Studies have shown that even such gentle treatments as syringe rinsing and passive ultrasonic irrigation with normal saline (0.9%) removed planktonic bacteria from root canals (Spoleti *et al.*, 2003). However, this does not reflect current understanding (Nair, 1987; Svensäter and Bergenholtz, 2004), and some studies have suggested that bacteria in biofilm formation may be up to 1000-fold more resistant to antimicrobial agents such as antibiotics than their planktonic counterparts (Svensäter and Bergenholtz, 2004). Work is therefore beginning to emerge in which the effects of drugs, medicaments and mechanical processes are evaluated on biofilm communities (Abdullah *et al.*, 2005). This has already confirmed the relative resistance of biofilms of oral origin to a range of agents such as chlorhexidine, amine fluoride, amoxicillin, doxycycline, and metronidazole (Shani *et al.*, 2000).

In endodontics, the susceptibility of biofilm bacteria to standard agents including sodium hypochlorite (NaOCl) and calcium hydroxide ($CaOH_2$) is of special interest. For example, NaOCl was used at different concentrations (1% and 6%) for either 1 or 5 minutes and was found that the 6% was more effective than the 1% NaOCl to remove *Enterococcus faecalis* biofilm (Dunavant *et al.*, 2006), and also other concentrations has been reported to be effective such as 2.5% for 15 and until 60 minutes (Spratt *et al.*,

2001) and 5.25% NaOCl for 5 minutes (Giardino *et al.*, 2007). Furthermore, the susceptibility of microorganisms in the biofilm to the irrigant depends on the type of microorganisms present (Spratt *et al.*, 2001). Planktonic *Enterococcus faecalis* was eradicated by different concentrations of NaOCl (0.5, 1, 2.5, 4 and 5.25%), revealing a relationship between the concentration of NaOCl and microbial reduction. The lower concentration of NaOCl (0.5%) took 30 minutes to destroy *Enterococcus faecalis*, 5.25% killed bacteria in 30 sec (Gomes *et al.*, 2001). The planktonic *Enterococcus faecalis* was also eradicated by combined use of 5.25% NaOCl and MTAD (an aqueous solution of 3% doxycycline, 4.25% citric acid, and 0.5% polysorbate 80 detergent) (Torabinejad *et al.*, 2003c).

These reports serve to highlight the great disparity of experimental parameters in disinfection studies, making direct comparison of biofilm and planktonic elimination by different agents difficult. Two observations of relevance are the generally increased effectiveness of higher concentration disinfectants, all other factors being equal, and the need for agents to make direct contact with the microorganisms they are seeking to kill. Increased concentration and methods of bringing irrigants into direct contact with bacteria may, however, be at a biological cost.

It is now well established that root canal infections represent complex communities of microorganisms that inhabit complex root canal spaces, and that microbial dispersal and killing may be challenging (Molven *et al.*, 1991; Barrieshi *et al.*, 1997; Nair *et al.*, 1999; Distel *et al.*, 2002; Hubble *et al.*, 2003; Svensäter and Bergenholtz, 2004; de Paz, 2007).

2.2.2 Complexities of the pulp canal space

Having established the tenacious, biofilm nature of established root canal infections, it is important to understand the anatomical complexity of the spaces in which they reside. Normal anatomical features of the pulp space include the pulp chamber with its pulp horns and root canal orifices and root canals with their accessory canals, lateral canals, fins, webs, anastomoses and apical foramina. This anatomy can be changed by the deposition of secondary and tertiary dentine and by the apical deposition of cementum (Walton and Vertucci, 2001).

Weine *et al* (1969) classified root canals systems into four basic types, but Vertucci *et al* (1984) subsequently classified them into eight configurations. The Vertucci *et al* (1984) classification may reflect the complex reality of canal systems in a way that the Weine *et al* (1969), system did not. Weine's 4 basic forms are:

Type I: A single root canal extend from the pulp chamber to the apex.

Type II: Separate root canals leave the pulp chamber and join short of the apex to form one canal.

Type II: Two separate root canals leave the pulp chamber remain separate to the site of exiting.

Type IV: Three separate and distinct root canals extend from the pulp chamber to the apex.

In their classification, Vertucci *et al* (1984) decalcified human teeth, injected them with hematoxylin dye and cleared them to determine the number and configurations of the root canals, the curvature of roots and root canals in all directions, the ramifications of main root canals, the location of the apical foramina, transverse anastomoses and the frequency of apical deltas (Vertucci *et al.*, 1974). Vertucci's 8 basic forms are:

Type I: A single canal extending from the pulp chamber to the apex.

Type II: Two separate canals leaving the pulp chamber and joining short of the apex to form one canal.

Type III: One canal leaving the pulp chamber and dividing into two in the root; the two then merge to exit as one canal.

Type IV: Two separate, distinct canals extending from the pulp chamber to the apex.

Type V: One canal leaving the pulp chamber and dividing short of the apex into two separate, distinct canals with separate apical foramina.

Type VI: Two separate canals leaving the pulp chamber, merging in the body of the root, and re-dividing short of the apex to exit as two distinct canals.

Type VII: One canal leaving the pulp chamber, dividing and re-joining in the body of the root, before and finally re-dividing into two distinct canals short of the apex.

Type VIII: Three separate, distinct canals extending from the pulp chamber to the apex.

Canal systems are, however, almost infinitely variable and can have multiple foramina, additional canals, fins, deltas, internal connections, loops, and C-shaped configurations. Furcation and lateral canals are also common (Lowman *et al.*, 1973; Burch and Hulen, 1974; De Deus, 1975). All of this highlights the complex, often curved spaces in which microorganisms and their substrate reside and the anatomical challenges which are implicit in cleaning them (Hess and Zurcher, 1925; Pineda and Kuttler, 1972; Vertucci, 1984; Vertucci *et al.*, 2006).

2.3 **Dentine as the tissue on which treatment is performed**

Endodontic procedures are conducted on dentine, and consideration of this tissue and its properties may be helpful before exploring the realities of root canal treatment its possible effects on dental tissues.

Dentine is located between the enamel or cementum of the external tooth and the pulp internally (Stock *et al.*, 2008). It is a hydrated, porous tissue containing dentinal tubules (El Feninat *et al.*, 1998; Nanci, 2008), resulting from the deposition of a collagenous matrix around the cytoplasmic processes of odontoblasts, which subsequently mineralises, encasing the odontoblast processes within the tissue (El Feninat *et al.*, 1998). In Figure 2.1 the hollow structure of dentinal tubules can be seen, and these dentinal tubules are considered as the characteristic feature of dentine and responsible for its permeability. The number and size of dentinal tubules is different at different locations on the dentine. Their course is S-shaped between the junction of dentine and enamel (DEJ) and the pulp, in the crown, and between the junction of dentine and cementum (CDJ) and the pulp, in the root (Garberoglio and Brännström, 1976). It is important during the preparation of research specimens from dentine surfaces to know the likely orientation of dentinal tubules, and to understand that this may have a bearing on the behaviour of the tissue.

Textbooks often provide descriptions of the structure and composition of dentine as if it was a uniform tissue, but this is not necessarily the case. Dentine can be divided into different types according to the site, structure, chemical composition, function and origin (Tjäderhane *et al.*, 2012). Reports during the last 84 years (from 1929 to 2013)

have used at least 19 terms to describe different types of dentine (Cox *et al.*, 1992), including mantle, primary, secondary, sclerotic, transparent, dead tract, circumpulpal, centripetal, dystrophic, irregular, irregular secondary, osteo, tertiary, irritation, protective, reparative, reactionary and interface dentine. It is beyond the scope of this literature review to describe the basis of the various classifications that have been proposed.

The predominant forms of dentine encountered within dental root canals and relevant to the current studies are:

1. Predentine
2. Globular dentine
3. Primary dentine
4. Peritubular dentine
5. Sclerotic dentine
6. Secondary dentine

Their location is shown in Figure 2.2 and Figure 2.3.

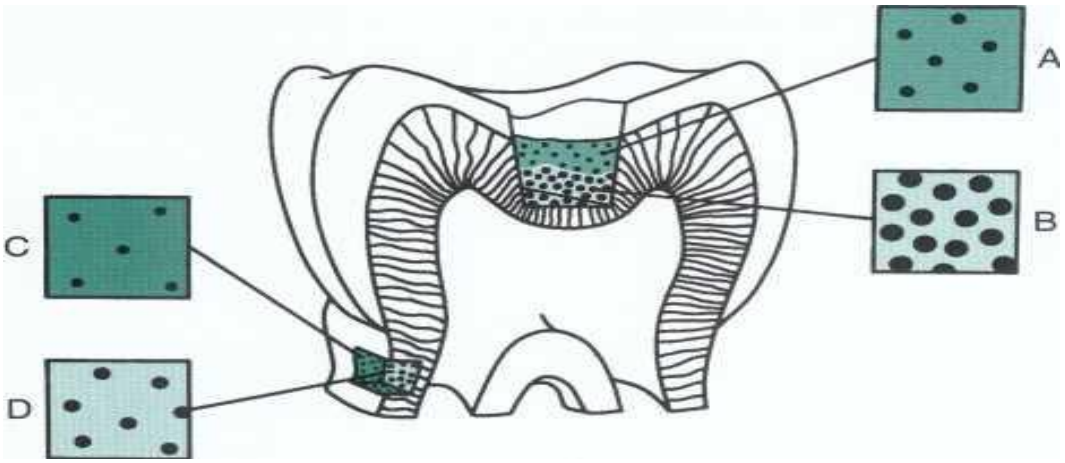

Figure 2.1. Illustration of dentinal tubules (A) which are narrower and more widely spaced at o the dentinoenamel junction compared to those in deeper areas (B). Dentinal tubules in superficial (C) and deep (D) root dentine are also narrower and more sparse than their counterparts in the coronal areas (Roberson *et al.*, 2006).

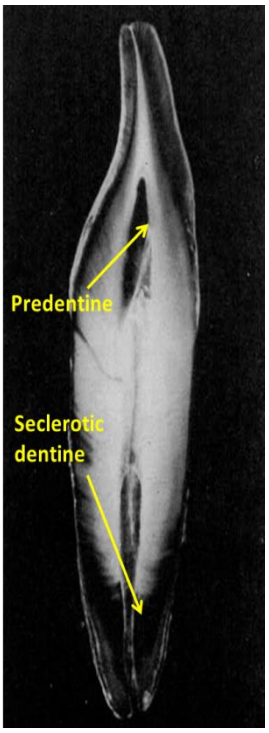

Figure 2.2. Photographic image by transmission electron microscope shows age-related root dentinal sclerosis starting from the apex of the root and progressing coronally. It shows the transition between transparent apical root dentine (dark) and opaque dentine (white) (Nalbandian *et al.*, 1960).

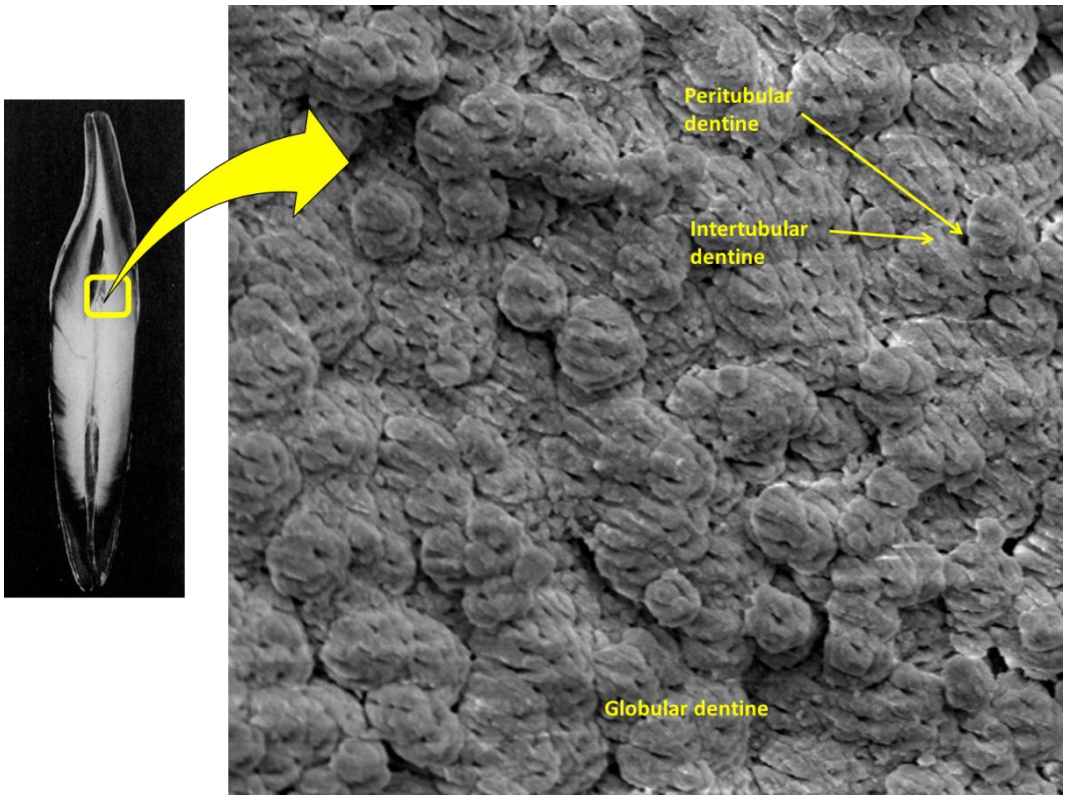

Figure 2.3. Photographic image by transmission electron microscope (Nalbandian *et al.*, 1960), and photographic image by SEM shows globular dentine, highly mineralized peritubular dentine contrasts with intertubular dentine which represents the bulk of dentine volume.

1. Predentine

Predentine is the most newly formed dentine which surrounds the pulp chamber and pulp canal. This inner-most dentine comprises un- or incompletely mineralized dentine matrix, whilst the remaining dentine that surrounds it is mineralized. Odontoblasts begin to secrete predentine matrix as they move concentrically inwards away from the dentinoenamel junction (DEJ), and this tissue forms the leading front of dentine matrix deposition as odontoblasts continue their formative activity. Its thickness varies from 10-30µm according to location and species of animal (Linde and Goldberg, 1993; Berkovitz *et al.*, 2002). The inner wall of the dental root canal is thus covered with a layer of predentine, and areas that do not come into contact with instruments during root canal treatment may remain covered with predentine.

2. Globular dentine

Globular dentine is the mineralized dentine positioned immediately beneath predentine. It has an irregular, 'lumpy' surface pattern as the formation of spheroidal foci of calcium-phosphate nuclei called "calcospherites" progresses. These calcospherites then fuse together to form primary dentine by a process of globular mineralization (Avery *et al.*, 2002; Bath-Balogh *et al.*, 2006) Figure 2.3. Areas of the root canal wall that are denuded of predentine by the application of protein-dissolving irrigants such as NaOCl may thus be expected to present a 'lumpy', globular surface (Haapasalo *et al.*, 2012).

3. Primary dentine

Primary dentine forms the bulk of the tooth and its roots, and comprises mineralised dentine. Primary dentine forms the primary shape of the tooth and the end-point of its secretion is said to be the time at which the tooth becomes functional and roots are completely formed (Linde and Goldberg, 1993; Berkovitz *et al.*, 2002; Nanci, 2008). The mineralised extracellular organic matrix of primary dentine consists, primarily of intertubular tissue, but with smaller amounts of peritubular dentine lining the walls of the dentinal tubules that course their way through the tissue (Nanci, 2008; Arola *et al.*, 2012; Tjäderhane *et al.*, 2012). Collectively, extracellular intertubular and peritubular dentine (Tjäderhane *et al.*, 2012) can be described as circumpulpal dentine (Linde and Goldberg, 1993).

3. Secondary dentine

following complete formation of primary dentine, the deposition of dentine continues physiologically at a slow rate, without the need for persistent stimuli and this dentine is called secondary dentine (Berkovitz *et al.*, 2002). A precise point can be determined in time between primary and secondary dentine, when primary dentine is no longer secreted and secondary dentine begins (Tjäderhane *et al.*, 2012).

4. Peritubular dentine

The peritubular (sometimes referred to as intra-tubular) dentine is a highly mineralised structure of dentine which has a thickness of approximately 0.5-0.8µm (Marshall *et al.*, 2001c). It is more highly mineralized than intertubular dentine and it contains no organic collagenous fibres in comparison with the intertubular dentine which surrounds it (Marshall *et al.*, 2001c) (Figure 2.3).

5. Sclerotic dentine

During maturation of the recently formed pertibular dentine, a more mineralized dentine is formed on the internal walls of dentinal tubules, particularly in the apical part of the root, where it may completely obliterate the tubules, forming translucent, sclerotic dentine (dentine sclerosis) (Vasiliadis *et al.*, 1983; Tjäderhane *et al.*, 2012).

Seclerotic dentine appears as transparent or translucent due to the obliteration of dentinal tubules with peritubular tissue (Nalbandian *et al.*, 1960). It may be divided according to the cause of its formation into 2 types either physiologic dentine sclerosis or reactive dentine (Tjäderhane *et al.*, 2012). The former results from aging, while the latter occurs as a result of external injuries such as caries tooth restoration and erosion. External injuries to the pulpo-dentinal complex such as caries, tooth restoration and erosion may result either in dentine sclerosis, the formation of dead tracts, or the secretion of reparative or reactionary tertiary dentine. Secretory responses, with the deposition of peritubular or tertiary dentine may be seen as defensive to protect the pulp from external insult. They are unlikely to involve the deep areas of dental root canals unless external root surfaces are exposed to the mouth. Sclerotic dentine affecting dental roots is more likely to be physiological, extending progressively from apical to cervical with increasing age (Stanley *et al.*, 1983) (Figure 2.2), and one of the

parameters explored in anthropological and forensic studies to age human remains (Morse *et al.*, 1991; El-Bakary *et al.*, 2010).

2.3.1 Structure and chemical composition of dentine

Dentine is composed of approximately 45% inorganic, 33% organic material (primarily type I collagen) and 22% water by volume, and approximately 70% inorganic, 20% organic material and 10% water by weight (Nanci, 2008).

Table 2.1 shows the chemical composition of human dentine. It should, however, be recognised that this represents an over-simplification, with different types of dentine expressing different proportions of these and other components.

Within the scaffold of matrix proteins secreted by odontoblasts, the remaining part of the organic phase is non-collagenous proteins and they are mostly glycoproteins and proteoglycans that cover the collagen fibrils and are associated with the inorganic phase. This is in addition to phosphoproteins, which could regulate biomineralization processes or could have an influence on inducing mineral nucleation and binding on the calcium phosphates (MacDougall *et al.*, 1992; Wiesmann *et al.*, 1993; Boskey, 1996; Bègue-Kirn *et al.*, 1998; Dahl *et al.*, 1998; Saito *et al.*, 1998; Wiesmann *et al.*, 2005). Nucleation of calcium phosphate crystallites starts between collagen fibrils and continues until they are completely mineralised (Höhling *et al.*, 1990; Plate *et al.*, 1992).

Most of the inorganic phase of the dental hard tissue consists of hydroxyapatite crystals, $Ca_{10}(PO_4)_6(OH)_2$, in which the major components are calcium and phosphorus (Dogan and Çalt, 2001; Arola *et al.*, 2012). However, there are some other atomic trace elements such as P, Cu, K, Cl, Zn, Fe, Ti, Sr, V, Mn, and Zr (Mjör, 1972). In addition, the inorganic mineral in dentine is carbonated apatite crystal which is similar to the mineral in bone and calcified tendon (Lees *et al.*, 1997; Wassen *et al.*, 2000).

Any differences in the composition of the constituents of the inorganic and organic phases of the dental hard tissue of human or animal may influence the composition, radiodensity and mechanical properties of these tissues (Fonseca *et al.*, 2004; Giannini *et al.*, 2004). Differences between human and animal dentine will be considered later.

Table 2.1. Typical constituents of human dentine (Nanci, 2008).

Dentine	wt%	v%
Inorganic	70%	45%
Organic	20%	33%
Water	10%	22%

2.3.2 Nanostructure of dentine

Dentine is a hydrated biological nano-composite tissue of hydroxyapatite crystallites with diameters of approximately 5 nm (Camps *et al.*, 2009), and located either in the gaps between collagen molecules (intrafibrillar) or attached to the type-I collagen fibrils (extrafibrillar) (Lees *et al.*, 1997; Wassen *et al.*, 2000). The morphology of the crystallites is plate-like and cylindrical inside the dentine and their dimensions are between 20 and 25 nm (Arsenault, 1989; Kinney *et al.*, 2001).

These crystals differ in enamel and dentine, being larger and more regular in enamel than dentine (90 nm and 31 nm respectively) (Hanlie *et al.*, 2006). Different studies have shown that the hydroxyapatite crystallites of intertubular dentine have a length of approximately 115 nm (+/-3) and width of approximately 10 nm (+/-3) (Waidyasekera *et al.*, 2010). At the dentinoenamel junction, the thickness of hydroxyapatite crystals (the filler phase) is approximately 50 nm in enamel and approximately 3 nm in dentine. In dentine these fillers are short, relatively flat and randomly organised. The coupling between these fillers at the dentinoenamel junction zone is incompletely understood (Fong *et al.*, 2000).

Studies used sequential demineralisation and deproteinization of dentine to allow imaging and investigation of collagen fibrils by Atomic Force Microscopy (AFM), since demineralisation results in dissolution of most of the mineral phase, and deproteinization results in gradual removal of the extracellular matrix proteins which can expose collagen fibrils (Marshall *et al.*, 2001c; Habelitz *et al.*, 2002a). Different diameters have been reported for type I collagen fibrils (hydrated and dehydrated) in dentine, ranging between 75 and 105 nm (Habelitz *et al.*, 2002a), and sometimes wider from 30-120 nm (Lin *et al.*, 1993). Some of this variation may be explained because

collagen fibrils have random orientation and are difficult to see in cross section perpendicular to the long axis of the collagen fibril (Lin *et al.*, 1993). However, other reasons such as tissue type, age and genetic variation should also be considered (Vesentini *et al.*, 2013). When collagen fibrils are dehydrated, they collapse and their dimension can be reduced to 10-40 % (Brodsky *et al.*, 1988). Generally the range in diameter of hydrated fibrils is less than for dehydrated fibrils (Garberoglio and Brännström, 1976; Pashley, 1991; Perdigão *et al.*, 1996; Habelitz *et al.*, 2002a) (Figure 2.4). Collagen fibrils with diameters of 83, 91 and 100 nm could have approximately 3200, 3550 and 3900 molecules (Holmes *et al.*, 2001). The collagen molecules are synthetized by the rough endoplasmic reticulum of the odontoblast during dentinogenesis and are extruded as triple helical collagen molecules (triple helix or tropocollagen) with a length of approximately 300 nm; three supercoiled polypeptide chains, bound together by water bridges and hydrophobic cross-links. Five tropocollagen molecules overlap each other longitudinally by about one quarter of their length to form a microfibril, which is about 4 nm (Scott, 2002; Vesentini *et al.*, 2013), or 8 nm in diameter, representing the building block of collagen fibrils (Prockop and Fertala, 1998; Habelitz *et al.*, 2002a). The microfibrils run parallel to each other to form collagen fibrils, so collagen fibrils growth occurs and this process is called fibrillogenesis (Prockop and Fertala, 1998; Habelitz *et al.*, 2002a). However, the arrangement of the microfibrils occurs in a pattern of staggered collagen microfibrils with gaps between the ends of successive collagen microfibrils, resulting in periodic alternating gaps and staggered zones (Bella *et al.*, 1995; Wess and Orgel, 2000) (Figure 2.5).

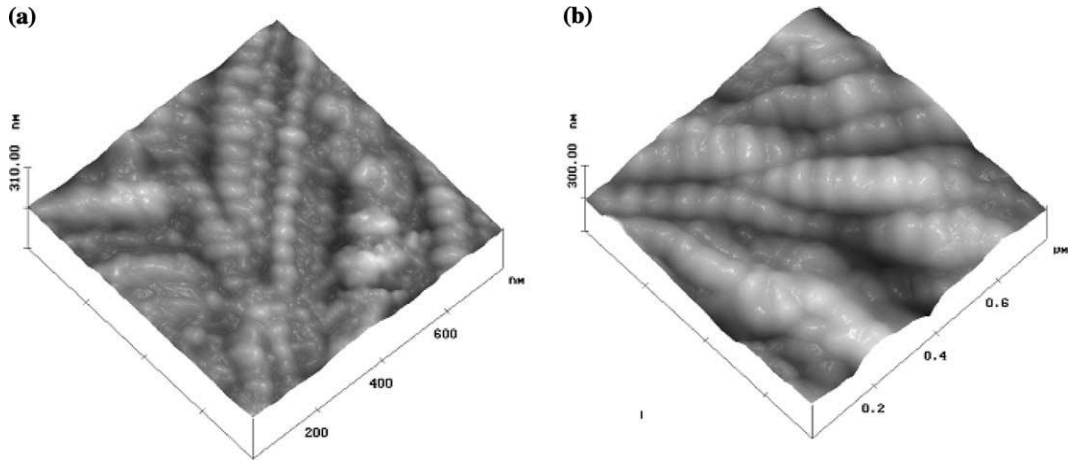

Figure 2.4. AFM images of human dentine collagen fibrils in which surface imaging was taken with a mixed-mode of the tapping and phase mode. There is a difference in the diameter of collage fibrils between image a (in liquid) and image b (in air) (Habelitz *et al.*, 2002a).

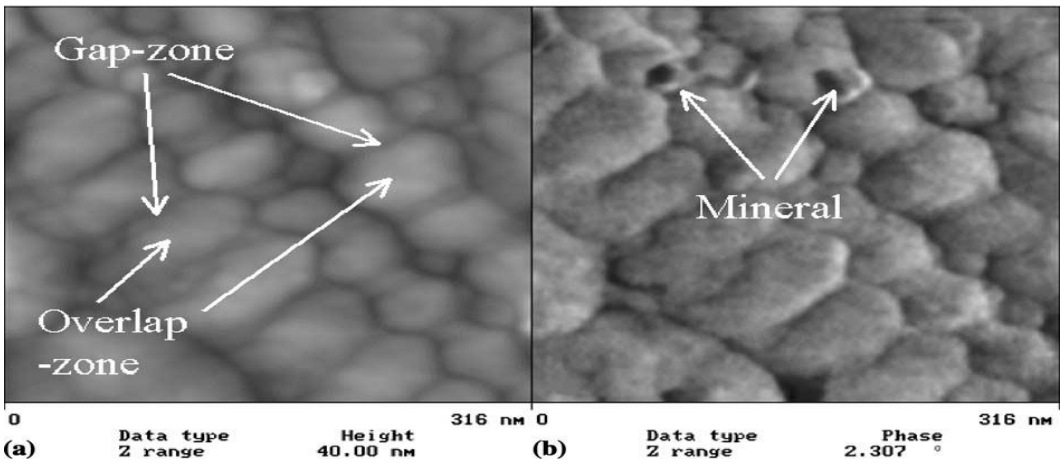

Figure 2.5. AFM images of human dentine collagen fibrils taken in liquid. (a) Tapping mode image: gap and overlap zones of adjacent fibrils interlock. (b) Phase mode image reveals the presence of mineral particles attached to fibrils (Habelitz *et al.*, 2002a).

2.4 Role, limitations and risks of mechanical instrumentation in root canal treatment

It was established in sections 2.2.1 and 2.2.2 that root canal infections represent complex communities of microorganisms that inhabit complex root canal spaces within dentine. Root canal treatment aims to eliminate microbial infection and substrate from pulp canal spaces, and the need for mechanical enlargement and irrigation/medication is apparent (Byström and Sunvqvist, 1985; Abbott, 2002). Mechanical enlargement (canal shaping), is undertaken to open access into the root canal system for the deep exchange of irrigants, to mechanically clean at least parts of the canal walls and to develop a shape that will promote the dense compaction of root canal filling materials whilst guarding against their over-extension. Generally, a smoothly tapering canal form, with its narrowest point apically and its widest point coronally has been the aim (Young *et al.*, 2007).

Mechanical enlargement of the root canal can be done by several techniques such as the balanced force technique in which flexible NiTi or stainless-steel K-type instruments (R-Flex-Files) with a modified tip are used. In this technique the instruments are introduced into the root canals with clockwise motion and apical advancement (placement phase) until slight torque was felt as the cutting edges engaged the canal wall, and then it was followed by counterclockwise rotation of maximum 120^0 with adequate apical pressure (cutting phase). The final phase is the removal of the instrument with clockwise rotation and withdrawal of the file from the root canal (Roane and Sabala, 1984; Roane *et al.*, 1985; Sabala *et al.*, 1988; Blum *et al.*, 1997).

The degree of canal enlargement remains contentious, with some systems imposing minimal canal tapering (Thompson and Dummer, 1997a; Thompson and Dummer, 1997b; Versümer *et al.*, 2002; Vaudt *et al.*, 2007), and others recommending that canals are opened widely (Peters *et al.*, 2003a; Peters *et al.*, 2003b; Sonntag *et al.*, 2007).

Without opening root canals to a very damaging degree, it is impossible for instruments to mechanically clean all walls of major root canals (Peters *et al.*, 2003a; Paqué *et al.*, 2009a), let alone their ramifications (Nair *et al.*, 2005). The shaping process is also fraught with dangers, which may compromise infection control and clinical outcomes,

many of which result from the straightening of inflexible instruments within curved canal spaces:

- Ledging
- Perforation
- Apical transportation
- Strip perforation
- Blockage with debris
- Instrument fracture

Furthermore, mechanical instrumentation produces a smear layer, which is composed of organic and inorganic particles (Moodnik *et al.*, 1776; McComb and Smith, 1975; Goldman *et al.*, 1981; Harrison, 1984; Mader *et al.*, 1984; Pashley *et al.*, 1988; Cengiz *et al.*, 1990; Baumgartner and Cuenin, 1992; Pashley, 1992), with an amorphous structure. More specifically, it comprises dentine shavings, remnants of vital or necrotic pulp and retained irrigant (Baumgartner and Mader, 1987; Pashley, 1992). In the case of infected pulp spaces, this smear layer may additionally contain microorganisms and their by-products (Hülsmann *et al.*, 2003b). Such material may be present on instrumented surfaces with a thickness of 1-2µm, with additional material packed inside dentinal tubules (at least where these may be open in the coronal and middle canal thirds) as smear plugs of up to 40µm (Mader *et al.*, 1984; Pashley, 1992), and yet more packed into canal ramifications, isthmuses, fins and uninstrumented parts of root canal walls (Paqué *et al.*, 2009b). It may also interfere with the penetration of disinfectant and irrigant agents, and their access to the biofilms that they must contact (Byström and Sunvqvist, 1985; Gençoğlu *et al.*, 1993; Pallares *et al.*, 1995).

Quantification by micro-computerised tomography (micro-CT) of the area of shaped-root canal walls by instruments showed that 35-50% of the root canal walls remain uninstrumented (Peters *et al.*, 2001a; Peters *et al.*, 2001b; Hubscher *et al.*, 2003; Peters *et al.*, 2003a).

2.5 **Need for irrigants to complement instrumentation**

Mechanical instrumentation therefore has its limitations, and must be supplemented by irrigation, to keep the canal system lubricated during instrumentation, to remove/kill microorganisms and dissolve/flush pulp remnants and predentine from major root canals and their ramifications. Irrigation has a major influence on root canal debridement, since it facilitates the cleaning of areas where instruments are not able to touch (Gulabivala *et al.*, 2005), disinfects root canal ramifications (Byström and Sundqvist, 1981; Byström and Sundqvist, 1983), removes smear layer and dentine debris (Baugh and Wallace, 2005). Irrigants should ideally be active agents that are effective in dissolving organic tissue and killing microorganisms in both planktonic and biofilm form (McComb and Smith, 1975; McComb *et al.*, 1976; Siqueira *et al.*, 1997; Siqueira *et al.*, 1999; Siqueira *et al.*, 2002; Haapasalo *et al.*, 2005; Hülsmann *et al.*, 2005; Zehnder, 2006; Mohammadi, 2008).

In fulfilling their functions, irrigant solutions should do minimal harm, in terms of tissue toxicity and the potential to damage hard and soft tissues (Harrison, 1984; Byström and Sunvqvist, 1985; Jeansonne and White, 1994; Berutti and Marini, 1996; Clarkson and Moule, 1998; Zehnder *et al.*, 2002; Zehnder, 2006; Yamazaki *et al.*, 2010). There is no single irrigant that has all of these properties (Haapasalo *et al.*, 2010), however, sodium hypochlorite (NaOCl) has been shown to be the closest to ideal (Abou-Rass and Patonai, 1982).

Different concentrations of NaOCl are used either alone or in combination, most commonly with the chelating agent, EDTA (Yamada *et al.*, 1983; Baumgartner and Ibay, 1987; Baumgartner and Mader, 1987; Cengiz *et al.*, 1990; Sen and Buyukyilmaz, 1998; Tatsuta *et al.*, 1999; Calt and Serper, 2002; Grandini *et al.*, 2002; Niu *et al.*, 2002; Grawehr *et al.*, 2003). Common irrigants will be discussed in the following sections.

2.5.1 Overview of common irrigant solutions

The following section will focus on number of commonly employed root canal irrigant agents, highlighting:

- What they are
- How they work

- Optimising their chemical performance
- Risks

2.5.1.1 Sodium Hypochlorite (NaOCl)

NaOCl is a non-specific deproteinating agent (Sauro *et al.*, 2009; Basrani and Haapasalo, 2012). It has been in widespread use since 1920 (Harrison, 1984; Zehnder, 2006). NaOCl has the ability to dissolve organic tissue, and the organic component of the smear layer. This is in addition to its lubricant action for endodontic instruments, its ability to flush debris from root canals and its antimicrobial action. Its shelf life is relatively long and it is inexpensive and easily prepared (Abou-Rass and Patonai, 1982; Harrison, 1984; Zehnder, 2006). Sodium hypochlorite has both sporicidal and virucidal effects, in addition to their wide-spectrum non-specific bactericidal and fungicidal ability (McDonnell and Russell, 1999; Zehnder, 2006).

Mechanism of action of NaOCl

NaOCl reaction with water

When NaOCl is added to water, a dynamic balance occurs as follows (Estrela *et al.*, 1999):

$NaOCl + H_2O \leftrightarrow NaOH + HOCl \leftrightarrow Na^+ + OH^- + H^+ + OCl^-$

Therefore, Na+ and the hypochlorite ions (OCl^-), result in an equilibrium with hypochlorous acid (HOCl), which is a weak acid (Estrela *et al.*, 2002; Basrani and Haapasalo, 2012) (Qian *et al.*, 2011). So, chlorine will be available in 2 forms, OCl^- and HOCl. At acidic and neutral pH (4 – 7) , the chlorine exists predominantly as HOCl, while at pH 9 and above it exists as OCl^- (McDonnell and Russell, 1999; Qian *et al.*, 2011).

The (HClO) is responsible for the antimicrobial action of NaOCl and it is called the active moiety because it is responsible for the inactivation of bacteria by chlorine releasing agents. The (HClO) contains the active chlorine gas (Cl_2) which is unstable and it is a strong oxidizing agent that responsible for the antimicrobial ability. It inhibits bacterial enzymes by irreversible oxidation of the sulphydryl groups (–SH) of bacterial essential enzymes (cysteine), therefore disrupting the metabolic function of bacteria (Moorer and Wesselink, 1982; Siqueira *et al.*, 1997). In addition, the chlorine is

responsible for the powerful organic tissue-dissolving ability of NaOCl (Penick and Osetek, 1970; Senia *et al.*, 1971; Hand *et al.*, 1978; Moorer and Wesselink, 1982; Harrison, 1984; Byström and Sunvqvist, 1985; Nakamura *et al.*, 1985; Jeansonne and White, 1994; Berutti and Marini, 1996; Siqueira *et al.*, 1997; Clarkson and Moule, 1998; McDonnell and Russell, 1999; Zehnder *et al.*, 2002; Young *et al.*, 2007; Yamazaki *et al.*, 2010).

The OCl^- is less effective in comparison with the undissolved HClO (McDonnell and Russell, 1999). Therefore, after decomposition of the NaOCl, it becomes most effective when the percentage of undissolved HOCl becomes higher than OCl^-, at low pH (McDonnell and Russell, 1999). On the other hand, alkaline pH is necessary for solution stability (Siqueira *et al.*, 1997).

NaOCl reaction with organic tissue

When NaOCl contacts organic tissue, it acts as a solvent and results in the formation of HOCl and the release of chlorine and the following reactions happen, saponification, amino acid neutralisation and chloramination (Estrela *et al.*, 2002; Kandaswamy and Venkateshbabu, 2010):

- NaOCl acts as an organic and fat solvent that degenerates fatty acids and transforms them into fatty acid salts (detergent) and glycerol (alcohol) (Estrela *et al.*, 2002), resulting in the dissolution of organic matter (Estrela *et al.*, 2002; Estrela *et al.*, 2003). As a result, a reduction in the surface tension of the solution takes place, because of the detergent (Spano *et al.*, 2001) Figure 2.6.

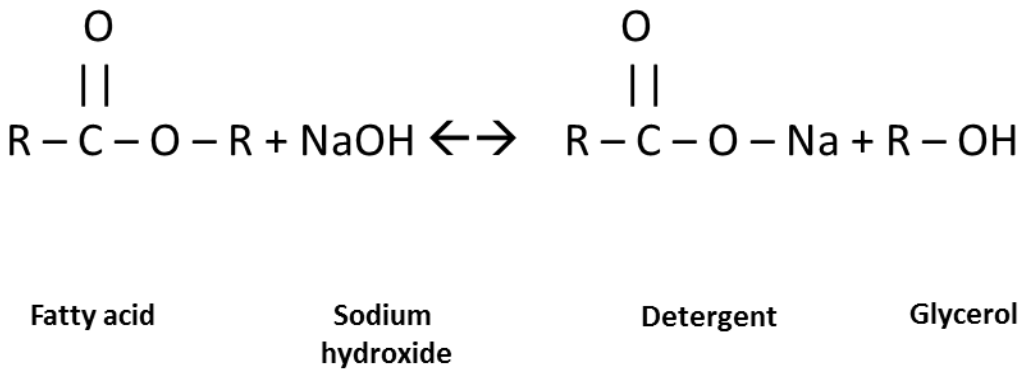

Figure 2.6. The saponification reaction of NaOCl and fatty acids which results in fatty acid salts (detergent) and glycerol (alcohol) (Kandaswamy and Venkateshbabu, 2010).

- NaOCl neutralises the amino acids, which results in the formation of salt and water (Figure 2.7), and with the consumption of hydroxyl ions (OH^-) the pH is reduced (Spano *et al.*, 2001; Estrela *et al.*, 2002).

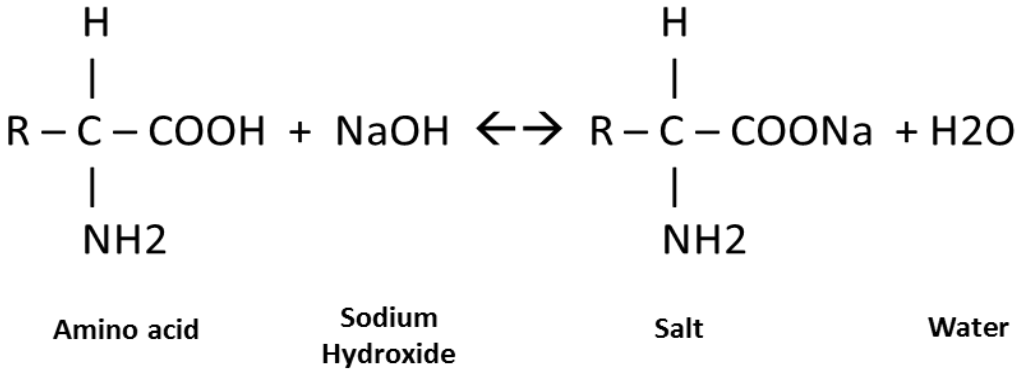

Figure 2.7 The reaction of amino acid neutralisation by NaOCl and the formation of salt and water (Kandaswamy and Venkateshbabu, 2010).

- The available chlorine in the form of HOCl releases and combines with the amino group NH of the protein through chloramination reaction and forms chloramines (Spano *et al.*, 2001) (Figure 2.8). So, amino acid degradation and hydrolysis result, which interfere with cell metabolism. The HOCl and OCl^- produce amino acid degradation and hydrolysis results (Estrela *et al.*, 2002).

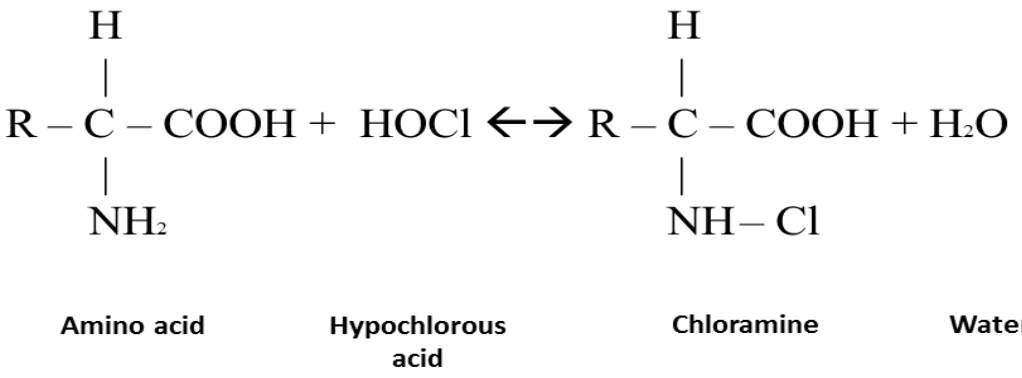

Figure 2.8 Chloramination process during the reaction of NaOCl with amino acids NH which results in chloramine and water (Kandaswamy and Venkateshbabu, 2010).

- In addition, NaOCl could result in the dissolution of collagen, for example in predentine, or collagen-mineral bonds, for example in mineralised dentine (Sim *et al.*, 2001), by fragmenting long polypeptide chains and chloramination of protein terminal groups and resulting in the loss of organic matrix and loss of magnesium and carbonate ions (Shellis, 1983; Davies *et al.*, 1993; Mohammadi, 2008). As a result, dissolution of organic components of dentine may occur (Saleh and Ettman, 1999).

For example, it was found that using sodium hypochlorite alone at any concentration until 9%, for a period until 1 h and at 37°C leaves the inorganic components intact, and it only affects the organic part of dentine (Marending *et al.*, 2007a). It was found that using NaOCl alone as root canal irrigant may dissolve organic material of dentine and expose the inorganic part of the smear layer which prevents further dissolution of the dentine, or it may dissolve the organic part and leave smear layer of mineralized tissue (Baumgartner and Mader, 1987; Baumgartner and Cuenin, 1992). This could be explained by the increase in Ca/P ratio of dentine surfaces after irrigation with NaOCl (Dogan and Çalt, 2001), and mineral accumulation in root dentine after treatment with NaOCl (Inaba *et al.*, 1996).

Increasing the efficacy of NaOCl:

The performance of NaOCl is enhanced by an increase in its concentration, temperature, frequent application or replenishment and agitation. It may be compromised by the presence of proteinacious material which could exhaust its abilities (Haapasalo *et al.*,

2012). So, it could produce a clean canal during or after instrumentation, but smear layer could still exist on canal walls which may compromise or limit its ability (Wayman *et al.*, 1979; Berg *et al.*, 1986).

The concentration of commercial NaOCl is represented by the concentration of the available chlorine (wt/v) (Zehnder *et al.*, 2002; Camps *et al.*, 2009), and the tissue-dissolving ability of NaOCl is a function of free available chlorine (Zehnder *et al.*, 2002), which can be measured by titration (Zehnder *et al.*, 2002; Camps *et al.*, 2009).

Generally, it is recommended to use a concentration of NaOCl between 0.5% and 5.25% (wt/v) (Hand *et al.*, 1978; Koskinen *et al.*, 1980b; Harrison, 1984; Baumgartner and Cuenin, 1992; Gomes *et al.*, 2001; Vianna *et al.*, 2004; Sena *et al.*, 2006; Stojicic *et al.*, 2010), however, there is no consensus with regards to the ideal concentration (Sim *et al.*, 2001; Retamozo *et al.*, 2010). Several researchers preferred 5.25% NaOCl (Baumgartner and Cuenin, 1992; Jeansonne and White, 1994; Berber *et al.*, 2006), whilst others preferred a concentration as low as 0.5% which still preserves antibacterial activity (Byström and Sundqvist, 1983), and may reduce the risks of chemical damage to dentine (Hu *et al.*, 2010).

It was found that NaOCl organic tissue dissolving ability increases with an increase in temperature (Cunningham and Balekjian, 1980; Moorer and Wesselink, 1982; Sirtes *et al.*, 2005). For example, a study found that both of 2.6% and 5.25% NaOCl can dissolve collagen, but the best regime was 5.25% NaOCl at 60°C in comparison with the same concentration at lower temperature (approximately 22°C) and with the lower concentration (2.6%) at the same temperature (Abou-Rass and Oglesby, 1981). Preheating of sodium hypochlorite (NaOCl) solution from 20°C to 45°C improves its non-vital pulp dissolving capacity and *Enterococcus faecalis* killing efficacy (Sirtes *et al.*, 2005). In addition, it has been stated that heating sodium hypochlorite decreases the surface tension and flow, which may also boost its tissue dissolving ability (Stojicic *et al.*, 2010).

NaOCl is more effective in cleaning wider areas of root canal than narrow areas (Senia *et al.*, 1971). Ram (1977) found that debris removal from root canals was a function of root canal diameter rather than type of irrigant used, so the size of root canal preparation may be important in influencing the flow and volume of irrigant (Ram, 1977).

It was observed that NaOCl dissolved the vital pulp tissue of bovine teeth faster than necrotic pulp tissue (Gordon *et al.*, 1981). These findings were corroborated by another study which observed that its maximum effect was on the vital tissues, whilst it had a moderate effect on necrotic tissues and a minimal effect on fixed tissue (Abou-Rass and Oglesby, 1981). Paradoxically, another study noticed that the NaOCl irrigant arrived at the apex of prepared root canals with necrotic pulp tissue more rapidly than in canals with vital pulp tissue (Salzgeber and Brilliant, 1977), which suggested that it dissolved necrotic tissue faster than vital tissue.

Risks of NaOCl

Whilst an increased concentration may influence the efficacy of NaOCl in microbial killing and tissue dissolution, this may also influence the potential to damage dentine (Byström and Sundqvist, 1983; Byström and Sunvqvist, 1985; Baumgartner and Cuenin, 1992; Grigoratos *et al.*, 2001; Sim *et al.*, 2001; Calt and Serper, 2002; Angker *et al.*, 2005). Root canal treated teeth are believed in many circumstances to be more susceptible to fracture than vital teeth (De Deus, 1975). Three main reasons have been cited: loss of tooth structure, change in the physical properties of dentine and changes in patient proprioception (Sobhani *et al.*, 2010). Above that, intra-canal irrigants, medicaments and materials could modify the physical and mechanical properties of dentine (Sim *et al.*, 2001), and dissolution of the organic part of dentine could result which could reduce the microhardness of dentine (Saleh and Ettman, 1999).

In addition to potential effects of NaOCl on hard tissues, it has a caustic effect on soft tissue and therefore, both patient and dentist should wear protective glasses. In addition, a rubber dam should be used to prevent its contact with oral mucosa. It should also not be forced into the periapical tissue, because it can cause a severe inflammatory reaction (Harrison, 1984; Zehnder, 2006). Sensitivity to hypochlorite is recognised but rare (Hostynek *et al.*, 1989).

2.5.1.2 Ethylenediamine Tetra-Acetic Acid (EDTA)

EDTA is a chelator that reacts with calcium ions in dentine (Weinreb and Meier, 1965). It was introduced in endodontics in 1957 by Nygaard-Ostby who recommended its concentration to be 15% with a pH of 7.3 (Nygaard, 1957). It has some antimicrobial

activity (Yoshida *et al.*, 1995). EDTA is an acronym of ethylenediaminetetraacetic acid, and is a polyamino carboxylic acid with the formula $[CH_2N\ (CH_2CO_2H)_2]_2$ (Basrani and Haapasalo, 2012) (Figure 2.9).

Mechanism of action of EDTA

EDTA forms stable complexes with di and tri cationic metal ions such as Ca^{2+} and Fe^{3+} and this stability comes from bonding between the organic substance (EDTA) and the central metal ion. EDTA has more than one pair of free electrons or approximately 6 binding sites ready to bind with metal ions (Putzer *et al.*, 2008; Basrani and Haapasalo, 2012). The crystalline field theory explains the chemical reaction between EDTA and calcium ions in which the attraction force between the central metal and the ligands is purely electrostatic, and the attraction force resulting from the metallic ion was higher than the repulsive force produced from atoms of the EDTA. So, EDTA forms stable complexes with calcium ions (Hülsmann *et al.*, 2003b). For example, in mineralised hard tissue such as bone, which is quite similar to dentine, the inorganic component is predominantly $Ca_3(PO_4)_2$, and EDTA can demineralise it with the formation of CaEDTA (Nikiforuk and Sreebny, 1953) (Figure 2.10).

As a result, carboxyl groups in the EDTA are ionised, and release hydrogen atoms which compete with calcium ions (Hülsmann *et al.*, 2003b). Chelators are widely used in medicine to bind and inactivate metallic ions, as in the case of metal poisoning when they can be used for excretion of excess or toxic ions (Flora and Pachauri, 2010).

When the antibacterial activity of 15% EDTA was compared with saline, it was found that the EDTA was more effective (Yoshida *et al.*, 1995). Its effect is exerted by extraction of bacterial surface proteins by combining with metal ions in the cell envelope of the bacteria, resulting in bacterial death (Haapasalo *et al.*, 2005; Basrani and Haapasalo, 2012). It could also be possible that EDTA plays a synergistic role with NaOCl in disinfecting and debriding root canals (Haapasalo *et al.*, 2003).

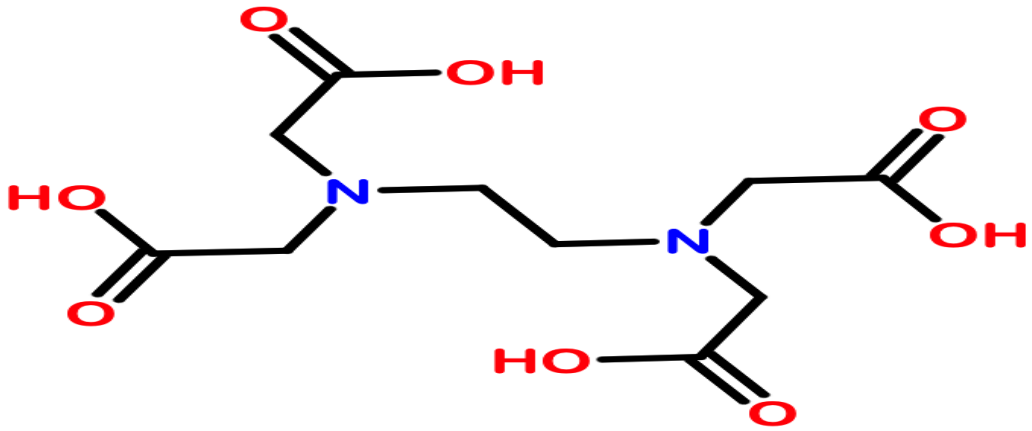

Figure 2.9. The chemical structure of EDTA.

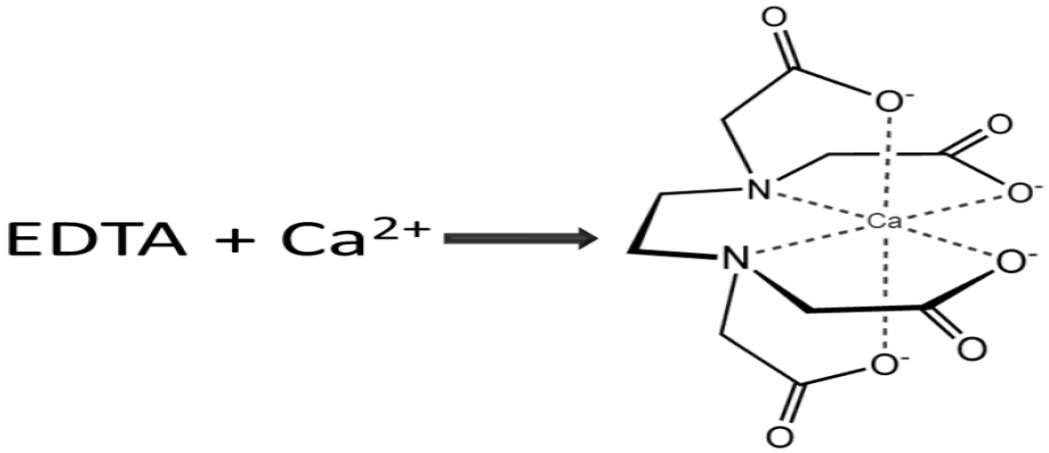

Figure 2.10. Metal ion (calcium) in dental hard tissue is sequestered by EDTA to form stable complexes of calcium edetate (CaEDTA) (Qian *et al.*, 2011).

Increasing the efficacy of EDTA

The effectiveness of the EDTA is dependent on pH (Cury *et al.*, 1981), concentration (Nikiforuk and Sreebny, 1953; Blomlöf *et al.*, 1997; Serper and Çalt, 2002), temperature (Nikiforuk and Sreebny, 1953) and time (Serper and Çalt, 2002).

In terms of pH, it was observed that EDTA at neutral pH (approximately pH 7–8) was more effective for hard tissue demineralisation than at higher pH (more than 10.5) (Nikiforuk and Sreebny, 1953). For example 0.25 M EDTA was used at different pHs (6.02, 7.49, 9.53 and 21.1) and at 25°C to demineralise rat bone, and it was observed that the time of demineralisation of the mineral part is reduced with increase pH of the EDTA (Nikiforuk and Sreebny, 1953).

The concentration of EDTA increases the effect of EDTA to a certain extent. For example, EDTA was used at different concentrations (0.2%, 0.4%, 0.6%, 0.8%, 1% , 1.2%, 1.4%, 1,6% and 1.8%) at 25°C and at pH 7.43 to analyse the rate of demineralisation of rat bone, and it was found that the time of demineralisation of the inorganic part is reduced sharply with increase concentration of the EDTA until 1% and more than this concentration the time reduced was nearly negligible (Nikiforuk and Sreebny, 1953).

Temperature increases the efficacy of EDTA. For example, 1% EDTA was used at different temperatures (4°C, 25°C, 37°C and 60°C) at approximately pH 7.4 for complete demineralisation of rat bone, and it was observed that increased temperature increased the rate of the demineralisation (Nikiforuk and Sreebny, 1953). Moreover, 17% EDTA at pH 7.43 for 1 minute and 10 minutes was employed to treat root dentine, and it was observed that the effectiveness of the EDTA was greater at the 10 minutes application than the 1 minute because it resulted in apparent destruction in the pertibular and intertubular dentine (Calt and Serper, 2002).

Other factors, such as the penetrability or wettability and surface area are important factors in determining the speed of demineralisation by EDTA (Nikiforuk and Sreebny, 1953). Conventional EDTA has a lower surface tension than hydrogen peroxide, normal saline and distilled water, but it was near to 2.5% or 5% NaOCl. The lower surface tension of EDTA and NaOCl enhance the wettability of root canal walls and the flow and penetration of these two irrigants into irregularities and dentinal tubules of the root

canal walls (Tasman *et al.*, 2000; Torabinejad *et al.*, 2002; Hülsmann *et al.*, 2003b). Above that, a detergent such cetavlon was added to the EDTA to form EDTAC to reduce the surface tension further (Hülsmann *et al.*, 2003b), and to improve the bactericidal efficacy of the EDTA (Sreebny and Nikiforuk, 1951; Nygaard, 1957; Goldberg and Abramovich, 1977; Guerisoli *et al.*, 2002; Hülsmann *et al.*, 2003b).

Furthermore, the demineralising efficiency of the EDTA also depends on the amount of the inorganic part of the mineralised tissue in relation to the organic substance in the mineralised tissue (Nikiforuk and Sreebny, 1953). When 0.5 M EDTA (approximately pH 7.4) was used for 4 weeks to demineralise rat teeth, it was observed that the speed of enamel demineralisation was much faster than dentine (Nikiforuk and Sreebny, 1953).

Finally, EDTA is available as a liquid or a paste and that the liquid EDTA form cleaned root canals much more effectively than the paste (Chen and Chang, 2011).

Risk of EDTA

EDTA is generally regarded to have low toxicity (Basrani and Haapasalo, 2012). The cytotoxicity effect of 1% and 0.1% concentrations of both alkaline and neutral EDTA was tested *in vitro* on fibroblasts derived from mouse skin for periods of 1 h, 3 h, 6 h, 12 h and 24 h. 1% solutions of both alkaline and neutral EDTA was severely cytotoxic at 1 h, while the 0.1% at both alkaline and neutral EDTA was moderately cytotoxic for 24 h period (Koulaouzidou *et al.*, 1999). Translation to the clinical setting is not always simple and the results of this investigation cannot provide definitive evidence that adverse tissue reactions would be noted *in vivo*.

Sen *et al* (2009) evaluated the potential erosive effects of 1%, 5%, 10% and 15% EDTA solutions (pH 7.2) after exposure of instrumented root canals for 1 minute. They concluded that a 1% EDTA solution was optimal as root canal irrigant, since it had the least erosive effect on dentine surfaces (Şen *et al.*, 2009). In addition, Calt *et al* (2000) recommended a 1 minute application of EDTA, since longer application could result in more erosion of intertubular and peritubular dentine (Calt and Serper, 2002).

2.5.1.3 Citric Acid (CA)

Citric acid is an organic acid ($C_6H_8O_7$) that may be used in liquid form as a root canal chelating agent (Yamaguchi *et al.*, 1996) (Figure 2.11). Antimicrobial properties have also been reported (Georgopoulou *et al.*, 1994; Yamaguchi *et al.*, 1996).

Mechanism of action of citric acid

Citric acid is a weak tri-basic carboxylic acid (2-OH-1,2,3-Propane Tricarboxylic Acid) which reacts with metals and forms stable complexes, which form non-ionic soluble chelates (calcium citrate) (Yamaguchi *et al.*, 1996; Dorozhkin, 1997; Scelza *et al.*, 2003; Papagianni, 2007). Citric acid then releases H^+ ions which attack the surface of hydroxyapatite crystals, leading to a sequence of ionic detachments from the surface of the hydroxyapatite into the surrounding medium. This results in the dissolution of the hydroxyapatite crystal (Dorozhkin, 1997) Figure 2.12.

De-Deus *et al* (2008) used co-site digital optical microscopy to evaluate the demineralising efficiency of 1% citric acid, 17% EDTA and 17% EDTAC on dentine. They found that dentine responded in different ways, and that citric acid was the most effective chelator, EDTA was intermediate and EDTAC was the least effective (De-Deus *et al.*, 2008). The mechanism of action of citric acid on killing bacteria is not well known (Basrani and Haapasalo, 2012).

Increasing the efficacy of citric acid

The chelating efficiency of citric acid increases with increasing concentration (Reis *et al.*, 2008), and lowering pH (Haznedaroglu, 2003). Increasing the time of application also increases effectiveness to some extent (Machado-Silveiro *et al.*, 2004).

The chelating effect of citric acid at 1% (pH 2.3), 5% (pH 2.0) and 10% (pH 1.8) was compared with environmental SEM and co-site optical microscopy (COSM) after application to dentine for 15, 30, 60, 180 and 300 sec application periods. Chelating efficacy on dentine was proportional to its concentration (Reis *et al.*, 2008). In addition, the concentration (µg/mL) of Ca^{2+} extracted from root dentine was analysed by inductively coupled plasma-emission spectrometry (ICP-AES) after treating root canals with 1% citric acid at pH 7.4 and pH 1.0. It was found that the lower pH citric acid removed more calcium than the higher pH solution. Citric acid was applied to slices of

cervical third root dentine at concentrations of 1% (pH 2.2) and 10% (pH 1.8) for 5, 10 and 15 minutes, and it was found that demineralisation was significantly time dependent. In addition, it was found that the decalcifying action of the 10% solution was approximately 2 times higher than the 1% solution (Machado-Silveiro *et al.*, 2004).

Risks of citric acid

Citric acid has been shown to be only mildly irritant to rat tissues, and less damaging than EDTA (Sousa *et al.*, 2005). This observation was corroborated by others working with cultured fibroblasts (Sceiza *et al.*, 2001). Within the context of the current work, citric acid is known to cause erosion of dentine, with measurable effects after 1 minute of application on both peritubular and intertubular dentine (Reis *et al.*, 2008).

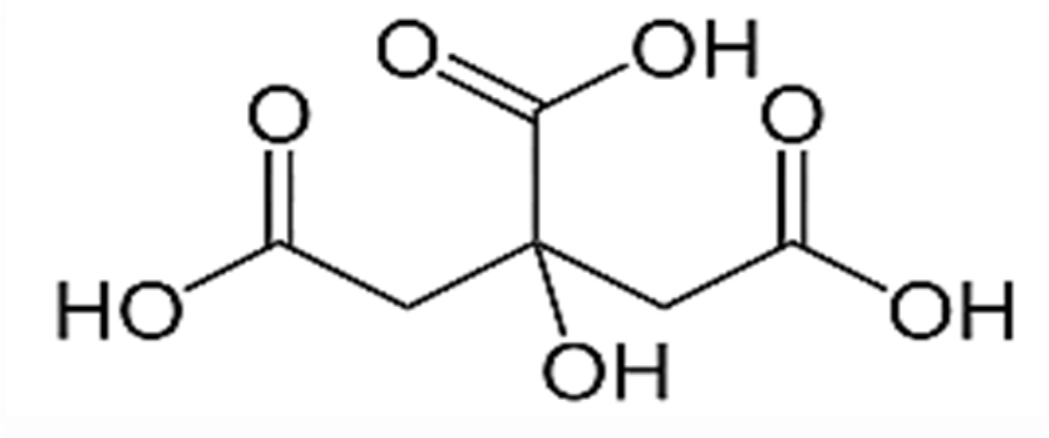

Figure 2.11. Structure of citric acid (Chemical Education Digital Library Staff, 2010).

$$Ca_{10}(PO_4)_6(OH)_2 + 2H^+ \rightarrow 6PO_4^{3-} + 2H_20 + 10Ca$$

Figure 2.12. The dissolution of hydroxyapatite by acid.

2.5.1.4 Chlorhexidine (CHX)

Chlorhexidine is a synthetic cationic bis-guanide which consists of two symmetrical 4-chlorophenyl rings and two bis-guanide groups, and they are connected by central hexam-ethylene chains (Greenstein *et al.*, 1986) (Figure 2.13). It was developed in the 1940s, is a strongly alkaline compound, and is used in the form of stable salts such as

chlorhexidine acetate and chlorhexidine hydrochloride or more commonly as chlorhexidine digluconate, which has greater solubility in water (Davies *et al.*, 1954; Foulkes, 1973; Zehnder, 2006).

The effect of chlorhexidine on microbial biofilms is significantly less than NaOCl, although it has wide range of antimicrobial activity against gram negative and gram positive bacteria and is effective as an antifungal agent especially against *Candida albicans* (Mohammadi and Abbott, 2009).

Mechanism of action of chlorhexidine

It is a cationic molecule (Mohammadi and Abbott, 2009), and can bind to anionic molecules such as phosphate present in the structure of hydroxyapatite on dentine surfaces (Kandaswamy and Venkateshbabu, 2010).

Chlorhexidine is a hydrophobic and lipophilic molecule which is positively charged and interacts with phospholipids and lipopolysaccharides on the bacterial cell membrane, entering into the cell through active or passive transport (Athanassiadis *et al.*, 2007). The antibacterial activity of chlorhexidine comes from the interaction between its positive charge and the negative charge of phosphate groups on microbial cell walls (Gomes *et al.*, 2003a; Gomes *et al.*, 2003b). As a result, the osmotic equilibrium of the cell wall changes, and consequently the permeability of the cell wall increases, allowing chlorhexidine penetration into the cell body (Mohammadi and Abbott, 2009). This damage of the cell membrane results in leakage of intracellular contents outside the cell, especially phosphate compounds such as adenosine triphosphate and nucleic acids (Siqueira Jr *et al.*, 2007) (Figure 2.14).

Chlorhexidine is widely used in periodontal treatment for plaque control and is a powerful antiseptic (Zamany *et al.*, 2003). In periodontal treatment, it is usually used at concentrations of 0.1-0.2%, but 0.2% chlorhexidine has less antibacterial efficacy than 2.5% NaOCl (Ringel *et al.*, 1982). In addition, it is less effective than 2.5% NaOCl in creating negative cultures (Ringel *et al.*, 1982). When it is used at 0.2% concentration, the low molecular weight substances can leak outside the bacterial cell, especially potassium and phosphorous, and when it is used at 2% concentration, it becomes bactericidal, as a result of precipitation of cytoplasmic contents (Gomes *et al.*, 2003b).

Therefore, in endodontic it was recommended to be used at the higher concentration of 2% (White *et al.*, 1997; Zamany *et al.*, 2003).

Chlorhexidine has a unique property of 'substantivity', which points to the residual antimicrobial activity of chlorehexidine (Rølla *et al.*, 1971; Dametto *et al.*, 2005; Mohammadi and Abbott, 2009). Substantivity means that chlorehexidine can bind to surrounding tissues and is slowly released for an extended period of time (Mohammadi and Abbott, 2009). For dentine surfaces, this arises because the positively charged chlorhexidine can adsorb onto the tissue surface (Athanassiadis *et al.*, 2007). The residual antimicrobial activity of chlorhexidine may last for 2 or 3 days (White *et al.*, 1997; Nelson Filho *et al.*, 1999).

Increasing the efficacy of chlorhexidine

It has been shown that chlorhexidine can work synergistically with hydrogen peroxide to reduce bacterial infection (Heling and Chandler, 1998). In addition, combinations of chlorhexidine with $Ca(OH)_2$ can result in a broad spectrum and long lasting antimicrobial effect (Waltimo *et al.*, 1999).

Risks of chlorhexidine

The chlorhexidine does not have tissue dissolving ability (Naenni *et al.*, 2004; Okino *et al.*, 2004), so it cannot be used alone to irrigate the root canal (Mohammadi and Abbott, 2009). If it is used in a combination with NaOCl, it is recommended to remove the remaining NaOCl by irrigation of the root canals either with alcohol or EDTA, because the interaction between NaOCl and chlorhexidine may result in the formation of a para-chloroaniline precipitate (PCA) which is believed to be carcinogenic (Chhabra *et al.*, 1991; Basrani *et al.*, 2010). Sensitivity to chlorhexidine is rare (Krautheim *et al.*, 2004), but anaphylactic reactions have been reported after application to mucous membranes (Krautheim *et al.*, 2004).

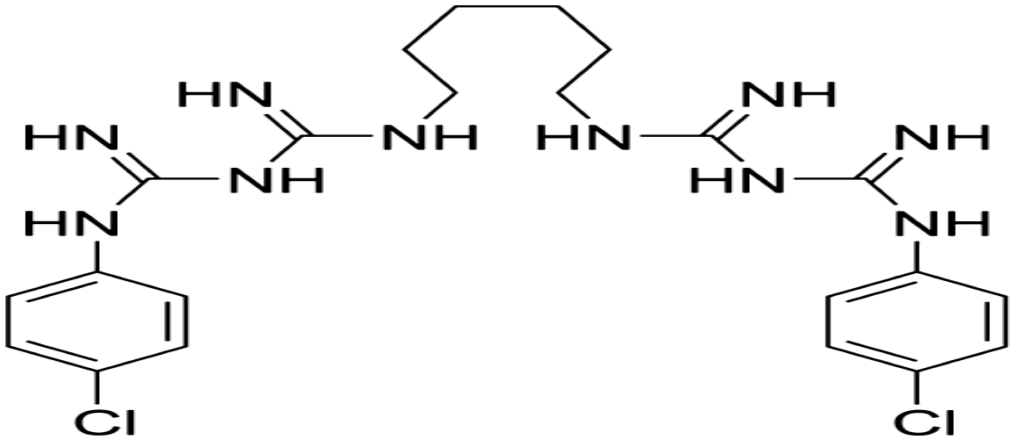

Figure 2.13. Structure of chlorhexidine digluconate (Basrani and Haapasalo, 2012).

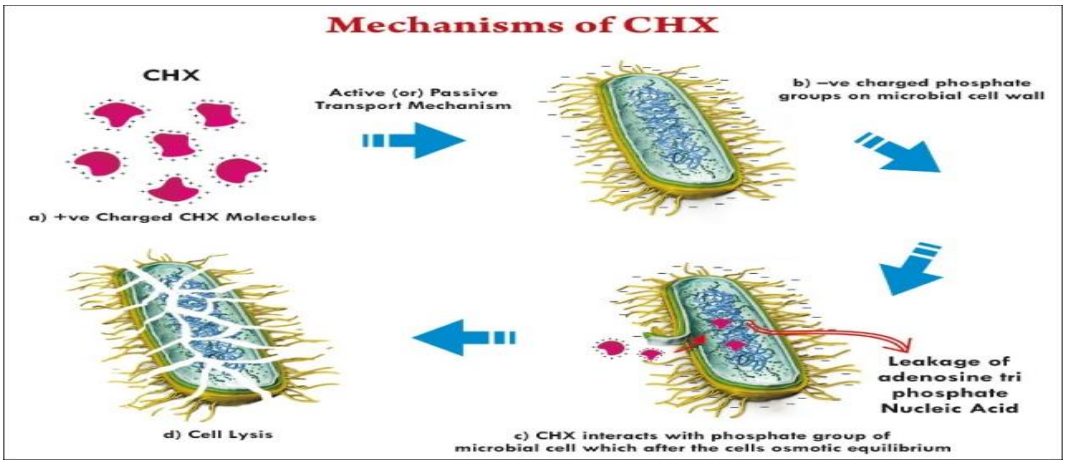

Figure 2.14. The mechanism of action of chlorhexidine (CHX) on bacteria (Kandaswamy and Venkateshbabu, 2010).

2.5.2 Alternative irrigant agents

A number of alternative irrigants have been utilised clinically and will be mentioned briefly.

2.5.2.1 Hydrogen peroxide (H_2O_2)

It is a contemporary bleaching agent which has the ability to bleach coloured organic and inorganic structures, including teeth (Chng *et al.*, 2005; Joiner, 2007). Hydrogen peroxide has been used alone or in combination with NaOCl in root canal irrigation (Heling and Chandler, 1998).

Joiner (2007) reported that hydrogen peroxide had no significant deleterious effects on the surface morphology, chemistry, or mechanical properties of enamel and dentine such as surface and subsurface microhardness. Risks have been associated with its use in non-vital bleaching, particularly involving thermocatalytic activation (Madison and Walton, 1990).

The nanomechanical properties of intertubular dentine were analysed by AFM after exposure to 30% hydrogen peroxide at 24°C for 24 h and revealed that the Young's modulus and nanohardness of intertubular dentine were significantly decreased (Titley *et al.*, 1988; Madison and Walton, 1990; Chng *et al.*, 2005; Joiner, 2007). Furthermore, the Ca/P ratios of dentine were changed following immersion of dentine specimens in 30% hydrogen peroxide at 37°C for 7 days to bleach them (Rotstein *et al.*, 1996). Whether such severe effects are encountered after more brief root canal irrigation with peroxide solutions is incompletely investigated. Recognised risks include the development of bubbles which may prevent effective deep irrigation or risk surgical emphysema.

2.5.2.2 Sterile water and saline

Distilled water or normal saline can be efficient irrigants for removal of loose debris from the cervical and middle thirds of the root canal, although these solutions are not efficient in the removal of a smear layer (Solovyeva and Dummer, 2000). Using tap water in conjunction with ultrasonic instrumentation to remove soft tissue from uninstrumented root canals is less effective than using NaOCl with ultrasonic instrumentation (Walker and del Rio, 1991). When normal saline was used with EDTA to remove debris and predentine from uninstrumented root canals, it was inefficient (Baumgartner and Mader, 1987). Normal saline is not recommended as a root canal irrigant, since it does not have antimicrobial properties (Ring *et al.*, 2008), and does not dissolve organic tissues (Senia *et al.*, 1971; Ring *et al.*, 2008). Distilled water is also unable to dissolve organic tissue (Senia *et al.*, 1971; Okino *et al.*, 2004).

2.5.3 Irrigant combinations

The most common combinations are:

- NaOCl with EDTA
- NaOCl with citric acid

Why combinations have been suggested

Individual irrigants may not be able to address both organic and inorganic components of smear layer removal and canal debridement (Wang and Spencer, 2002). NaOCl is able to address organic elements, whilst demineralizing agents act on inorganic matter, softening the dentine surface and sub-surface to facilitate its shaping, contributing to smear layer removal and assisting the detachment of biofilms (Harrison, 1984; Titley *et al.*, 1994; Tay and Pashley, 2001).

How combinations work together

When the deproteinating agent NaOCl is applied alone to dentine, two possible actions may occur. Either the organic portion of the dentine could be removed to such extent that it exposes large amounts of inorganic mineral, which could act as a barrier to further dissolution by the NaOCl. In this way, the effects of further NaOCl application could be limited. Alternatively, there could be some sort of chemical equilibrium which could stop further dissolution of the organic substance (Sim *et al.*, 2001; Rajasingham *et al.*, 2010).

When the chelating agent EDTA is applied alone to dentine, similar but opposite rate limiting effects may occur (Dogan and Çalt, 2001; Pérez-Heredia *et al.*, 2008), with the organic component of the dentine matrix being revealed by the demineralising agent which is no longer able to be active on the surface (Apostolopoulos and Buonocore, 1966; Baumgartner and Mader, 1987). Alternatively, the chelator may remove the smear layer without having a significant effect on the underlying dentine (Gwinnett, 1984).

Both EDTA and citric acid have often been recommended to supplement the action of NaOCl in clinical endodontics (Koskinen *et al.*, 1980a; Yamada *et al.*, 1983; Baumgartner *et al.*, 1984; Baumgartner and Mader, 1987; Czonstkowsky *et al.*, 1990; Baumgartner and Cuenin, 1992; Dogan and Çalt, 2001; Balaji and Sajjan, 2002;

Guerisoli *et al.*, 2002; Grawehr *et al.*, 2003; Haapasalo *et al.*, 2005), and their helpful synergism is recognised (Baumgartner and Ibay, 1987; Baumgartner and Mader, 1987; Grawehr *et al.*, 2003).

2.5.3.1 NaOCl with EDTA

A combination of NaOCl and EDTA resulted in more effective root canal cleaning than the use of either agent alone, removing pulpal debris and predentine and exposing the globular surface of the dentine in non-instrumented areas of root canals. Smear layer was also removed from instrumented areas of the canals (Baumgartner and Mader, 1987; Pérez-Heredia *et al.*, 2006).

In addition, Byström and Sunvqvist (1985) found that the combination of NaOCl and EDTA was more effective in canal disinfection than NaOCl alone (Byström and Sunvqvist, 1985). The enhanced effect in eliminating *Enterococcus faecalis* biofilms from root canals has also been recognised (Soares *et al.*, 2010).

It is recommended in the clinical setting that they are not mixed, since NaOCl is inactivated by EDTA (Grawehr *et al.*, 2003; Violich and Chandler, 2010), probably due to the loss of available chlorine (Baumgartner and Ibay, 1987; Grawehr *et al.*, 2003; Gulabivala *et al.*, 2005; Zehnder *et al.*, 2005; Rossi-Fedele *et al.*, 2012).

Protocols suggesting the use of NaOCl during instrumentation, with the application of EDTA to remove smear layer, before final NaOCl disinfection have been described (Yamada *et al.*, 1983; Grawehr *et al.*, 2003; Marending *et al.*, 2007b).

Risks of NaOCl and EDTA combination

The sequential use of 17% EDTA for 2.5 minutes at pH 7.7 followed by 5.25 % NaOCl for 2.5 minutes was reported to reduce the microhardness of dentine significantly (Eldeniz *et al.*, 2005). Similar findings have been reported by others (Sayin *et al.*, 2007).

Dentine erosion has also been noted when a combination of 15% EDTA for 3 minutes followed by 6% NaOCl for 2 minutes was applied to root canals. The severity of damage to dentine depended on the time of the application of each solution, with reduced exposure time to EDTA being important if damage was to be limited (Niu *et*

al., 2002). Similar finding were observed after using 17% EDTA (pH 7.0) for 30 sec followed by 5.25% NaOCl (Qian *et al.*, 2011).

When dentine was treated with 2.5% NaOCl for 21 minutes, followed by 17% EDTA for 3 minutes, and then followed by 2.5% NaOCl for 3 minutes (total exposure to the 2.5% NaOCl = 24 minutes), the flexural strength of dentine was reduced significantly, but the elastic modulus was unaffected (Marending *et al.*, 2007a; Marending *et al.*, 2007b).

2.5.3.2 NaOCl with citric acid

The combination of NaOCl and citric acid has been shown to be more effective in root canal debridement than the use of either agent alone (Haznedaroglu, 2003; Pérez-Heredia *et al.*, 2006). Wayman (1979) established that the sequential use of 10% of citric acid solution and 2.5% NaOCl solution followed by 10% citric acid achieved the best results (Wayman *et al.*, 1979). Furthermore, the effectiveness of removal of smear layer by different concentrations of citric acid solutions (5%, 10%, 25% and 50%) at low pH (1.9, 1.8, 1.5 and 1.1), respectively, were compared with the same concentrations buffered to high pH (6.0). It was found that the 5% and 10% concentrations were significantly more effective than the same concentrations at high pH. Thus pH may be more important than the concentration of citric acid (Haznedaroglu, 2003). Low concentrations at original, non-buffered pH may be most effective.

Like EDTA, citric acid should be not mixed with the NaOCl, since it too may reduce the available chlorine and thus the antimicrobial and tissue-dissolving effects of the NaOCl (Baumgartner and Ibay, 1987; Zehnder *et al.*, 2005; Rossi-Fedele *et al.*, 2012).

Risks of NaOCl and citric acid combination

Treatment of root dentine with 9% citric acid for 2.5 minutes at pH 1.3 followed by 5.25 % NaOCl for 2.5 minutes resulted in reduced microhardness of the dentine and significantly increased the roughness of the dentine surface significantly (Eldeniz *et al.*, 2005). Dentine erosion was also noted when dentine was treated with 10% citric acid for 5 minutes followed by 5.25% NaOCl for 1 minute or 5 minutes (Qian *et al.*, 2011).

The use of chelating agents with NaOCl may thus represent a two-edged sword, enhancing the potential to clean root canals, but with the risk that the dentine may be weakened by the combined chemical effects.

2.5.4 The delivery and activation of irrigant solutions

Irrigant solutions have traditionally been delivered to root canals with a syringe and some form of needle. The depth of insertion of the irrigant needle has an impact on the exchange of irrigant solution (Abou-Rass and Piccinino, 1982; Boutsioukis *et al.*, 2010b), and the design of its tip has an impact on irrigant hydrodynamics (Boutsioukis *et al.*, 2010c; Shen *et al.*, 2010; Devi and Abbott, 2012; Snjaric *et al.*, 2012; Park *et al.*, 2013).

2.5.4.1 Syringe delivery

Irrigant is delivered most commonly by the application of positive pressure to a disposable syringe with a side-ported needle (Desai and Himel, 2009). A recognised risk is the possibility of introducing irrigant into the periapical tissues (Brown *et al.*, 1995; Fukumoto *et al.*, 2006; Zairi and Lambrianidis, 2008; Desai and Himel, 2009; Boutsioukis *et al.*, 2010c; Verhaagen *et al.*, 2012), causing tissue damage and postoperative pain (Ehrich *et al.*, 1993). Mercifully, such events occur rarely, and limiting factors include the closed system in which irrigation takes place and the effects of tissue pressure at the periapex. Investigations and discussions of root canal irrigation must recognise the effects of closed-systems (Usman *et al.*, 2004; Gutarts *et al.*, 2005; Burleson *et al.*, 2007), and the limitations this may impose on fluid exchange beyond the tip of the irrigating needle (Chow, 1983; Sedgley *et al.*, 2005). Vapour lock, caused by gas entrapment is also recognised to limit irrigant penetration (Senia *et al.*, 1971; Dovgyallo *et al.*, 1989; Migoun and Azouni, 1996; Pesse *et al.*, 2005; de Gregorio *et al.*, 2009). *In vitro*, studies have sought to simulate the clinical scenario by closing the apex of the root. Apices may for example be embedded in polyvinysiloxane impression material, and studies in which this is undertaken are associated with less effective irrigant exchange than those conducted open systems (Baumgartner and Mader, 1987; O'Connell *et al.*, 2000; Albrecht *et al.*, 2004).

This was confirmed recently by Tay *et al.* (2010) who found that there was a difference in root canal cleanliness between closed and open root canal systems in apical, middle and coronal thirds, using conventional syringe irrigation with side-vented needles (Tay *et al.*, 2010).

Irrigant replacement in a positive-pressure conventional syringe system may be limited to 1-1.5 mm beyond the needle tip and may require a high flow rate to generate turbulent fluid flow for effective agitation (Chow, 1983; Sedgley *et al.*, 2005; Boutsioukis *et al.*, 2009; Gao *et al.*, 2009). The position of the tip of the needle could be either slightly coronally to the binding point or at the point of resistance felt by the operator. In addition, this position can be estimated when a needle with known external diameter (D_N) is inserted in a root canal with known apical preparation size (A) and taper (T) using the equation ($L= (D_N - A) / T$), where L represents the distance from the working length (WL) (Boutsioukis *et al.*, 2013a). Irrigation needle size is measured according to the ISO 9626:1991/ Amd.1:2001 specifications (ISO 9626 2001) (Boutsioukis *et al.*, 2013a), and usually falls within the range of 21-30 G, with external diameters of 0.8-0.3 mm respectively (Boutsioukis *et al.*, 2007a; Boutsioukis *et al.*, 2007b; Boutsioukis *et al.*, 2010c; Boutsioukis *et al.*, 2013a). The apical part of the root canal is often recommended to be enlarged to at least size 35-40 (0.35-0.4mm diameter) in order to facilitate needle placement to within 1-2 mm of working length (Ram, 1977; Usman *et al.*, 2004; Zehnder, 2006; Hsieh *et al.*, 2007; Huang *et al.*, 2008). Use of a small diameter needle may logically allow penetration to within 1 mm of working length, but the problem of vapour lock is still not solved and may limit exchange in the apical third (Tay *et al.*, 2010). Irrigation with chelating agents results in demineralisation and a mesh of collagen (fibrillar network of collagen) could form in the apical part of root dentine surface which could trap debris during root canal irrigation, if the irrigation could not produce adequate turbulent flow (Tay *et al.*, 2007) (Tay *et al.*, 2010). Although syringe irrigation represents normal practice for most dentists, the limitations of fluid exchange in closed systems and heightened awareness of canal ramifications have encouraged the pursuit of more effective methods of irrigant delivery and turnover. 'Activated' irrigation, employing a variety of methods to improve irrigant penetration, flow and hopefully effectiveness has become a strong

research focus in recent years (McGill, 2008; Desai and Himel, 2009; van der Sluis *et al.*, 2010). Examples will be described in the following pages.

2.5.4.2 Manual dynamic exchange

Irrigant volume and flow are important factors which influence the efficacy of root canal debridement (Chow, 1983). Several techniques have been used in order to increase the flow and distribution of irrigants into awkward places inside the root canal system (Gu *et al.*, 2009). In this method, after instrumentation of the root canal, a gutta-percha point is repeatedly inserted to the full working length of the canal and pumped up and down at low amplitude and at approximately 100 cycles per minute (McGill, 2008).

The unpublished experiment by Pierre Machtou in 2003 showed that agitation of irrigant by a well-fitting gutta-percha point enhanced irrigant penetration and replacement apically in comparison with static irrigation (Huang *et al.*, 2008). Dynamic manual agitation of irrigant with gutta-percha point could have the potential to displace the apical vapour lock (gas entrapment) from a closed system (Liao and Zhao, 2003; McGill, 2008).

2.5.4.3 Passive ultrasonic activation

Ultrasonic devices were introduced to dentistry in the 1950s and later adopted for endodontic applications (Richman, 1957), both for cutting dentine, and activating NaOCl solutions (Martin *et al.*, 1980; Cunningham and Martin, 1982; Cunningham *et al.*, 1982; Cameron, 1986b; Alaçam, 1987; Cameron, 1987). Ultrasonic files oscillate at 25-30 kHz, which is beyond the limit of human hearing, and work in transverse vibration (Walmsley, 1987). Attempts to shape canals ultrasonically have not been well reviewed, frequently resulting in damaged and irregular canal shapes and apical perforation (Lumley *et al.*, 1992; Peters, 2004). Nevertheless, they are effective in root canal irrigation (Weller *et al.*, 1980; Ahmad *et al.*, 1987a), and ultrasound is popularly adopted in passive ultrasonic irrigation (PUI). In this technique, after canal shaping, the canal is flooded with irrigant. In the next step, a small file (size 15) or smooth wire is introduced into the centre of the canal until it reaches the apical region and activated

ultrasonically. The file moves freely and as a result, the irrigant moves easily into the apical part of the canal, with efforts to avoid potentially damaging wall contact (Weller *et al.*, 1980; Ahmad *et al.*, 1987a; Ahmad *et al.*, 1987b; Ahmad *et al.*, 1988; Krell *et al.*, 1988). Its effects may be due to acoustic streaming and cavitation (Ahmad *et al.*, 1987a) and may result in more efficient cleaning in comparison with root canal irrigation without PUI (Ahmad *et al.*, 1987a; Ahmad *et al.*, 1987b; Ahmad *et al.*, 1988; Lumley *et al.*, 1991; Ahmad *et al.*, 1992; Roy *et al.*, 1994).

Van der Sluis *et al* (2007) reviewed the literature between 1965 and 2007 on the comparative efficiency of PUI and conventional syringe irrigation of root canals, and found that PUI was more effective than conventional syringe irrigation in removing organic tissue, planktonic bacteria, and dentine debris from the root canals (van der Sluis *et al.*, 2007).

An ultrasonically activated irrigating needle has been developed to work as an adjunctive device for root canal debridement (Burleson *et al.*, 2007; Desai and Himel, 2009). This needle may be connected to a MiniEndo piezoelectric ultrasonic unit and can be set to the highest power setting without needle breakage, and with a constant stream of NaOCl. It has been suggested that a constant stream of ultrasonically activated NaOCl may allow shorter irrigation times in comparison with conventional syringe irrigation, making it attractive clinically (Gutarts *et al.*, 2005). It has been identified that combining this system with hand and rotary instruments removed vital pulp tissue from canals and isthmuses more effectively than hand and rotary instrumentation alone (Gutarts *et al.*, 2005; Burleson *et al.*, 2007; Desai and Himel, 2009).

2.5.4.4 Endoactivator

The Endoactivator (sonic activation) is a recently introduced device, consisting of a small battery operated cordless hand piece, which delivers sonic energy through disposable nylon tips. It has been shown that EndoActivator and passive ultrasonic activation of the endodontic file inside pre-shaped canals resulted in a significantly cleaner canal in comparison with hand instrumentation alone (Jensen *et al.*, 1999; Desai and Himel, 2009; Uroz-Torres *et al.*, 2010). In addition, there was no difference in the

cleaning efficiency between the EndoActivator and passive ultrasonic techniques (Jensen *et al.*, 1999; Desai and Himel, 2009; Uroz-Torres *et al.*, 2010). One advantage of the EndoActivator is that the nylon tips do not cut dentine (Uroz-Torres *et al.*, 2010).

2.5.4.5 EndoVac

This system depends on producing a negative pressure irrigation system and has three major components. The first part is the master delivery tip , which simultaneously delivers and evacuates the irrigant into and from the canal. The second part is the macrocannula, which is used to extract the irrigant out of the chamber, coronal third and middle third of the canal. The final part is the microcannula, which is used to remove the irrigant and debris from the full working length of the canal, cervical, middle and apical thirds through 12 microscopic holes by negative pressure.

This system has been identified to debride root canals significantly better than positive pressure irrigation with the endodontic syringe. In addition, it can deliver the irrigant to the apical part of the root canal and with more safety in comparison with conventional syringe irrigation in which irrigant extrusion into the periapical tissues is a real consideration (Nielsen and Baumgartner, 2007; Mitchell *et al.*, 2010). Another study has shown that EndoVac alone could have comparable effects on pulp space infections to the combination of positive pressure conventional syringe irrigation and intracanal antibiotic dressing. EndoVac could therefore eliminate the need for intracanal dressing (Cohenca *et al.*, 2010).

EndoVac appears to have the maximum ability to deliver irrigant safely into the full working length of the root canal without apical extrusion into the periapical tissues (Tay *et al.*, 2010), but the wall shear stress produced by the irrigant is lower than for some other systems (Chen *et al.*, 2013).

The theoretical and *in vitro* benefits of activated irrigation are gaining recognition, but clinical evidence on treatment outcomes is limited.

2.6 Important properties of dentine

Dentine possesses a number of important properties that affect its mechanical behaviour, and some of these could potentially be affected by clinical interventions such as endodontic irrigation. A number of properties have been analysed after endodontic treatment:

2.6.1 Chemical properties

Ca/P ratio of dentine

The Ca/P ratio (wt/wt) is the relative concentration of calcium (wt%) and phosphate (wt%) (Hanlie *et al.*, 2006; Arnold and Gaengler, 2007). This property is usually measured by energy-dispersive X-ray analysis (EDAX) which can quantify the concentration of elements such as Ca, P and C on the dentine surface, each as a relative amount of the total element content (100%) in weight percent (Dogan and Çalt, 2001; Arnold *et al.*, 2003; Arnold and Gaengler, 2007).

The Ca/P ratio in dentine and enamel can be similar. For example, it was shown that the Ca/P ratio of both fully mineralised dentine and enamel was approximately 2.1 (Arnold and Gaengler, 2007), a finding reinforced by another study that determined both to be approximately 2.13 (Falla-Sotelo *et al.*, 2005). Another study found that the Ca/P ratio of root dentine was approximately 1.72 (Dogan and Çalt, 2001). The Ca/P of hydroxyapatite apatite is between 2.0 and 2.3 (Arnold *et al.*, 2003; Falla-Sotelo *et al.*, 2005). For predentine, the Ca/P ratio was found to be approximately 2.5, highlighting variations in mineralizing and mature dentine (Arnold and Gaengler, 2007).

Variation is also apparent in different types and anatomical locations of dentine (Hennequin *et al.*, 1994; Hennequin and Douillard, 1995; Dogan and Çalt, 2001), while there is less variation in enamel (Dogan and Çalt, 2001; Arnold and Gaengler, 2007).

2.6.2 Mechanical properties of dentine

Dentine Hardness

Many authors have measured the hardness of dentine, historically with microhardness based instruments (Kinney *et al.*, 1996a; Marshall *et al.*, 1997), but more recently using

higher sensitivity instruments capable of measuring at the nanoscale (Kinney *et al.*, 1996a; Kinney *et al.*, 1996b). While there are some differences in the magnitude of the hardnesses reported in these studies, mostly due to differences in measurement type (e.g. Vickers (Craig *et al.*, 1959; Fusayama and Maeda, 1969; Pashley *et al.*, 1985; Kinney *et al.*, 1996a; Fuentes *et al.*, 2003) or Knoop (Craig *et al.*, 1959; Fuentes *et al.*, 2003; Kinney JH *et al.*, 2003; Oyen, 2006) hardness testers) and the applied force, generally the hardness of dentine can be ranked as softest closest to the pulp and hardest nearest the DEJ (Craig *et al.*, 1959; Fuentes *et al.*, 2003; Kinney JH *et al.*, 2003; Oyen, 2006). Carious dentine is significantly softer than healthy dentine (Craig *et al.*, 1959), with sclerotic dentine surrounding carious lesions being the hardest of all dentine (Craig *et al.*, 1959).

It is likely that these differences in hardness relate to differences in Ca:P ratio and tubule density in each type of dentine (Kinney *et al.*, 1996b), particularly when the hardnesses of mineralised tissues are considered. Enamel is significantly harder than dentine (343 compared to 68 Knoop hardness numbers), while there is no significant difference between the hardness of dentine and cortical bone (Hodge and McKay, 1933; Craig and Peyton, 1958; Xu *et al.*, 1998; Kinney JH *et al.*, 2003; Oyen, 2006; Nanci, 2008). However, with the development of more sensitive nanohardness testers, it has become apparent that there are differences in the hardness of peritubular dentine and inter tubular dentine that may also account for some of the differences in hardness reported for the different types of dentine (Kinney *et al.*, 1996a). Using AFM-based nanoindentation, in which high spatial resolution can be paired with high force sensitivity, it has been established that peritubular dentine is four-times harder than intertubular dentine. Additionally, intertubular dentine exhibits some location dependant hardness, typically between 0.12 and 0.18 GPa near the pulp and 0.49 and 0.52 GPa at the DEJ, while, the hardness of peritubular dentine is constant (between 2.23 and 2.54 GPa) irrespective of location (Kinney *et al.*, 1996a; Kinney *et al.*, 1996b). The value of the microhardness of dentine does not depend on tubular orientation, so this property is isotropic (Wang and Weiner, 1998a; Kinney *et al.*, 1999; Arola *et al.*, 2012).

Elastic modulus

Different studies have measured elastic modulus of dentine, previously using microhardness based instruments (Meredith *et al.*, 1996; Jantarat *et al.*, 2002), and more recently using higher sensitivity instruments capable of measuring at the nanoscale (Kinney *et al.*, 1996a). For example, it was found that the elastic modulus of dentine increased from the dentinoenamel junction toward the pulp, ranging from 8.7 to 11.2 GPa (Meredith *et al.*, 1996). Using the higher sensitivity instruments via AFM , it was found that the elastic modulus of peritubular dentine was approximately 29.8 GPa near the dentinoenamel junction and near the pulp. The elastic modulus of intertubular dentine ranged from 17.7 to 21.1 GPa, the lowest value being near the pulp (Kinney *et al.*, 1996a). This property is isotropic and its value does not depend on tubular orientation (Wang and Weiner, 1998a; Kinney *et al.*, 1999; Arola *et al.*, 2012).

Ultimate tensile strength (UTS)

Ultimate tensile strength is another property of dentine in which the inorganic part of dentine (hydroxyapatite crystals) is responsible for this property (Marshall G. W., 1993). In addition, its value depends on tubular orientation, so it is not isotropic (Wang and Weiner, 1998a; Kinney *et al.*, 1999; Arola *et al.*, 2012). Spatial variation in strength is also influenced by tubular density, as in some studies it was shown that coronal dentine near to the pulp was weaker than dentine near the enamel (Carvalho *et al.*, 2001; Inoue *et al.*, 2003; Giannini *et al.*, 2004). In addition, regional variation could have an effect on the UTS of dentine. For example, a study showed that the middle third of root dentine had a significantly higher UTS than the cervical third of root dentine (Mannocci *et al.*, 2004). Age of teeth could also result in reduction of UTS of dentine (Arola *et al.*, 2012). Furthermore, studies done on human and bovine teeth found that the tensile strength of radicular dentine was higher than coronal dentine (Sano *et al.*, 1994; Inoue *et al.*, 2002).

A wide range of UTS values has been reported; 40 GPa, 58 GPa and 65 GPa, which was attributed to differences in the cross sectional diameter of specimens selected in these studies (Carvalho *et al.*, 2001; Lertchirakarn *et al.*, 2001; Inoue *et al.*, 2003). In addition, it was shown that the value of UTS of fully demineralised dentine reduced to 30% of the fully mineralised dentine (Sano *et al.*, 1994).

Ultimate tensile strength of a tooth is the maximum stress that this tissue can withstand before failing or breaking (Lertchirakarn *et al.*, 2001). Marshall (1993) suggested that the inorganic part of dentine (hydroxyapatite crystals) was responsible for this property (Marshall G. W., 1993). Studies have shown that strength depends on tubular orientation, while hardness and elastic properties of dentine are isotropic (Wang and Weiner, 1998a; Kinney *et al.*, 1999; Carvalho *et al.*, 2001; Arola *et al.*, 2012). Therefore, if the dentinal tubules are aligned with the direction of loading, so they are parallel to the direction of maximum tensile strength, and the strength is significantly lower (Lertchirakarn *et al.*, 2001). If on the other hand they are perpendicular, then they are perpendicular to the direction of maximum tensile stress and the strength is significantly higher (Lertchirakarn *et al.*, 2001).

Toughness

Fracture toughness of dentine is another property which represents the ability of a material to resist fracture caused by extension of an existing crack (Arola *et al.*, 2012), and its value depends on the orientation of dentinal tubules and tubular density (Rasmussen *et al.*, 1976; Rasmussen, 1984). Therefore, different values of fracture toughness have been reported in the literature. For example, it was found that it was approximately 2.1 MPa $m^{0.5}$ for coronal dentine and 4.8 MPa $m^{0.5}$ for radicular dentine (Wang, 2005), while another study found that fracture toughness of coronal or radicular dentine was similar, approximately 1.79 MPa $m^{0.5}$ (Imbeni *et al.*, 2003). However this value was approximately 42% lower than the value reported elsewhere in the literature (approximately 3.08 MPa $m^{0.5}$), and recognising the confounding effects of the angulation of fracture in relation to the direction of dentinal tubules (Imbeni *et al.*, 2003). The fracture toughness of dentine could also be changed with aging of teeth due to deposition of mineral in dentinal tubules which increases the peritubular dentine thickness and relative mineral content (Arola *et al.*, 2012).

Testing devices that have been employed to study the physical properties of dentine specimens will next be considered.

2.7 **Devices for analysing dentine**

Several techniques have been used to analyse dentine.

2.7.1 SEM

Scanning electron microscopy (SEM) has been widely applied for structural and histological analysis of dentine (Kessler *et al.*, 1983; Stamos *et al.*, 1987; Bolanos *et al.*, 1988; Loushine *et al.*, 1989; Hülsmann *et al.*, 1997; Teixeira *et al.*, 2005). SEM is particularly valuable for qualitative, but not quantitative analysis of dentine surfaces (Radmacher *et al.*, 1992; El Feninat *et al.*, 2001a; El Feninat *et al.*, 2001b; Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b; Nirmala and Subba Reddy, 2011).

The SEM is a basic type of electron microscope which is used for Ultrastructural investigation of a sample. It works under vacuum by hitting a specimen with an electron beam to form an image and it focuses the electron beam and magnifies the image of the specimen using electromagnetic lenses, from approximately 10 to 100,000 times. It depends in its work on wavelength of 0.005 nm and an accelerating voltage of 50 kV. When the accelerating voltage is increased, the velocity of electrons is increased, the wavelength decreased and the resolving power is increased. The image of the specimen is formed from the secondary electron after hitting of the specimen, and it provides a three-dimensional image (Stadtländer, 2007).

Advantages

SEM has been used to observe the detailed structure of dentine collagen (Arsenault, 1989; Lin *et al.*, 1993; Perdigão *et al.*, 1999). In addition, the cleanliness of the root canal walls has been investigated after root canal debridement (Salman *et al.*, 2010). The interface between the root canal materials and dentine has also been analysed to evaluate marginal adaptation of different root canal filling materials onto dentine surfaces (Upadhyay *et al.*, 2011) and to evaluate microleakage (Xavier *et al.*, 2005). The resin dentine interface zone was also been evaluated by the SEM to evaluate the efficacy of bonding of different adhesive techniques onto the root canal walls (Ferrari *et al.*, 2001). Furthermore, the SEM also has been used to evaluate different techniques

used for removal of mechanically-created smear layer on horizontally cut root dentine (Shellis and Curtis, 2010).

Disadvantages

The SEM can analyse conductive surfaces, but not non-conductive surfaces (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b), and it cannot monitor dynamic processes (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b). In addition, it cannot analyse biological samples in a liquid environment (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b). An examination of the sample with SEM depends on a vacuum, which can result in the drying of the sample and structure alterations (Reedy *et al.*, 1983; El Feninat *et al.*, 2001a; Donald, 2003; Daskalova *et al.*, 2010).

Imaging of the sample by the SEM can only be undertaken once, due to the fact that the sample requires physical and chemical fixation, and a surface coating. In addition, during the steps of samples preparation for the SEM there is a need to dehydrate specimens with critical point dryer before the surface coating, so artefacts can result from the dehydration of the sample (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b).

2.7.2 EDAX

Energy dispersive x-ray analysis (EDAX) is a method which combines with SEM to allow quantitative analysis of chemical elements on the surface of the specimen (Dogan and Çalt, 2001; Ayo-Yusuf *et al.*, 2005).

In this technique, electrons produced by the probe of the EDAX machine collide with electrons of the elements of the specimen. An electron in a specific shell of the constituent elements of the specimen, for example in level K, L or M, is ejected from its shell, when the energy of the incident electron is high enough to eject them. The number of ejected electrons depends on the atomic number of the target element, which could have shells or subshells and result in larger numbers of x-rays released (Ingram *et al.*, 1999).

The ejected electron leaves a space which is replaced by an electron from a higher shell, according to the quantum theory. This transition results in an x-ray with specific frequency. For example, in Ca two x-ray transitions occur in which 2 electrons are

transmitted to level K, one from level L and the other one from level M. The x-rays produced are Kα and Kβ respectively (Figure 2.15). The wavelength or the energy of the characteristic x-ray for each element is detected by a special detector. In energy dispersive –x-ray analysis (EDAX) the energy of the x-ray is detected and measured and an energy dispersive spectrum of elements within the specimen is produced, with specific peaks (Ingram *et al.*, 1999).

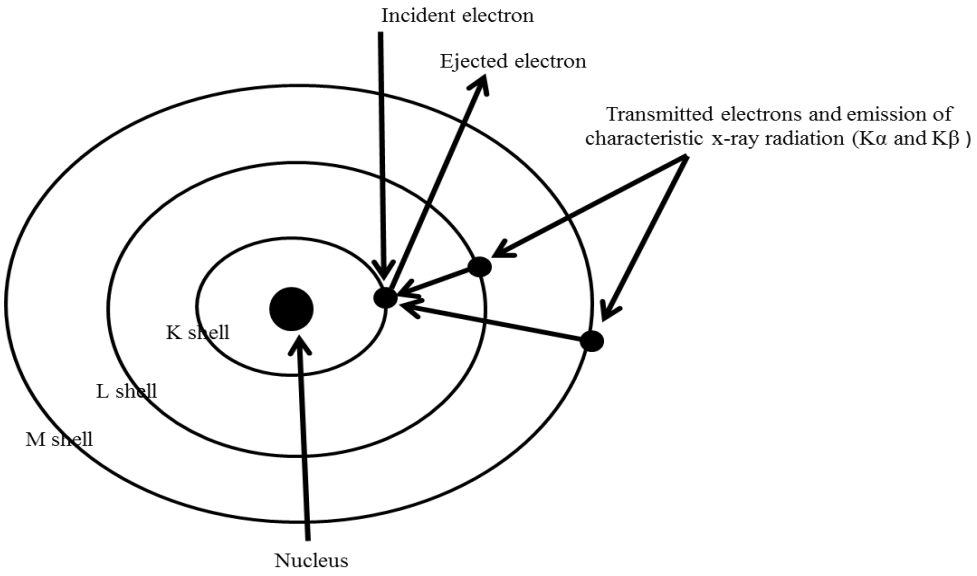

Figure 2.15. A simplified diagram showing the production of x-ray radiation by collision of incident electrons with those of the inner shells of an atom.

Advantages

EDAX is an SEM based technique. It is a relatively rapid and it does not require chemical preparation as for SEM (Ayo-Yusuf *et al.*, 2005). Disadvantages of traditional methods of element analysis, such as chemical analysis, include the destruction of solid materials such as bone or tooth in order to analyse its elemental content, and large volumes of material are often needed to analyse its elements separately (Akesson *et al.*, 1994). EDAX can be applied to small specimens, without first dissolving them, and can instantly measure the quantity of elements in a solid material (Akesson *et al.*, 1994). For example, it has been used to quantify the amount of Ca and P in dentine (Daley *et al.*, 1990; Dogan and Çalt, 2001) and bone (Obrant and Odselius, 1985), and the amount of tin in dentine and enamel (Schlueter *et al.*, 2009; Ganss *et al.*, 2010).

Disadvantages

The EDAX machine works under low-vacuum, and examination of the sample could result in drying of the sample and structural alterations, because the analysis is conducted under vacuum conditions (Donald, 2003; Daskalova *et al.*, 2010).

2.7.3 ESEM

Environmental scanning electron microscopy (ESEM) is able to carry out the same analysis as the EDAX and can make an image of the surface in exactly the same way as the SEM. It can combine both the work of the EDAX and the SEM (Gilbert and Doherty, 1993; de Wet *et al.*, 2000; Manero *et al.*, 2003; Earl *et al.*, 2011).

Advantages

This method is also relatively rapid and non-destructive in comparison to the SEM and it does not require chemical preparation and dehydration of the sample (Gilbert and Doherty, 1993; de Wet *et al.*, 2000; Manero *et al.*, 2003; Earl *et al.*, 2011).

Disadvantages

Under vacuum of the ESEM machine, drying of the sample could happen and this could result in structure alterations of the sample.

2.7.4 TEM

Transmission electron microscopy (TEM) has been used to observe the morphology of dentine (Van Meerbeek *et al.*, 1993a; Habelitz *et al.*, 2002a; Hashimoto *et al.*, 2003b; Nalla *et al.*, 2005). It is a basic type of electron microscope which can be used for ultrastructural investigation of a sample. It transmits electrons through ultra-thin slices of material and the electrons interact with the slice to form two-dimensional images which are formed from the transmitted, deflected, backscattered and secondary electrons and emitted photons, when the primary beam hits the slice. In addition, it can magnify the image of the specimen from approximately 500 to 500,000 times (Stadtländer, 2007).

Advantages

The TEM can image the sample with higher magnification and higher resolution than the SEM (Hashimoto *et al.*, 2003a; Stadtländer, 2007). The TEM has been used to evaluate the quality of the sealing efficacy of the root canal by different types of root canal filling materials (Tay *et al.*, 2005). In addition, the morphology and structure of biofilm cells has been analysed by the TEM (Ran *et al.*, 2013). Furthermore, the TEM has been used to observe the microstructure of dentine at nanostructure scale and the structural features of collagen fibrils in high resolution (Van Meerbeek *et al.*, 1993a; Habelitz *et al.*, 2002a; Nalla *et al.*, 2005). Vitrified ice sections were used to solve the problem of collapse of collagen fibrils which could happen from the dehydration of the specimen (Beniash *et al.*, 2000). In addition, a focused ion-beam method (FIB) has been used instead of the microtomy method to prepare slices from a sample to be analysed by the TEM, since the FIB method reduces the artificial damage on the sample and allows more precise preparation site than the microtomy (Nalla *et al.*, 2005).

The FIB method uses a beam of ions of sufficient energy to prepare slices from a sample, while the microtomy method prepares electron-transparent slices from mineralised tissues by mechanical sectioning with diamond knife (Hashimoto *et al.*, 2003a; Nalla *et al.*, 2005). In addition, metallic stain-coating is not needed for the sample to be analysed by the TEM (Hashimoto *et al.*, 2003a).

Disadvantages

It needs a complicated procedure of sample preparation by partial decalcification of the sample with aqueous EDTA solution and it often needs staining to resolve the zones of the gap and overlap of collagen molecules (Van Meerbeek *et al.*, 1993a; Habelitz *et al.*, 2002a; Nalla *et al.*, 2005). In addition, artefacts could result from the dehydration of the sample during it preparation for the TEM (Dechichi *et al.*, 2007). Artificial damage to the sample could result from the use of the microtomy method (Cairney and Munroe, 2003; Nalla *et al.*, 2005).

2.7.5 CLSM

Confocal laser scanning microscopy (CLSM) can be used to analyse the surface of dentine (Kimura *et al.*, 2000; Bitter *et al.*, 2004).

Advantages

The main advantage is that the sample does not need to be dry and it can be left humid during the analysis. Consequently, artefacts will not happen from sample shrinkage (Bitter *et al.*, 2004).

Disadvantages

The disadvantage of the CLSM is that it cannot record the detailed structure of the surface of the specimen.

2.7.6 Microhardness tester

Microhardness tester can be used to analyse the microhardness of dentine and the 2 common types of microhardness are Knoop hardness number (KHN) and Vickers hardness number (VHN) (Craig and Peyton, 1958; Gutiérrez-Salazar and Reyes-Gasga, 2003).

Advantages

This method can be used to assess the microhardness of a structure before and after treatment (Saleh and Ettman, 1999), so the data canal be analysed on the same specimen with less variable.

Disadvantages

The microindentation measurements of dentine such as the hardness are changed according to the site of measurement between enamel and pulp and according to the density of dentinal tubules. Therefore, it was not possible to determine if this change was due to the density of dentinal tubules or the difference in material properties of dentine constituents (Craig *et al.*, 1959; Fusayama and Maeda, 1969; Pashley *et al.*, 1985; Kinney *et al.*, 1996a). In addition, the size of the microindenter is large in relation to the microstructure of dentine and therefore, it measures the average value of this complex hydrated biological composite structure (Pashley *et al.*, 1985; Marshall *et al.*, 1997). Unfortunately, a similar problem was encountered in the measurement of elastic properties of dentine such as Young's modulus and as a result, gives way to stress. The hardness is related to these two mechanical properties (Kinney *et al.*, 1996a; Marshall *et al.*, 1997). Regrettably, the values provided by the literature vary widely and it is

unknown whether this is related to differences in the local structure or a variation in the testing methods that were used (Marshall *et al.*, 1997).

2.7.7 Nanoindenter

Nanoindentation has been used as common technique for investigation of localised mechanical properties of biological hard tissues (Kinney *et al.*, 1996a; Rho *et al.*, 1997; Zysset *et al.*, 1999; Habelitz *et al.*, 2001).

Nanoindentation technique can measure mechanical properties of tooth structure such as nanohardness and elastic modulus (Willems *et al.*, 1993; Rho *et al.*, 1997; Zysset *et al.*, 1999; Cuy *et al.*, 2002; Habelitz *et al.*, 2002b; Ge *et al.*, 2005; Oyen, 2006; Low *et al.*, 2008).

Advantages

Nanoindentation can analyse the specimen without influence from specimen morphology and tubular orientation (Kinney *et al.*, 1996a; Rho *et al.*, 1997; Zysset *et al.*, 1999; Habelitz *et al.*, 2001). It can measure the mechanical properties of a specimen and is assumed to be representative of all the specimens, because the machine analyses less than 1μm thickness on the surface of the specimen (Habelitz *et al.*, 2002b)**.** In addition, the nanoindentation measurement can be carried out on enamel and intertubular dentine (Van Meerbeek *et al.*, 1993b; Willems *et al.*, 1993), which uses smaller indenting styli, so it can analyse the mechanical properties of a material with a smaller applied load and a finer spatial resolution such as Young's modulus can be obtained by it (Doerner and Nix, 1986).

Disadvantages

This technique could change the surface of the sample, because the analysing tip could induce change in the surface during the measurement so the sample could be measured only one time (Angker and Swain, 2006). Consideration has to be given to the fact that any change in the surface layer of mineralised tissue due to the storage medium can influence the results of the specimen analysis (Habelitz *et al.*, 2002b), although this is a general problem for the storage of specimens before analysis (Strawn *et al.*, 1996).

2.7.8 SPM and AFM

Scanning probe microscopy (SPM) and atomic force microscopy (AFM) (Radmacher *et al.*, 1992; Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b) can measure the mechanical properties of dentine such as tensile and flexural tests and micro-indentation and nano-indentation (micro to nanometre scales) (Hodge and McKay, 1933; Craig and Peyton, 1958; Rasmussen *et al.*, 1976; Fox, 1980; Waters, 1980; Rasmussen and Patchin, 1984; El Mowafy and Watts, 1986; Lin and Douglas, 1994; Kinney *et al.*, 1996b; Meredith *et al.*, 1996; Wang and Weiner, 1998b; Xu *et al.*, 1998).

Both AFM and the SPM belong to the same scanning probe microscopy family of instruments. The scanning probe microscope (SPM) is used to take an image of surface structures at nanoscale resolution including biological tissues such as dentine (Radmacher *et al.*, 1992; Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b).

Advantages

The AFM can image the same sample several times and as a result, it is a non-destructive method (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b). In addition, it can analyse material properties such as roughness and elasticity of hydrated dentine and changes in these properties both before and after treatment as well as the changes during storage of the dentine. AFM can be used to analyse both conductive and non-conductive surfaces (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b).

The AFM provides a topographical 3-dimensional image of the sample with a minimum 0.0001µm vertical resolution and 0.0001µm lateral resolutions. The AFM can supply a real time image, and therefore, it can monitor dynamic processes, and it can monitor biological samples *in vitro*. Furthermore, it can analyse biological samples in a liquid environment (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b).

An examination of the sample with the AFM does not need vacuum conditions (Donald, 2003; Daskalova *et al.*, 2010). In addition, there is no need to dehydrate specimens for AFM analysis. So, artefacts can be reduced when the AFM is used (Kubinek *et al.*, 2007a; Kubinek *et al.*, 2007b).

AFM has been used widely in the last few decades to analyse mineralised tissues including dentine (Marshall *et al.*, 2001c). The changes in their structure caused by

different materials such as demineralizing agents can be analysed in real time either in a liquid or dry environment (Marshall Jr *et al.*, 1993; Marshall *et al.*, 1995; Marshall Jr *et al.*, 1997; El Feninat *et al.*, 1998; Marshall *et al.*, 1998; Silikas *et al.*, 1999). Furthermore, it has been used to analyse the nanomechanical properties of normal dentine in addition to demineralised dentine (Kinney *et al.*, 1996a; Kinney *et al.*, 1996b; Balooch *et al.*, 1998; Marshall *et al.*, 2001a). In addition, it has been used to analyse NaOCl deproteinization of dentine (Perdigão *et al.*, 1999).

Disadvantages

Distortion or artefacts of the image could result from air bubbles, contaminants within the liquid cell or movement of the sample (Finke *et al.*, 2000; Marshall *et al.*, 2001b; Marshall *et al.*, 2004).

2.8 Evidence for damage to dentine

Generally, chemomechanical treatment of teeth could affect longevity of the tooth structure after doing root canal treatment, since both of the mechanical and chemical treatment together could interact cumulatively to increase the possibility of a tooth fracture by damaging of dentine.

2.8.1 Evidence for damage to dentine by root canal treatment procedures

The reduction of dentine volume caused by endodontic access and canal enlargement may reduce the strength of the tooth and resulting in a tooth that may be less resistant to applied forces and susceptible to strain or facture (Hansen and Asmussen, 1979; Howe and McKendry, 1990; Pantvisai and Messer, 1995; Wilcox *et al.*, 1997; Gulabivala *et al.*, 2000; Regan *et al.*, 2000; Marchi *et al.*, 2008). Clinical studies reinforce such views (Cavel *et al.*, 1985; Hansen and Asmussen, 1990). By contrast, Reeh *et al* (1989) found different results, since they found that endodontic procedures; access preparation, instrumentation and root canal obturation resulted in small effects on tooth stiffness (approximately 5% reduction) in comparison with non-treated teeth (Reeh *et al.*, 1989).

Controversy persists, whilst the science of measurements for the teeth is improving and methods of analysis influence results (Kinney JH *et al.*, 2003). It could be concluded that preservation of internal tooth structure is relevant to the preservation of fracture resistance (Trabert *et al.*, 1978; Tjan and Whang, 1985).

2.8.2 Evidence for chemical damage to dentine by irrigant solutions

The irrigation of root canals with water or normal saline does not have an effect upon the mechanical properties of dentine and does not make a significant chemical change to the root dentine surface (Grigoratos *et al.*, 2001; Sim *et al.*, 2001; Driscoll *et al.*, 2002). However, these solutions are equally unlikely to play an active role in the dissolution of pulp debris or killing microorganisms (Okino *et al.*, 2004; Ring *et al.*, 2008). The same may not be true for irrigation with active agents such as NaOCl and EDTA, though the topic remains controversial (Arola *et al.*, 2012).

For example, when NaOCl solution was used alone at 3% and 5%, the modulus of elasticity and flexural strength of dentine specimens were reduced (Grigoratos *et al.*, 2001). A repeated observation was found following exposure to 5.25% NaOCl (Sim *et al.*, 2001), although a study found that the elastic modulus of dentine was unaffected following treatment with 2.5% NaOCl for 24 minutes, in which only the flexural strength of dentine was reduced significantly (Marending *et al.*, 2007a; Marending *et al.*, 2007b).

Reduction in dentine microhardness was also noticed after irrigation with 5% NaOCl for 2 minutes (Toledano *et al.*, 2005), with 2.5% and 6% NaOCl for more than 10 minutes (Slutzky-Goldberg *et al.*, 2004), with 2.5 % NaOCl and 5.25% NaOCl for 15 minutes (Ari *et al.*, 2004), and with 5% NaOCl for the very long period of 2 days (Fuentes *et al.*, 2004). It was also noted that the effect of higher concentration of NaOCl such as 6% on the microhardness of dentine was greater than the lower concentration such as 2.5% NaOCl (Slutzky-Goldberg *et al.*, 2004). The tensile strength was also reduced significantly when dentine specimens were immersed in 5% NaOCl for 2 days (Fuentes *et al.*, 2004). Not all of these observations are clinically relevant.

Hu *et al* (2010) recommended using 0.5 % NaOCl for root canal irrigation, because they analysed human dentine by Fourier transform infrared spectroscopy technique (ATR-

FTIR) and found that prolonged use of the 0.5 % NaOCl had less damaging deproteinating effects on dentine than higher concentrations (Hu *et al.*, 2010).

The EDTA was used alone to treat root dentine at 17% concentration for 1 minute (Saleh and Ettman, 1999), for 5 minutes (Sayin *et al.*, 2007), and for 15 minutes (Ari *et al.*, 2004), and it was found that the microhardness of root dentine was reduced significantly. Similar results have been observed following exposure of dentine to 15% EDTA for 5 minutes (Cruz-Filho *et al.*, 2011). It was also observed that both of the microhardness of dentine and the tensile strength were reduced significantly when dentine was treated with 0.5 M EDTA (pH 7.0) for 5 days (Fuentes *et al.*, 2004), although a study used 17% EDTA for 5 sequential irrigation periods of 30 minutes on dentine specimens and found no significant changes in dentine surface strain (Rajasingham *et al.*, 2010).

When citric acid was used alone to treat root dentine at different pHs (0.8, 1.1 1.3, 1.5 and 1.7), it was found that there was a significant loss in calcium and phosphorous. It was also observed that the demineralizing efficacy of pH 0.8 and pH 1.7 citric acid was similar, but the efficacy of both of them was less than with pH 1.1 citric acid (Hennequin *et al.*, 1994). Applying citric acid at pH 1.0 to external root surfaces also liberated Ca and P, but not Mg, and this loss varied significantly between different depths of dentine from the external root surface (Hennequin and Douillard, 1995). When 10% citric acid was applied for 5 minutes, it resulted in a significant reduction in microhardness of dentine (Cruz-Filho *et al.*, 2011).

Alternating use of NaOCl and chelating agent results in more effect on dentine than using an irrigant alone (Rajasingham *et al.*, 2010), though this again may be controversial, with others showing that 5% NaOCl alone increased the strain of dentine significantly more than when its use was alternated with 17% EDTA (Sobhani *et al.*, 2010).

It remains uncertain whether the benefits and risks of combined irrigation outweigh those of utilising NaOCl alone.

Limitations

There is no ideal root canal irrigant (Haapasalo *et al.*, 2010). In teeth with open apices, the balance between canal cleaning and safety is very important. As a result, there is a need for a novel regime to remove substrate to clean the root canals and control infection, whilst minimising the risk to the patient (Al-Kilani *et al.*, 2003). In addition, some of the investigations on biomechanical properties of dentine claimed that endodontic treatment did not make teeth brittle (Huang *et al.*, 1992; Sedgley and Messer, 1992).

All of these findings are contradictory or equivocal and till now there is no definitive proof for mechanical weakening of dentine by irrigants (Sim *et al.*, 2001; Gulabivala *et al.*, 2005). These contradictory findings could also result from other variables such the period of time teeth left after removing their pulps and until receiving treatment and also age of the teeth (Arola *et al.*, 2012). In addition, the type of teeth tested in these studies could be different and also the beams of dentine bathed in solution may behave differently from dentine adjacent to irrigated canals. There are many unanswered questions.

2.9 Methods of scoring the cleanliness of root canal

The effects of an intervention can be evaluated by the application of a scoring scale. In some circumstances, the intervention can be measured objectively on a continuous scale, such as changes in length, mass, or volume. In other circumstances, qualitative, categorical parameters are employed.

The effects of root canal instrumentation and irrigation have commonly been scored on categorical, qualitative scales, viewing SEM images to assess cleanliness and presence of smear layer. A variety of methods are available based on 3 (Turek and Langeland, 1982; Goldberg *et al.*, 1988; Ciucchi *et al.*, 1989; Walker and del Rio, 1991; Bertrand *et al.*, 1999; Torabinejad *et al.*, 2003a; Torabinejad *et al.*, 2003b; Lui *et al.*, 2007; Blank-Gonçalves *et al.*, 2011), 4 (Ahmad *et al.*, 1987b; Ahmad *et al.*, 1987a; Ahmad *et al.*, 1988; Baker *et al.*, 1988; Haikel and Allemann, 1988; Keir *et al.*, 1990; Prati *et al.*, 1994; Wu and Wesselink, 1995), 5 (Lumley *et al.*, 1992; Hülsmann *et al.*, 1997;

Hulsmann *et al.*, 1999; Hülsmann *et al.*, 2003a) or 7 categories (Cheung and Stock, 1993).

Three category scoring systems

The 3 category system used by Bertrand *et al.* (1999) and Ciucchi *et al.* (1989) (Ciucchi *et al.*, 1989; Bertrand *et al.*, 1999) for smear layer assessment is as follows:

0: no visible tubule orifices, surface rated totally smeared.

5: scattered open tubule orifices, surface rated partially free of smear layer.

10: regularly distributed open tubule orifices.

The 3 category system used by Torabinejad *et al* (2003) (Torabinejad *et al.*, 2003a), for smear layer is:

1: no smear layer. No smear layer on the surface of the root canals; all tubules were clean and open.

2: moderate smear layer. No smear layer on the surface of root canal, but tubules contained debris.

3: heavy smear layer. Smear layer covered the root canal surface and the tubules.

Blank-Gonçalves *et al* (2001) scored their specimens using the 3 score system of Torabinejad *et al* (2003) (Torabinejad *et al.*, 2003a; Blank-Gonçalves *et al.*, 2011), but their scores were 0, 1 and 2 instead of 1, 2 and 3.

Torabinejad *et al* (2003) (Torabinejad *et al.*, 2003a), also applied a 3 category scale for the degree of erosion of dentinal tubules:

1: no erosion. All tubules looked normal in appearance and size.

2: moderate erosion. The peritubular dentin was eroded.

3: severe erosion. The intertubular dentin was destroyed, and tubules were connected to each other.

There is no information available about the description of 3 category systems used by Turek and Langeland (1982), Goldberg *et al* (1988), Walker and del Rio (1991) and Lui *et al* (2007) (Turek and Langeland, 1982; Goldberg *et al.*, 1988; Walker and del Rio, 1991; Lui *et al.*, 2007).

Four category scoring systems

The 4 category system used by Wu and Wesselink (1995) for residual canal debris in longitudinal grooves created on inner canal walls following a variety of irrigation regimes (Wu and Wesselink, 1995) was as follows:

0: debris did not intersect a horizontal line of a grid used on a specimen through stereomicroscope.

1: debris intersected a horizontal line, but did not intersect any vertical line.

2: debris intersected a horizontal line and one vertical line.

3: debris intersected a horizontal line and the 2 vertical lines.

The 4 category system used by Ahmad *et al* (1987) and Ahmed *et al* (1988) (Ahmad *et al.*, 1987b; Ahmad *et al.*, 1987a; Ahmad *et al.*, 1988), for canal debris was:

0: no superficial debris.

1: minimal amount debris.

2: moderate amount debris.

3: heavy amount of debris.

And for smear layer:

0: no smear layer with all tubules opened

1: little smear layer with more that 50% of the tubules opened

2: moderate smear layer with less thatn 50% of the tubules opened

3: heavy smear layer with outlines of the tubules obliterated

There is no information available about the 4 category scoring systems used by Prati *et al* (1994), Keir *et al* (1990), Baker *et al* (1988), and Haikel and Allemann (1988) (Baker *et al.*, 1988; Haikel and Allemann, 1988; Keir *et al.*, 1990; Prati *et al.*, 1994).

Five category scoring systems

The 5 category systems used by Hülsmann *et al* (1997) in their studies (Hülsmann *et al.*, 1997; Hulsmann *et al.*, 1999; Hülsmann *et al.*, 2003a), for scoring of remaining debris and smear layer were:

For remaining debris:

Score 1: Clean root canal wall with only few small debris particles.

Score 2: Few small agglomerates of debris.

Score 3: Many agglomerates of debris covering less than 50% of the root canal wall.

Score 4: Many agglomerates of debris covering more than 50% of the root canal wall.

Score 5: Many agglomerates of debris covering all or nearly all of the root canal wall.

For smear layer:

Score 1: No smear layer with open dentinal tubules.

Score 2: Small amount of smear layer and some open dentinal tubules.

Score 3: Homogenous smear layer covering the root canal wall and only few open dentinal tubules.

Score 4: Homogenous smear layer that covers the entire root canal wall and no open dentinal tubules.

Score 5: Heavy and non-homogenous smear layer that covers the entire root canal wall.

There is no information available about the 5 category scoring system used by Lumley *et al* (1992).

Seven category scoring systems

There is no information available about the 7 category scoring system used by Cheung and Stock (1993) (Cheung and Stock, 1993).

Important considerations in the design of a scoring system include consistency and validity, while the application of a scoring scale requires (George *et al.*, 2008):

- Selection of representative, not just interesting image fields in the images
- Consistent image magnification and focus
- Coding of images
- Blinding of examiners to control bias
- Calibration of examiners for inter and intra-examiner consistency

- Consistent viewing conditions
- Appropriate analytical techniques, depending on the nature of the data.

Therefore, to avoid any possibility of bias in the calibration of the images either it is recommended to blind the examiner (Blank-Gonçalves *et al.*, 2011), or coding of the images. To manage the possibility of disagreement between intra and inter-examiner calibration and the reliability of calibration, a consensus agreement is recommended (Blank-Gonçalves *et al.*, 2011).

A study compared the Hülsmann *et al* (1997) scoring system (Hülsmann *et al.*, 1997), and a digital imaging technique using Image Pro-Plus software (Media Cybernetics, Bethesda, MD) for smear layer removal from the apical third of root canals. Inversing the scores of Hülsmann *et al* (1997) score system was done by making the highest score as the best and the lowest score as the worst scores. Firstly, the 3 observers were calibrated with analysis by Cohen's Kappa test. After that, the SEM images were calibrated with the digital imaging technique. After scoring, it was found that the results were similar between the 2 methods (George *et al.*, 2008). It is unknown from the literature whether the Hülsmann *et al.* (1997) scoring system is applicable to instrumented and non-instrumented root canals in which surfaces have not been denuded of predentine and coated with smear layer.

2.10 **Suitability of animal dentine and dental roots for endodontic research**

It is becoming difficult in many countries to secure sufficient extracted human teeth for teaching and research (Nakamichi *et al.*, 1983; Hülsmann *et al.*, 2005). This is a problem for many areas of research, including the bonding of adhesive resins to dental tissues, and as early as 1983, investigations were being conducted to evaluate bovine teeth as an alternative to human teeth (Nakamichi *et al.*, 1983).

Using human dentine in the form of slices or pressed dentine chips may have advantages of increasing sample sizes, but variability is inevitable and *in vivo* conditions may not be modelled well (Maroli *et al.*, 1992; Schmalz *et al.*, 2001). But even with good access to human teeth, it can be difficult to secure large groups with standardised features including root canal length and width; size, position and

angulation of apical constriction and width, length, radius and location of root canal curvature. Other factors include the age of the donor, the length and nature of storage conditions, variations in the hardness of dentine at different locations of the same specimen, irregular calcifications of dentine and inclusions such as pulp stones (Hülsmann *et al.*, 2005). The potential to collect large numbers of teeth that are similar in terms of age, development status, time and conditions of extraction and storage, make animal sources such as sheep and cows attractive to researchers. But the question is whether these benefits of consistency and sample size are overshadowed by important differences in size, composition, structure and physical properties.

2.10.1 Volume of root canal

In endodontic research, the conformation and volume of the pulp space may have a significant impact on outcomes. Wide canals may, for example, be difficult to shape like human molar canals, and the volumes and dynamics of irrigant application may be very different (Macedo *et al.*, 2010). The wide bovine incisor root canal is such an example which could have larger volume of root canal space than human root. So, more available free chlorine and a higher rate of consumption of the reactant were expected in the case of bovine compared with human teeth (Macedo *et al.*, 2010).

2.10.2 Density and diameter of dentinal tubules

In human teeth, the density of dentinal tubules at the wall of the pulp chamber is reported to be approximately 45,000 tubules mm^{-2}, reducing to 29,500 tubules mm^{-2} in the middle of the dentine and 20,000 tubules mm^{-2} at the DEJ. In addition, the average lumen diameter of dentinal tubules is approximately 2.5µm at the wall of the pulp chamber, 1.2µm in the middle of the dentine and 0.9µm at the DEJ (Garberoglio and Brännström, 1976).

In animal teeth (rat, cat, dog and monkey) the density of dentinal tubules at the wall of the pulp may be as high as 50,000-90,000 tubules mm^{-2}, 37,000-50,000 tubules mm^{-2} in the middle of the dentine and 10,000-25,000 tubules mm^{-2} at the DEJ. At the junction of the coronal and the radicular parts (cervical), it is approximately 42,000 tubules mm^{-2}, while it is approximately 8000 tubules mm^{-2} on the radicular part at the pulpal side

(Forssell-Ahlberg *et al.*, 1975). In addition, the average lumen diameter of dentinal tubules is 1.7 – 2.8μm at the wall of the pulp, 1.0 – 1.3μm in the middle of the dentine and it is 0.6 – 0.9μm at the DEJ, since the tubules in deeper areas of dentine contain lower levels of peritubular dentine (Forssell-Ahlberg *et al.*, 1975). Therefore, each dentinal tubule appears as an inverted cone shaped with its base on the pulpal side (Pashley, 1991; Pashley, 1996) Figure 2.1.

Bovine teeth were also investigated and the number and diameter of dentinal tubules of the coronal (at the pulpal side of dentine and at the middle dentine) and root dentine were compared with the number and diameter of dentinal tubules of the coronal dentine of human primary and permanents teeth (Schilke *et al.*, 2000).

It was found that the density of dentinal tubules was between 20,980 tubules mm^{-2} and 17,310 tubules mm^{-2} in the coronal dentine of bovine teeth; between 24,162 tubules mm^{-2} and 18,243 tubules mm^{-2} in the coronal dentine of human primary teeth and between 21.343 tubules mm^{-2} and 18,781 tubules mm^{-2} in the coronal dentine of human permanent teeth, at the pulpal side and in the middle part of the dentine respectively. The density of dentinal tubules was between 23,738 tubules mm^{-2} and 23,760 tubules mm^{-2} in the root dentine of bovine teeth, at the pulpal side and in the middle part of dentine respectively (Schilke *et al.*, 2000).

The diameter of dentinal tubules was found to be between 3.5μm and 2.85μm in the coronal dentine of bovine teeth, between 2.82μm and 2.55μm in the coronal dentine of human primary teeth and between 2.9μm and 2.65μm in the coronal dentine of human permanent teeth at the pulpal side and in the middle part of the dentine respectively. In addition, the diameter of dentinal tubules was between 3.23μm and 3.1μm in the root dentine of bovine teeth, at the pulpal side and in the middle part of the dentine respectively (Schilke *et al.*, 2000). Unfortunately, there has been no published study about the diameter and density of dentinal tubules of ovine teeth.

Inter-species differences may raise concerns about the suitability of animal dentine for dental, including endodontic research, but the realities of sample collection mean that the use of animal teeth may be an inescapable reality. For this reason, teeth used as models for human specimens should be characterised well so their potential weaknesses are recognised and controlled for when possible.

2.10.3 Chemical and physical composition of human and animal teeth

The different chemical and physical composition of the dental hard tissues of animal and human teeth could result in different responses to experimental interventions. In addition to that, the different biological composition of human and animal dental pulps could produce different results when they are treated with root canal irrigants. For example, in a study it was suggested that the Ca/P ratio of dentine varied between rat and human teeth which could be mainly due to differences in Ca content resulting from the differences in Mg content. The Ca/P ratio of primary human dentine was 2.071; in newly erupted permanent human dentine it was 2.048; in old permanent human dentine it was 2.091; in elephant dentine it was 1.675; in dog dentine it was 1.952; in horse dentine it was 1.931; in 1 year old ditto rat it was 1.917; in guinea-pig molar it was 1.748; in guinea-pig incisor it was 1.831; in hare molar it was 1.891; in hare incisor it was 1.753; in rabbit molar it was 1.775 and in rabbit incisor it was 1.761. In addition, the wide difference in mineral constituents between teeth of different species, between different teeth of the same species, between bone and teeth of the same species and between the different tissue of the same tooth could result from variation in calcification process which is a specific cell process and not a precipitation from solutions (Murray, 1936).

Generally, the formula of hydroxyapatite crystals of human teeth is $Ca_{10}(PO4)_6(OH)_4$ with incorporation of some other trace elements which could give some information about habitat environment and dietary intake (Falla-Sotelo *et al.*, 2005). It was suggested that the exact formula for dentine is $(OH)_2Ca_6[(P_{5.6}C_{0.5})O_{24}](Ca_{2.7}Mg_{0.5}C_{0.6})$, while for enamel is $(OH)_2Ca_6[(P_{5.8}C_{0.2})O_{24}](Ca_{3.1}Mg_{0.1}C_{0.5})$ (Gruner *et al.*, 1937). Mg can replace Ca in the apatite of bone and teeth and it is equivalent to it, so the Ca/P ratio changes (Murray, 1936). In a study it was shown that Mg concentration increased in dentine as the distance from the dentinoenamel junction increased towards predentine. This study showed that Mg played an important role during mineralization (Johnson, 1972). Hydroxyapatite mineral has different isomorphous forms such as $CaF_2[Ca_3(PO4)_2]_x$ fluorapatite, $CaCl_2[Ca_3(PO4)_2]_x$ chlorapatite, $Ca(OH)_2[Ca_3(PO4)_2]_x$ hydroxyapatite, $CaCO_3[Ca_3(PO4)_2]_x$ carbonate-apatite and $CaO[Ca_3(PO4)_2]_x$ oxyapatite. In which x=3 except in $CaCO_3[Ca_3(PO4)_2]_x$ it is between 2 and 3 (Gruner *et al.*, 1937).

In a study undertaken on the circumpulpal dentine of crown and root of rat incisors, their hardness and microradiographs were analysed. In addition, the Ca, Mg and PO_4 were analysed, and as a result, found that a variation in the non-collagenous organic matrix can result in variation in these inorganic contents. For example, a relatively higher content of phosphorylated phosphoproteins in coronal dentine was associated with a higher Ca and lower Mg contents in comparison with root dentine (Steinfort *et al.*, 1990). Similar differences may occur in human and other animal teeth. If so, this may result in high variation in the Ca/P ratios or mechanical properties such as stiffness between different teeth for the same species and at different positions from root canal lumen, before or after treatment with root canal irrigants.

2.10.4 Sterilisation and storage procedures

The storage of teeth can result in microbial invasion or degeneration of organic components of the dentine, which could have an effect upon the permeability of teeth (Outhwaite *et al.*, 1976; Pashley, 1985). Furthermore, the mechanical properties of the dentine can be affected, if they are stored in solutions such as deionized water and calcium chloride buffered saline solution (Habelitz *et al.*, 2002b). In this case, the modulus of elasticity and hardness of dentine and enamel can reduce as a possible result of chemical dissolution, tissue demineralization and softening of enamel and dentine by the storage medium (Habelitz *et al.*, 2002b).

Conversely, it has been demonstrated that the disinfection of teeth and dentine slices by the use of autoclaving can prevent bacterial invasion, and this may be of special interest to those working on the permeability of dentine (Pashley, 1985), or with techniques such as cell culture, where the impact of infection can be serious (Schmalz *et al.*, 2001).

Schmalz *et al* (2001) did a preliminary study and identified that bacterial invasion could be prevented by either autoclaving or 70% ethanol sterilization (Schmalz *et al.*, 2001), although the 70% ethanol could change the chemical and optical properties of dentine (Strawn *et al.*, 1996). It was claimed that the effect of sterilization of the teeth on dentine permeability was similar for both human and bovine teeth (Schmalz *et al.*, 2001). In laboratory testing situations, there is a need to prevent biofouling of stored

teeth and to control for risks associated with blood-borne viruses, whilst minimising the risks of tissue damage. Chloramine-T solutions are the current standard.

2.10.5 Some experimental situations of using animal teeth

In recent times, bovine teeth have been the most common animal teeth to be used in many areas of dental research. They are easily obtained from animals slaughtered for human consumption, in large numbers, and in a uniform and controlled conditions (Schmalz *et al.*, 2001; Al-Kilani *et al.*, 2003; Fonseca *et al.*, 2004; Rahman *et al.*, 2005; Tanaka *et al.*, 2008; Yassen *et al.*, 2011). In addition, ethics committees agree to their use as an alternative to human teeth, although there is still some concern about data obtained from animal teeth and whether the results are transferrable to the human situation. Bovine teeth have been used in adhesion and dentine barrier tests (Nakamichi *et al.*, 1983; Saunders, 1988; Retief *et al.*, 1990; Ruse and Smith, 1991; Schmalz and Schweikl, 1994; Schilke *et al.*, 1999), in microleakage tests (Reeves *et al.*, 1995), in trace elements studies (Hamada, 1989) and in morphological characteristics studies (Schilke *et al.*, 2000). Immature ovine teeth have also been used in studies on the chemical dissolution of pulp debris from root canals (Al-Kilani *et al.*, 2003; Rahman *et al.*, 2005).

Guidelines are not currently available to help researchers choose the best animal tooth available to conduct different types of investigation. Better characterisation of ovine and bovine dentine will thus form an important part of the current thesis.

2.11 **Unanswered questions emerging from this literature review**

Although it has been suggested that 0.5%, 1%, 2.5% and 5.25% concentrations of NaOCl are efficient in removing root canal debris, it is unclear which concentration is optimal for efficient debridement whilst having minimal effects on mechanical properties of dentine (Byström and Sundqvist, 1983; Sim *et al.*, 2001; Pascon *et al.*, 2009; Hu *et al.*, 2010; Huang *et al.*, 2012).

The experiments included within this thesis will investigate the following questions:

1. Are ovine and bovine root dentine good alternatives to human dentine for endodontic research?
2. Can root dentine be analysed at different depths from the lumen of the root canal?
3. What is the effect of NaOCl at different concentrations, different exposure times and different temperatures on the cleanliness of root canals?
4. What effects do irrigation regimes with NaOCl have on the walls of dental root canals and/or at different depths from the root canal lumen, in terms of Ca/P ratios?
5. What effects do irrigation regimes with NaOCl have on the dentine of root canals at different depths from the root canal lumen, in terms of mechanical properties such as dentine stiffness?
6. What effects do chelating agents such as EDTA and citric acid have on the dentine surface of root canal walls in terms of mechanical properties such as dentine stiffness?

Chapter 3: The validation of animal teeth as a model for human teeth

3.1.1 Introduction

There is little doubt that human enamel and dentine are the most suitable choice as substrates for studies investigating the interactions of materials and treatment techniques with human dental tissues (Cecchin *et al.*, 2010). However, due to the difficulty in obtaining large, uniform and good quality samples of extracted human teeth, it has become necessary to seek alternatives, such as ovine and bovine teeth. They were investigated in several studies including tests of root canal irrigants (Al-Kilani *et al.*, 2003), tissue micro hardness (Castanho *et al.*, 2011), adhesion of composite resins to enamel (Nakamichi *et al.*, 1983), the organic and inorganic content (Tanaka *et al.*, 2008), and other physical properties (Lin and Douglas, 1994). Over the last few years, the availability and large size of bovine teeth has encouraged their use for adhesion studies on enamel and dentine (Schilke *et al.*, 2000).

Large bovine teeth present a number of advantages for laboratory research such as the ability to prepare more than one specimen from the same tooth, giving the ability to sometimes prepare both test and control specimens from the same tooth. Teeth taken from animals belonging to the same herd may in addition give uniformity in terms of similar genetic lineage and dietary exposure, with the possibility of greater homogeneity of tissues, compared to a sample of teeth taken from a diverse group of animals (including human). Other factors include the uniformity of age of teeth taken from a flock sacrificed at the slaughter house, and control over the timing of extraction and storage conditions before use. Managing all of these variables in a collection of human teeth is extremely challenging.

However, only a few studies have been done to investigate whether animal teeth are suitable substitutes for human teeth in hard tissue research (Schilke *et al.*, 2000; Fonseca *et al.*, 2004; Wegehaupt *et al.*, 2008; Lopes *et al.*, 2009). Little has been published about the structure of bovine teeth such as the morphology, density and diameter of dentinal tubules (Schilke *et al.*, 2000; Camargo *et al.*, 2008; Lopes *et al.*, 2009). One study found that the density and diameter of dentinal tubules of human and

bovine teeth were relatively similar. However, this study analysed the dentine in the circumpulpal and middle regions, but not close to the dentinoenamel junction. In addition, it analysed the coronal and root dentine of bovine incisors, but only the coronal dentine of human third molars (Schilke *et al.*, 2000). Comparisons for root dentine were therefore not possible. The study of Lopes *et al* (2009) showed that there were some structural differences between bovine and human dentine in terms of the diameter and density of dentinal tubules, but did not investigate root dentine specifically (Lopes *et al.*, 2009).

Two studies were performed using ovine teeth in order to evaluate endodontic irrigants, in which the authors stated that the collected ovine teeth were a suitable model for human teeth, but without undertaking any investigations on tissue structure and composition. Rather, the authors pointed to the large samples of uniform mammalian teeth that could be harvested in one session and held under identical storage condition until they were used. The authors also pointed to issues of cross infection control that may be less of a concern with ovine than human teeth (Al-Kilani *et al.*, 2003; Rahman *et al.*, 2005).

Therefore, the present series of studies sought to provide information on the nature of ovine and bovine dentine in order to inform researchers on their suitability and limitations as models for human teeth. The structure of human, ovine and bovine teeth was investigated in terms of the diameter and density of dentinal tubules in root dentine, the Ca/P ratio and stiffness of root dentine, and estimations were also made of relative pulp canal volume.

3.2 Diameter and density of dentinal tubules

3.2.1 Aim

To characterise whether ovine and bovine incisors are a potential model for human teeth in endodontic studies, in terms of diameter and density of dentinal tubules. This study tested the null hypotheses that there was no difference in the diameter and density of dentinal tubules in human root dentine compared with ovine and bovine root dentine.

3.2.2 Materials and methods

3.2.2.1 Teeth collection and storage

Forty two teeth (n=14) (14 human premolars, 14 ovine incisors and 14 bovine incisors) were used for this study. The ovine (from animals 2-3 years old) and bovine (from animals18-20 months old) incisors were harvested from freshly culled sheep and cows from an abattoir in Newcastle upon Tyne, while human premolar teeth (from individuals 12-14 years old) were collected from the Newcastle Dental Hospital.

The teeth were cleaned of periodontal tissues with a no 22 scalpel blade (Swann Morton, Sheffield, England) and stored in 1% chloramine-T (wt/v) (SIGMA-ALDRICH, UK) at 4˚C until they were needed.

3.2.2.2 Specimen preparation

The teeth were de-coronated with a low speed saw at 5 rpm, under constant cold water irrigation (Testbourne Model 650, South bay Technology (SBT), INC.). Their pulp tissue was grossly extirpated with a medium size barbed broach (Maillefer, Ballaigues, Switzerland), and the root canals were irrigated with 3ml distilled water using a Luer-Loc syringe with a 27-gauge Monoject endodontic needle (Monoject, Gosport, UK).

3.2.2.3 Storage of the samples and SEM preparation

The roots were then split into two halves by grooving them longitudinally with a flex diamond disc (Skilldenta, Skillbond®, Skillbond Direct Ltd Dudley House, UK) until just before the lumen and then cracking apart with a chisel.

One half of each specimen was fixed in 2% glutaraldehyde solution in Sorenson's phosphate buffer (pH 7.3) overnight for SEM analysis. After that, the specimens were dehydrated in a series of ascending concentrations of ethanol solutions: 25%, 50%, 75% each for half hour, then 100% ethanol for 1 hour, before storage in fresh 100% ethanol (25%, 50%, 75% and 100% EtOH to water 100%) and transferred into a critical point drier (Tsousimis Samori 780 CPD, Tsousimis Research Group., Rockville, Maryland, USA). After mounting the samples on aluminium stubs, they were gold coated (Polaron E5100 cool sputter coater 12nm, Hertfordshire, England) on their internal (root canal) surfaces for SEM assessment (Cambridge S240, Cambridge, England) at 8 KV accelerating voltage.

3.2.2.4 Analysis of the of the samples by the SEM

These halves were subjected to analysis by SEM at 2000x magnification and measured with ImageJ software (Abràmoff *et al.*, 2004; Schneider *et al.*, 2012) to assess the dentinal tubular diameter and density on the root canal wall. The SEM image was transferred into a computer to be analysed digitally by the ImageJ software. The scale (in µm) on the SEM image was used to transfer the units of the ImageJ from pixels into µm to measure the diameter and to determine an area with dimension of µm * µm. This area was used to calculate the number of dentinal tubules per μm^2, before transforming into tubules per mm^2, by multiplying the value in µm by 10^6.

The measurement was done throughout the length of the canal wall by assuming that there was no difference in the size and distribution of tubules between the coronal, middle and apical thirds. In this measurement, only 3 tubules per image were selected. Differences between human, ovine and bovine teeth were analysed in SPSS 19.0 (SPSS Inc, Chicago, IL).

3.2.3 Results

Three areas were scored on each sample, making intended data points of 42 for each group. However, cellular debris on a number of specimens precluded analysis, and made the number of data points variable amongst the groups (Table 1.5).

Figure 3.1, Figure 3.2 and Figure 3.3 show the structure of the canal walls of ovine, bovine and human teeth respectively. The predentine of bovine teeth was observed to be smoother than in human and ovine teeth, since the collagenous network seems to be more prominent in the human and ovine predentine. The data collected on the diameter and density of the dentinal tubules were not normally distributed (Table 3.1 and Table 3.2), and were consequently analysed using the Kruskal-Wallis test ($p<0.05$).

The median tubular densities (generally for whole root length) were 25000 tubules mm^{-2} for ovine teeth, 27500 tubules mm^{-2} for bovine teeth and 25000 tubules mm^{-2} for human teeth. The trend showed that tubular density was the lowest for ovine teeth, which was similar to human teeth, while for bovine teeth it was the highest (Table 3.3 and Figure 3.4). Analysis revealed that there were no significant differences between the tubular densities of the three species ($p>0.05$).

The median tubular diameter was 2.1µm for ovine teeth, 1.8µm for bovine teeth and 2.2µm for human teeth. The trend showed that the diameter of dentinal tubules was lowest for bovine teeth, ovine teeth were in the middle, and for human teeth it was the highest (Table 3.4 and Figure 3.5). The differences were again not significant ($p>0.05$). Therefore, both the density and diameter of dentinal tubules on the root canal walls of ovine and bovine teeth were not significantly different from each other or from human teeth.

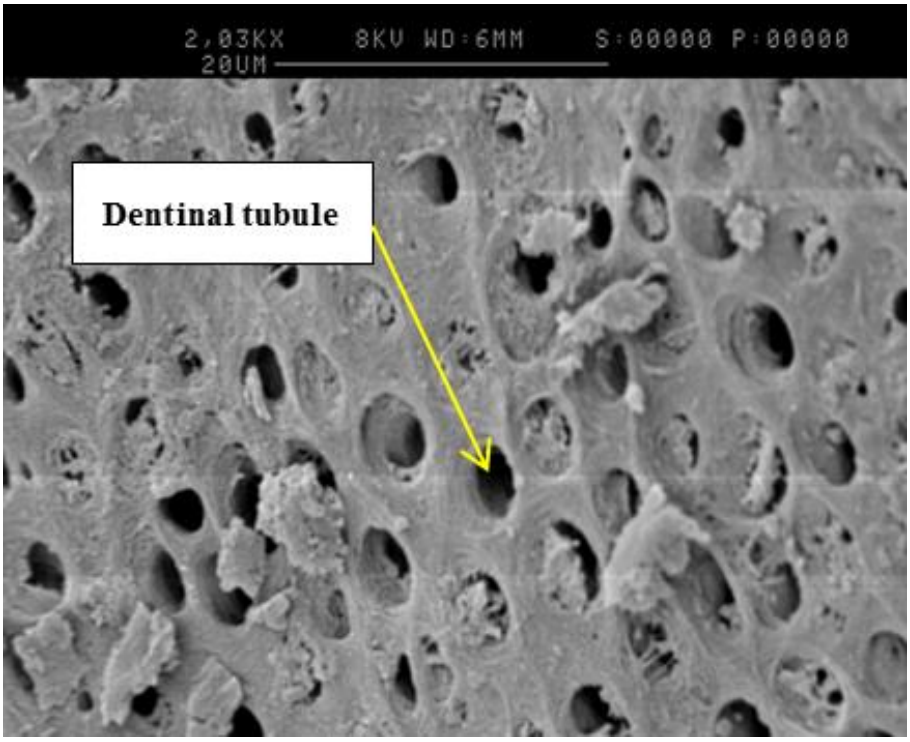

Figure 3.1. SEM image (2000x magnification) to show the structure of root canal dentine walls in the cervical third of ovine teeth in a native, non chemically-treated state.

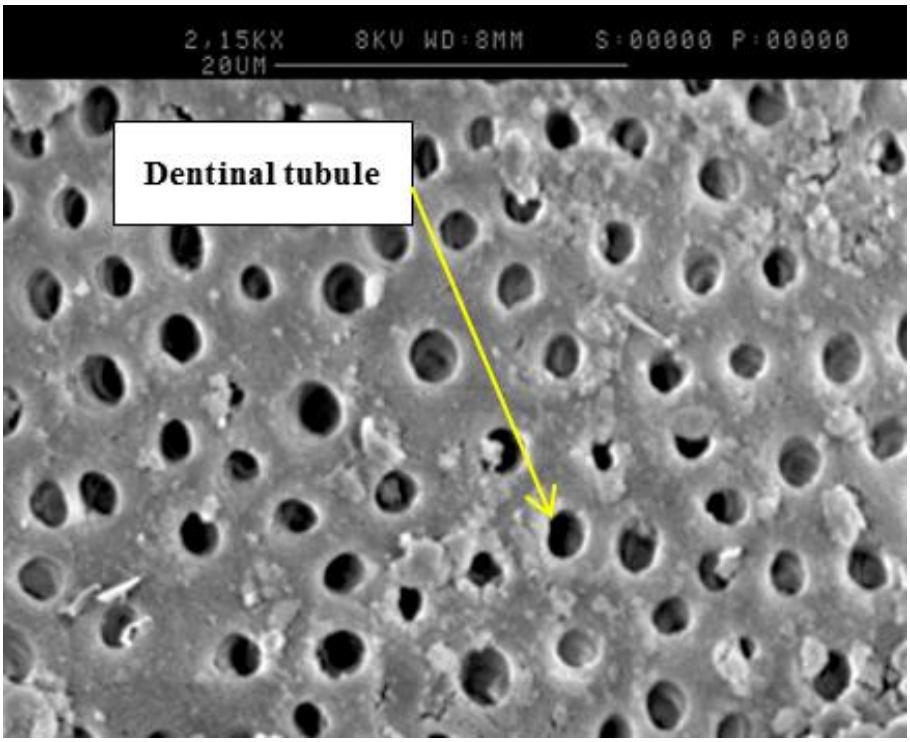

Figure 3.2. SEM image (2000x magnification) to show the structure of root canal dentine walls in the cervical third of bovine teeth in a native, non chemically-treated state.

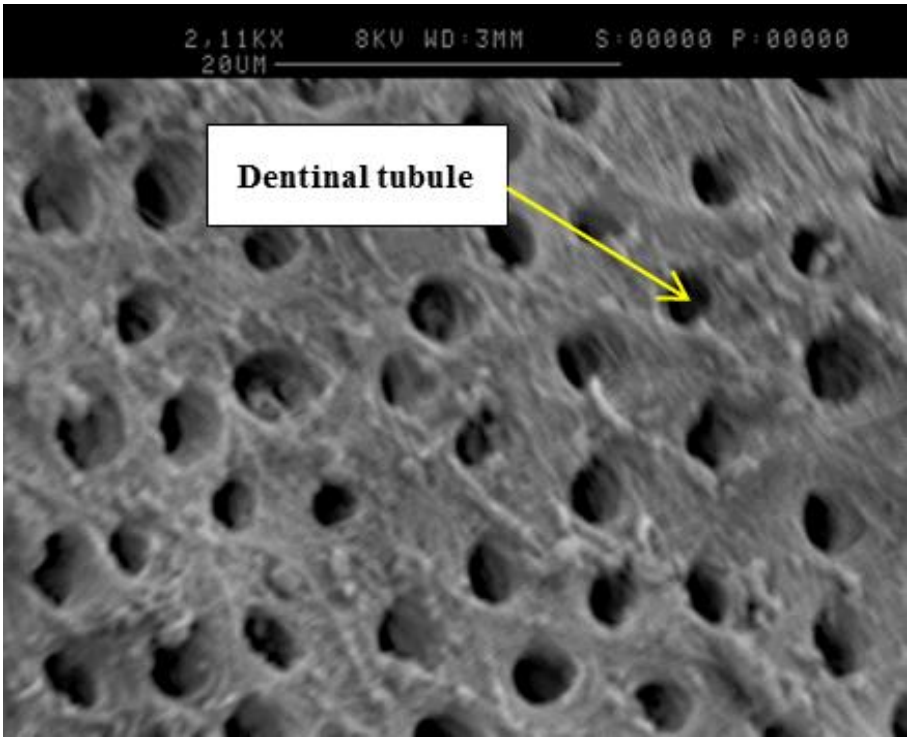

Figure 3.3. SEM image (2000x magnification) to show the structure of root canal dentine walls in the cervical third of human teeth, in a native, non chemically-treated state.

Table 3.1. Normality test of the data of dentinal tubule density (combined cervical, middle and apical thirds) in ovine, bovine and human root canals ($p<0.05$).

Tests of Normality

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Dentinal tubules density (no. of dentinal tubules/sq mm)	Ovine teeth	.186	37	.002	.933	37	.028
	Bovine teeth	.114	38	.200*	.963	38	.238
	Human teeth	.158	28	.073	.932	28	.071

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 3.2. Normality test of the data of variance dentinal tubule diameter (combined cervical, middle and apical thirds) in ovine, bovine and human root canals ($p<0.05$).

Tests of Normality

	Group	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Dentinal tubule diameter (micron)	Ovine teeth	.156	37	.023	.851	37	.000
	Bovine teeth	.119	38	.190	.944	38	.055
	Human teeth	.101	28	.200*	.936	28	.089

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table 3.3. Median (upper and lower quartiles) of the dentinal tubule density in ovine, bovine and human root canals. The difference found was not significant ($p<0.05$. Kruskal-Wallis test).

Tooth type	Number of analysed data	Median density of dentinal tubules (tubules mm^{-2})	Upper & lower quartiles
Ovine teeth	**37***	**25000**	**32500 & 20000**
Bovine teeth	**38***	**27500**	**35625 & 20000**
Human teeth	**28***	**25000**	**35000 & 2000**

* The data should include 42 samples for each group, since number of specimens was 14 (n = 14). However, the number of specimens that could be assessed was not uniform, since pulp debris remained in a number of specimens, making it impossible to see the dentine walls. So, the number of data was different in each group.

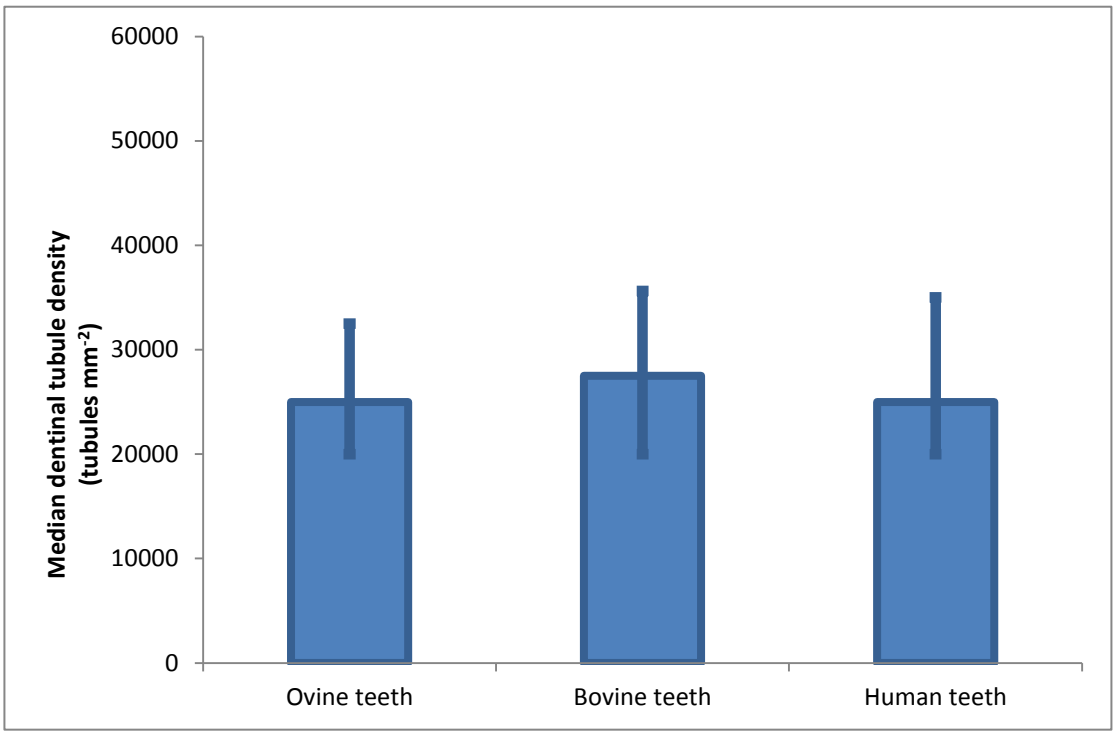

Figure 3.4. Median dentinal tubule density in ovine, bovine and human root canals. The difference found is not significant ($p<0.05$, Kruskal-Wallis test). The error bars are the upper and lower quartiles for each group.

Table 3.4. Median (upper and lower quartiles) of the dentinal tubule diameter (µm) in ovine, bovine and human root canals. The difference was not significant ($p<0.05$. Kruskal-Wallis test).

Tooth type	Number of analysed data	Median diameter of dentinal tubules (µm)	Upper & lower quartiles
Ovine teeth	**37***	**2.1**	**2.5 & 1.8**
Bovine teeth	**38***	**1.8**	**2.4 & 1.5**
Human teeth	**28***	**2.2**	**2.5 & 1.9**

* The data should include 42 samples for each group, since number of specimens was 14 (n = 14). However, the number of specimens that could be assessed was not uniform, because pulp debris remained in a number of specimens, making it impossible to see the dentine walls. So, the number of data was different in each group.

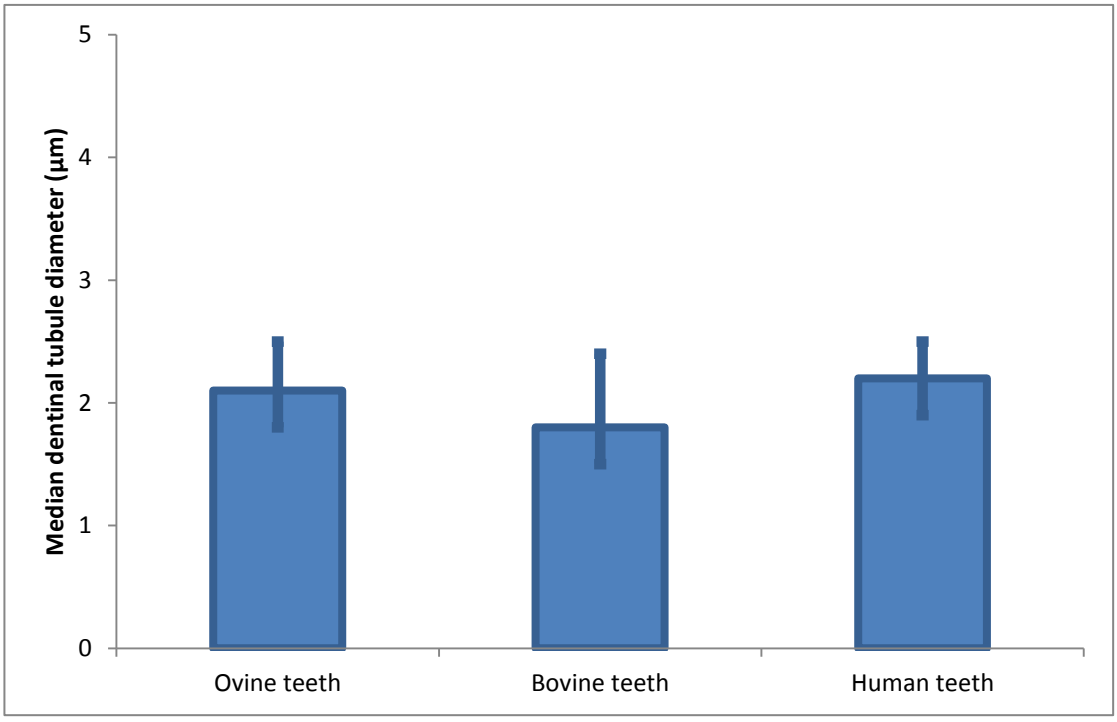

Figure 3.5. Median dentinal tubule diameter (µm) in ovine, bovine and human root canals. The difference is not significant ($p<0.05$, Kruskal-Wallis test). The error bars are the upper and lower quartiles for each group.

3.2.4 Discussion

The complexity of collecting enough human teeth in one session (Mellberg, 1992), the increase in precautionary measures to control biohazards (Rueggeberg, 1991), ethical issues (Skene, 2002) and the difficulty in ensuring uniformity (Zero, 1995) has led to the search for an alternative model to human teeth for use in dental research. Bovine teeth are commonly used as an alternative to human teeth (Yassen *et al.*, 2011), whilst few studies used ovine teeth (Al-Kilani *et al.*, 2003; Rahman *et al.*, 2005). The extraction of ovine teeth is simple and much easier than extracting bovine teeth. Therefore, both of these two types were selected to ascertain whether it is possible to increase the number of alternative species, in order to have a best model for human teeth.

Some data were lost due to difficulties in scoring root canal walls that were covered with pulpal debris. Cellular material could perhaps have been removed by further mechanical and/or chemical means, but the focus of this work was to score surfaces as close to their native state as possible. This resulted in unequal groups for analysis and limitation in the number of collected data from each group, and it is recognised that this could have an impact on the results. Therefore, the number of collected data was increased by analysing 3 areas on each specimen, in the cervical, middle and apical thirds, and assuming the diameter and the density of the dentinal tubules on the canal wall were similar at all thirds in any species. Consequently, if they changed from one third to another, it was assumed that the change would be equally represented in all species. This assumption was not systematically tested.

The results of this study showed that the diameter and the density of dentinal tubules in human root dentine were not significantly different from those in ovine and bovine teeth. Thus, based on this parameter, it would be possible to use ovine and/or bovine teeth as a model for human teeth in endodontic research. The results of this experiment concurred with those of Schilke *et al* (2000) which found that the diameter and density of dentinal tubules in bovine teeth were not significantly different from human teeth (Schilke *et al.*, 2000).

Other studies have made contradictory findings (Dutra-Correa *et al.*, 2007; Lopes *et al.*, 2009). For example, it was found that that the average diameter of dentinal tubules in

human coronal dentine was approximately 2.42µm, 2.99µm, 2.94µm at the pulpal wall, in the middle of the dentine and at the dentinoenamel junction respectively. Comparable values for bovine teeth were 4.21µm, 3.98µm and 3.14µm (Lopes *et al.*, 2009). These differences were significantly different, though it should be noted that these values concerned coronal, not root dentine. Others have shown greater differences in the diameter of dentinal tubules in bovine coronal dentine, measured as 2.71µm at the pulpal wall, 4.62 in the middle area of dentine, and 5.21µm at the dentinoenamel junction (Dutra-Correa *et al.*, 2007). This study also noted a difference in the thickness of peritubular dentine between bovine and human coronal dentine.

In addition, the average density of dentinal tubules of human teeth (22329 tubules mm^{-2}) was shown in some studies to be significantly greater than in bovine teeth (15964 tubules mm^{-2}), regardless of the region of dentine (Lopes *et al.*, 2009). Greater variation in the density of dentinal tubules in different regions of bovine coronal dentine were again noted by others, being approximately 50310 tubules mm^{-2} at the pulpal wall, 29433 tubules mm^{-2} at the middle area of dentine and 18772 tubules mm^{-2} at the dentinoenamel junction (Dutra-Correa *et al.*, 2007).

Schilke *et al* (2000) found non-significant differences when they compared the coronal parts of the human and bovine teeth. By contrast, they found significant differences in the diameter and density of dentinal tubules between bovine root dentine and human coronal dentine (Schilke *et al.*, 2000).

Unfortunately, Schilke *et al* (2000) did not compare bovine with human root dentine. In addition, their investigation was conducted after treating the dentine with EDTA and NaOCl (Schilke *et al.*, 2000). The current study avoided the application of such agents that may alter the diameter of dentinal tubules, remove predentine or demineralise the tissue.

The effects of chemical treatments are recognised. After treating dentine with 35% phosphoric acid, Dutra-Correa *et al*. noted that tubules became wider, and attributed this to the dissolution of peritubular dentine (Dutra-Correa *et al.*, 2007). This increase in tubular diameter was also found in a study comparing monkey, rat, cat and dog dentine, with the recommendation to analyse the dentine in its un-decalcified state (Forssell-

Ahlberg *et al.*, 1975). Etching dentine to expose the dentinal tubules could change the diameter of dentinal tubules (Lopes *et al.*, 2009).

Forssell-Ahlberg *et al* (1975) used plastic film to cover the SEM images to calculate the diameter and density of dentinal tubules, while in our experiment digital image analysis was undertaken with ImageJ software (Forssell-Ahlberg *et al.*, 1975). Although no systematic comparison of methods was conducted, it is anticipated that the digital method may be more accurate.

The tooth types compared in this study were not uniform. Premolars are the most readily obtained human teeth, but neither sheep nor cows possess premolars. Sheep do not possess maxillary incisors. Some caution may therefore be needed in making comparisons.

3.2.5 Conclusion

Within the limitations of this study, it could be concluded that human teeth were not significantly different from ovine and bovine in terms of tubular density and diameter in root dentine. The null hypothesis tested was therefore upheld.

3.3 **Estimation of root canal volume**

3.3.1 Aims

To characterise whether ovine and bovine mandibular incisors are a potential model for human teeth in endodontic studies, in terms of root canal volume. This study tested the null hypothesis that volume of human root canals was not significantly different from ovine and bovine root canals.

3.3.2 Materials and methods

3.3.2.1 Teeth collection and storage

See section 3.2.2 for tooth collection and storage. Fifteen teeth (5 human premolars, 5 ovine incisors and 5 bovine incisors) were included in each study. All of the teeth included had immature roots with open apices.

3.3.2.2 Specimen preparation

See section 3.2.2 for specimen preparation. All the root lengths were standardized to 13 mm by keeping the apex and sectioning the root at 13 mm from the apex.

3.3.2.3 Estimation of root canal volume by filling brim-fill with fluid

Fifteen samples (5 human premolar, 5 ovine incisors and 5 bovine incisors) were included in this study:

Group 1: ovine roots (n=5).

Group 2: bovine roots (n=5).

Group 3: human roots (n=5).

A medium size barbed broach (Maillefer, Ballaigues, Switzerland) was used to grossly remove pulp tissue. In this method, any fluid in each root canal was aspirated using a 3ml Luer-Loc syringe with a 27-gauge Monoject endodontic needle (Monoject, Gosport, UK). The root canal was then dried further with 6 paper points size 25, and the apex of the root was sealed with soft red wax (Metrodent Ltd, Huddersfield, UK) to ensure that the water did not run out of the apex. After that, the root was weighed in its empty state by digital scales (Mettler, Type AE 240, Switzerland) to 5 decimal places. Distilled water was then injected into the root canal until brim-full using a 3ml Luer-Loc syringe with a 27-gauge Monoject endodontic needle (Monoject, Gosport, UK), and the root was weighed again in the filled state by digital scales (Mettler, Type AE 240, Switzerland). This process was repeated five times for each root, and after each time the root canal was dried with 6 paper points size 25.

The volume of the pulp space was calculated by subtracting the weight of each sample empty from the same sample brim-filled with distilled water. The volume of water was calculated by dividing the mass of water by its density, assuming the density of water to be 1 g cm^{-3}. Results were analysed in SPSS 19.0 (SPSS Inc, Chicago, IL), and one-way ANOVA ($p<0.05$) was used to compare between all of the three groups (1, 2 and 3).

3.3.2.4 Estimation of root canal volume by digital imaging

Fifteen more samples (5 human premolar, 5 ovine incisors and 5 bovine incisors) were divided into 3 groups:

Group 1: ovine roots (n=5).

Group 2: bovine roots (n=5).

Group 3: human roots (n=5).

A digital x-ray was taken for each of the roots in bucco-lingual plane and estimations were made of canal shape. From the radiographic images of each type of tooth, a conical outline was proposed for the root canal space of each tooth, and the apical end of each root was ignored on each specimen to make the estimation similar (Figure 3.6).

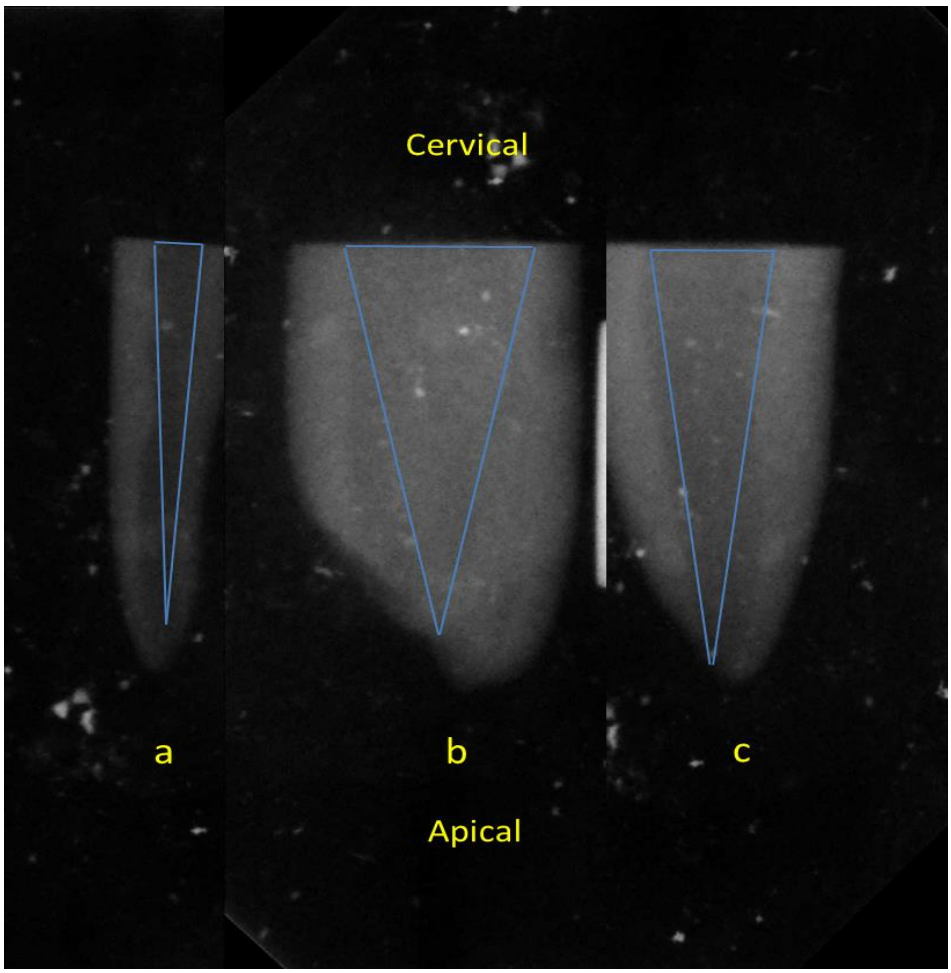

Figure 3.6. Radiographic images show the bucco-lingual dimensions of root canal of 3 species, a. ovine, b. bovine and c. human teeth, respectively. They show an imaginary outline of the root canals. All are at the same magnification.

A cervical slice with 2 mm thickness was prepared horizontally from each of these roots with a low speed saw at 5 rpm, under constant cold water irrigation (Testbourne Model 650, South bay Technology (SBT), INC.). A medium size barbed broach (Maillefer, Ballaigues, Switzerland) was used to check that the lumen of slices was cleared from any remnants of pulp debris. A plastic scale was placed at the same level with the cervical slice, to be used as a reference scale later, and then a digital camera was used to take a photograph of the specimen with the plastic scale (Figure 3.7, Figure 3.8 and Figure 3.9). Images were then analysed digitally with ImageJ software, with sensitivity of 3 decimals. The plastic scale served to transfer the units of the ImageJ from pixels into cm to measure the area of cervical sections in units of cm, so the ruler depicted is to indicate scale and was not used for the measurements. The canal volume was then estimated for each species. The volume of the root canal was calculated by measuring the area of the lumen of the cervical slice, and multiplying by the height of the root canal (13 mm) and divided by 3. So, the volume of root canal area of the lumen of the cervical slice * height of the root which was proposed as the height of a cone * 1/3, (v = area * h * 1/3).

The data were analysed in SPSS 19.00 (SPSS Inc, Chicago, IL), the comparison was done between the groups 1, 2 and 3 using the parametric one way ANOVA test ($p<0.05$).

Figure 3.7. Photographic image of a horizontal section from the cervical third of the root of an ovine tooth with a plastic scale, the ruler depicted is to indicate scale and was not used for the measurements.

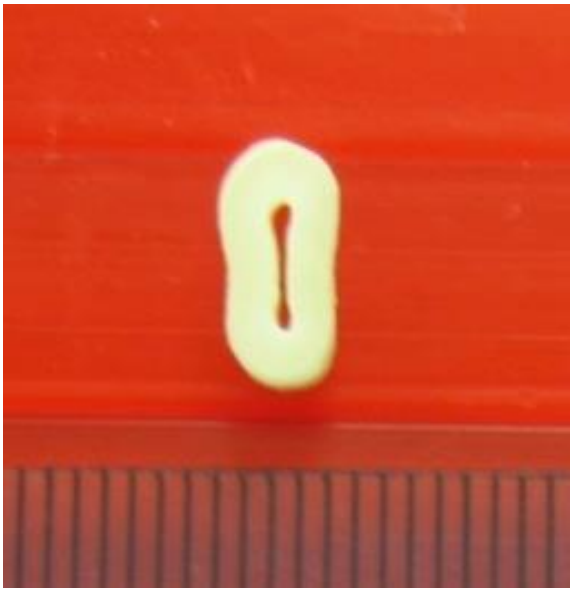

Figure 3.8. Photographic image of a horizontal section from the cervical third of the root of a bovine tooth with a plastic scale, the ruler depicted is to indicate scale and was not used for the measurements.

Figure 3.9. Photographic image of a horizontal section from the cervical third of the root of a human tooth with a plastic scale, the ruler depicted is to indicate scale and was not used for the measurements.

3.3.3 Results

3.3.3.1 Estimation of root canal volume by filling brim-fill with fluid

In this method, the data of all species were normally distributed, and were analysed using the one-way ANOVA test ($p<0.05$). The data show that the root canal volumes of human and ovine teeth were approximately half those of bovine teeth (Table 3.5. In addition, there was a significant difference found between human and bovine and between ovine and bovine teeth ($p<0.05$), while there was no significant difference between human and ovine ($p>0.05$), (Figure 3.10).

Table 3.5. Mean volume and standard deviation of ovine, bovine and human root canal spaces measured using distilled water.

Tooth type	Mean volume (SD) (mm^3)
Ovine	**9.7 (3)**
Bovine	**19.5 (5.4)**
Human	**7.8 (2.8)**

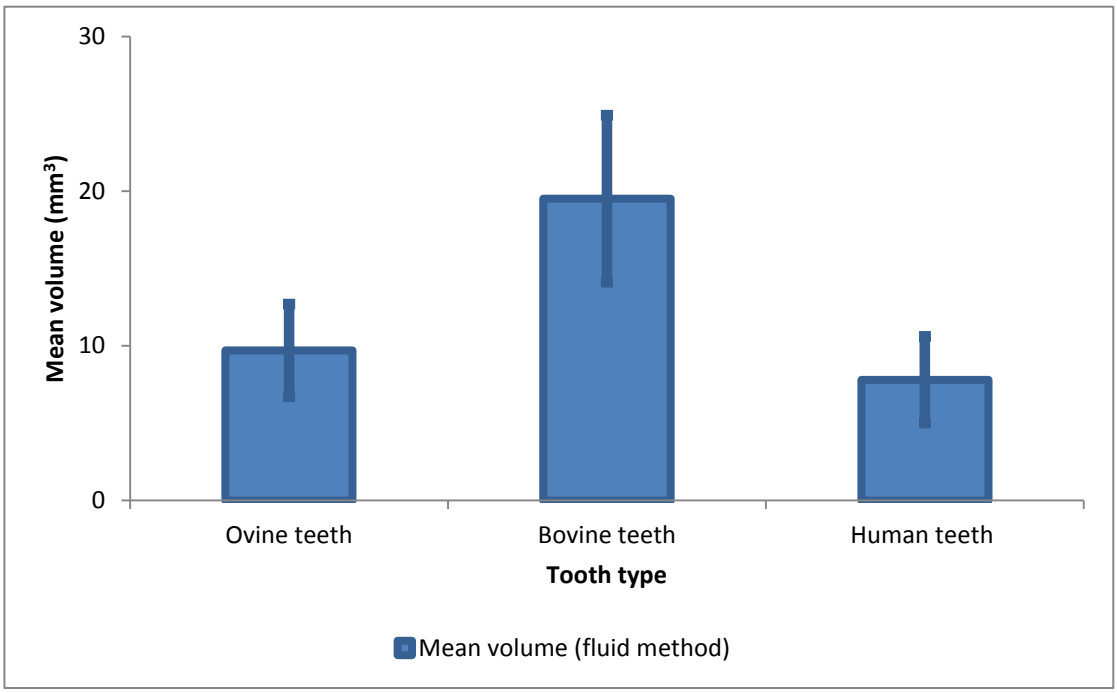

Figure 3.10. Mean volume of ovine, bovine and human root canal spaces measured using distilled water. The difference was significant ($p<0.05$, One way ANOVA). The error bars are the standard deviation values for each group. The number of samples was five for each group, and each sample was measured 5 times.

3.3.3.2 Estimation of root canal volume by digital imaging

In this experiment, there was a significant difference found in the volume of the root canal spaces between human and bovine teeth ($p<0.05$), while the differences between human and ovine and between ovine and bovine were not significant ($p>0.05$) (Table 3.6). The results did, however, show the same trends as the previous study of volumetric analysis using fluid (Figure 3.11).

The estimated volume of bovine root canal spaces (Table 1.8) was double that of human and ovine. Variation was highest in the ovine group Table 3.6.

Table 3.6. Mean estimated volume and standard deviation of ovine, bovine and human root canal spaces measured digitally using ImageJ software.

Tooth type	Mean volume (SD) (mm^3)
Ovine	**3.99 (2.9)**
Bovine	**7.2 (2.3)**
Human	**3.0 (1.0)**

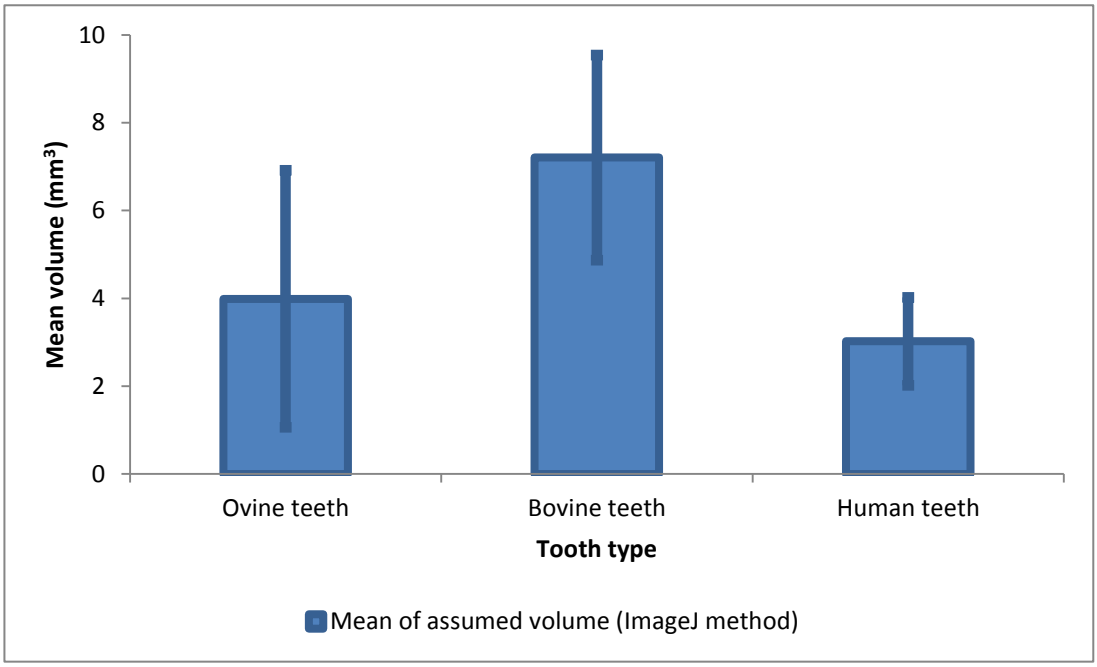

Figure 3.11. Mean assumed volume of ovine, bovine and human root canal spaces measured digitally using ImageJ software. The difference was significant ($p<0.05$, One way ANOVA). The error bars are the standard deviation values for each group.

3.3.4 Discussion

To assess whether animal teeth may serve as good models for human teeth in endodontic research, it is important to understand whether their volumes are comparable. This may be particularly relevant in studies about root canal irrigation, where canal surface area and dimension may influence the soft-tissue eliminating capacity of specified irrigant volumes, and influence the fluid dynamics caused by irrigation. Several studies have focussed on the shape and anatomical variations of human teeth, whilst only a few studies have been published on the volume of the human root canal space (Fanibunda, 1986; Madarati *et al.*, 2009; Macedo *et al.*, 2010).

Previous studies injected a silicone impression material into prepared root canals to study the morphology of the root canal (Davis *et al.*, 1972; Barker *et al.*, 1973). In both of these studies, the authors used a clear resin to reproduce a translucent replica of the teeth and then acrylic resin was injected into their pulp canal space. In a further study, a transparent resin was used as a replica of the tooth, and dye was injected into the root canal to produce a replica of the pulp space inside the clear resin (Fisher *et al.*, 1975). Furthermore, a study used Indian ink to outline the root canal morphology, before the teeth were demineralised and cleared to show variations in the configurations of root canals (Çalişkan *et al.*, 1995; Neelakantan *et al.*, 2011). One criticism of these techniques is that part of the pulpal wall was destroyed by grinding the teeth and by cutting an access cavity into the root canals. As a result, they are considered to be destructive methods (Fanibunda, 1986). Fanibunda (1986) in his method of measuring the root canal volume using the silicone impression material preserved the integrity of the pulpal wall (cutting only an access cavity without preparing the inside the root canals), whilst at the same time he produced a replica of the root canal space. However, this method could be also destructive, since the teeth were eroded by immersion in 50% hydrochloric acid. Therefore, there is a need for less destructive method to measure the volume of the root canal space. Two such methods were employed in the current work.

The first method estimated the root canal volume by injecting brim-full with fluid, while, the second method estimated root canal space volume after digital imaging. In the first method, there was a significant difference found between human and bovine and between ovine and bovine teeth, while there was no significant difference between human and ovine ($p>0.05$). In the second method, there was a significant difference

between human and bovine teeth ($p<0.05$), while between human and ovine and between ovine and bovine there was no significant difference ($p>0.05$). Neither of the methods used in this work have been reported previously, and both identified similar trends.

The imaging method made significant assumptions by ignoring any anatomical variation inside the root canal space, and did not account for the frustum-like configuration of pulp spaces. The fluid method may in some respects be more accurate, but the influence of factors including air entrapment, diffusion of fluid into dentinal tubules and incomplete canal drying cannot be fully assessed. In addition, the contact angle, surface tension and the wettability of the liquid could have an effect on the penetration of the liquid totally or partially into the root canal space (Nikiforuk and Sreebny, 1953; Tasman *et al.*, 2000).

In comparison with the study done by Fanibunda (1986), the fluid method is less destructive, allowing the experiment to be repeated more than once on the same sample. The imaging method was more destructive, requiring horizontal slices to be cut from the cervical third of the root.

The accuracy of measuring an object on a photographic image or radiograph was assessed in a pilot study by measuring diameter of a cylinder with a plastic ruler. The cylinder and a plastic scale were placed together on a table at the same height and photographed together, and the plastic scale used as a reference for image analysis software. The method was found to be reliable and consistent.

Standardising the root specimens at 13 mm eliminated some of the variation within this investigation on grossly quite dissimilarly sized teeth. Variation in the cross-sectional profile of the roots could not be so readily controlled and as Figure 3.7, Figure 3.8 and Figure 3.9 demonstrate ovine canals have a triangular cross-section, bovine have a nearly elliptical cross-section, and human have a circular cross-section. The influence of these shapes on fluid dynamics was not studied in this work.

Probably the most accurate method of estimating pulp canal volume would be with 3D imaging, but such techniques were not available.

3.3.5 Conclusion

Within the limitation of this study, the null hypothesis was partially rejected. The volume of human root canals was significantly different from bovine, but not significantly different from ovine teeth.

3.4 **Analyses of Ca/P ratio of root dentine by EDAX**

3.4.1 Aims

To investigate the variation in the calcium: phosphorus ratio in human, ovine and bovine root dentine specimens at a range of distances from the root canal lumen. This study tested the null hypotheses that Ca/P ratios in human root dentine were no different from those of ovine and bovine root dentine.

3.4.2 Materials and methods

3.4.2.1 Teeth collection and storage

See section 3.2.2 for tooth collection and storage. Fifteen teeth (5 human premolars, 5 ovine incisors and 5 bovine incisors) were used for this study.

3.4.2.2 Specimen preparation

See section 3.2.2 for specimen preparation.

3.4.2.3 Storage of the samples and EDAX preparation

Horizontal sections were prepared from the cervical third of each root with a low speed circular diamond saw at 5rpm under constant water irrigation (Testbourne Model 650, South bay Technology (SBT), INC.), and stored in 1% chloramine-T (wt/v) at 4˚C.

Next, each section was embedded in polystyrene based clear resin (Bondaglass, Bondaglass-Voss Ltd.), (Sigma-Aldrich), in a 30 mm cylindrical sample mould (BUEHLER® USA). The embedded specimens in the clear resin were sequentially polished with grades P600, P800, P1000 and P1200 abrasive papers (Norton) with universal polisher (Metaserv), followed by aluminium oxide (Al_2O_3), 1µm, 0.3µm and

0.05µm (BUEHLER® USA) with rotary polishing machine (Metaserv Rotary Pregrinder) to remove the saw-generated smear layer. Subsequently, each specimen was transferred into 2% glutaraldehyde fixative overnight before the Energy dispersive x-ray (EDAX) analysis (JEOL JSM 5300LV).

Specimens were polished at each stage for 20 sec. The macro polisher speed was 60 rpm and specimens were held against the polishing disk by hand, with an effort to maintain uniform pressure, and under continuous irrigation with distilled water. All specimens were prepared by the same operator. The micro polisher speed was 120 rpm, with specimens presented to the disc under constant light pressure, and under continuous irrigation with the diamond or aluminium oxide paste.

3.4.2.4 Analysis of the of the samples by the EDAX

After mounting, they were analysed by energy dispersive x-ray spectrometry (EDAX) (JEOL JSM 5300LV). Ca/P ratios were determined at 0µm, 100µm, 200µm, 300µm and 1000µm from the root canal lumen (Figure 3.12). The Ca/P ratio was calculated by dividing the percentage content of the Ca by the percentage content of P. Results were then analysed using the Kruskal-Wallis and Mann-Whitney U tests ($p<0.05$).

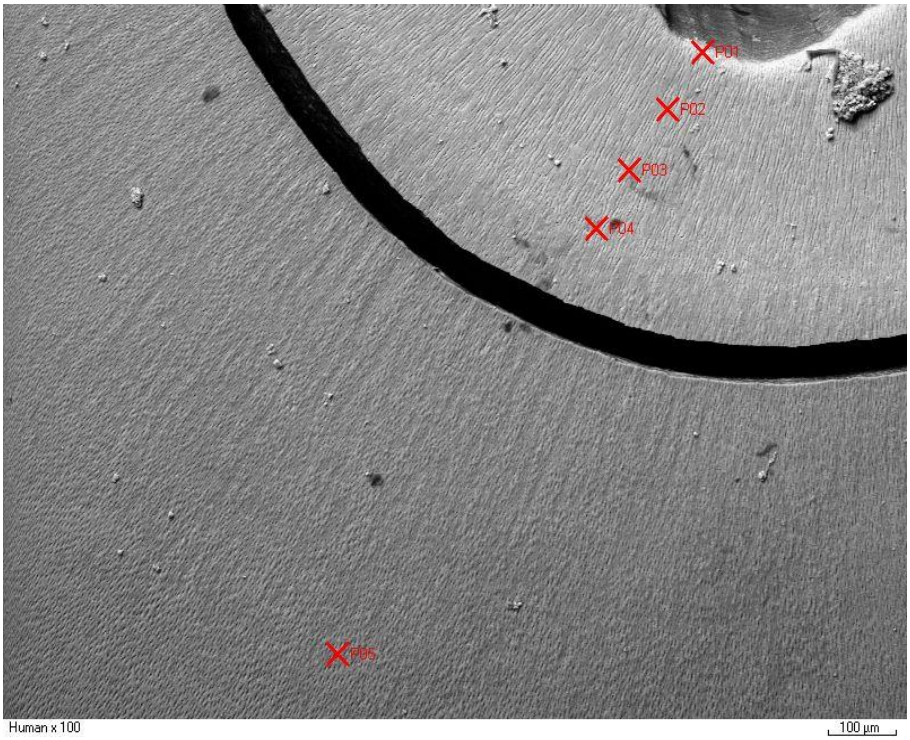

Figure 3.12. Typical SEM photograph showing locations at which EDAX measurements were taken at intervals from the canal lumen (x), 0µm, 100µm, 200µm, 300µm and 1000µm. Note: the black curved-line is a crack resulting from drying of the sample within the vacuum chamber of the EDAX machine.

3.4.3 Results

Figure 3.13 and Figure 3.14 show the typical spectra of minerals including Ca, P, C, Mg and Cl on ovine and human cervical root dentine analysed by EDAX machine, respectively. No spectra are presented for bovine teeth (data lost following the retirement of a colleague). Table 3.7 shows the median and upper and lower quartiles of Ca/P ratios of the cervical thirds of ovine, bovine and human roots at 0µm, 100µm, 200µm and 300µm and 1000µm from canal lumen. Figure 3.15 shows the median Ca/P ratios of ovine, bovine and human dentine at increasing distances from the root canal lumen. It shows a trend in which the Ca/P ratio of ovine had the lowest value, Ca/P ratio of bovine as a middle value and Ca/P ratio of human as the highest value, at all distances from the lumen except at 1000µm. At 1000µm, the Ca/P ratios of bovine and human dentine were equal.

Variability between the 3 species was high at the first 4 points from the root canal lumen, while it decreased at 1000µm. In human dentine, the variation in the Ca/P ratios was higher at 200 µm than at other areas for the same species, and higher than the other

2 species in the same area and all other areas. There were significant differences in the Ca/P ratios between ovine and bovine dentine, and between ovine and human dentine at points up to 300µm from the canal lumen ($p<0.05$). The Ca/P ratio of bovine dentine was significantly different from that of human at the root canal lumen (0µm) only ($p<0.05$) (Figure 3.15).

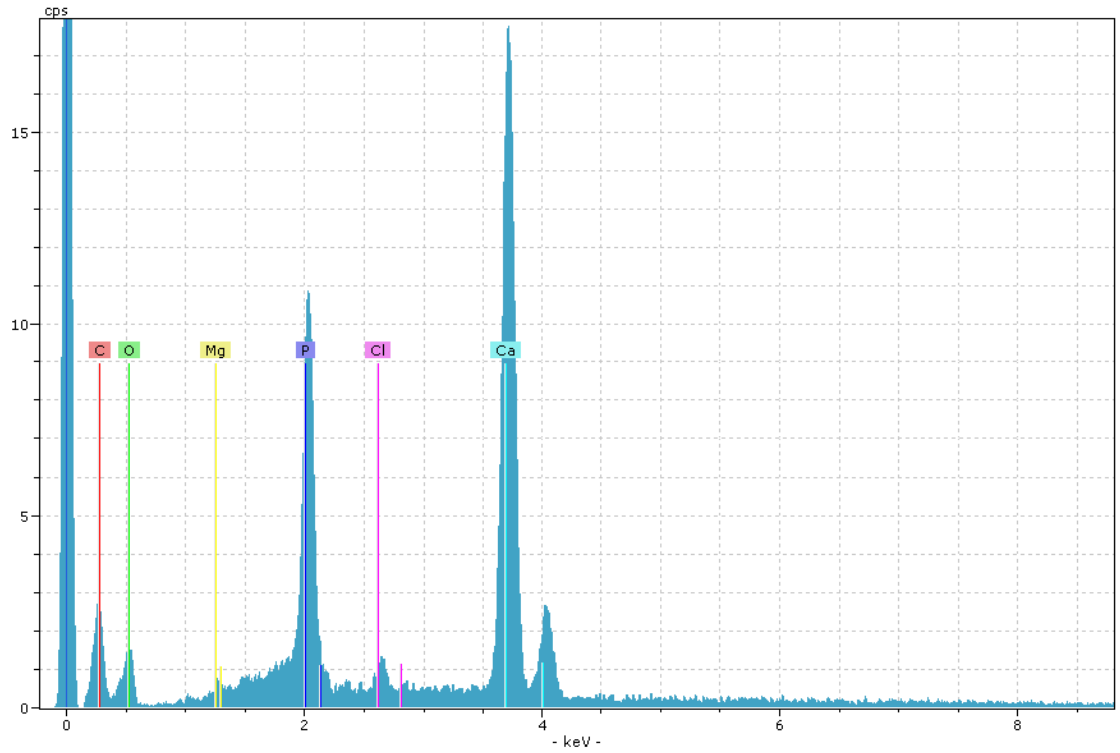

Figure 3.13. Typical EDAX spectra of ovine cervical root dentine.

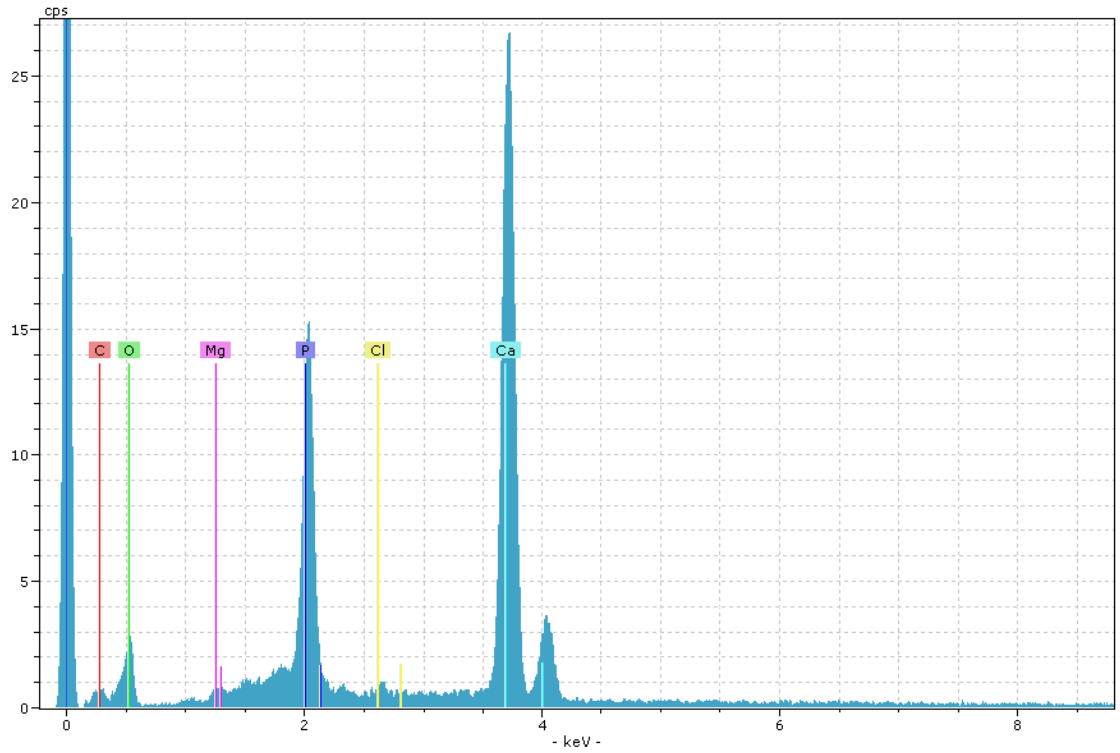

Figure 3.14. Typical EDAX spectra of human cervical root dentine.

Table 3.7. Median (upper and lower quartiles) of the Ca/P ratios of ovine, bovine and human teeth at, 0µm, 100µm, 200µm, 300µm and 1000µm from root canal lumen.

Group	At 0µm	At 100µm	At 200µm	At 300µm	At 1000µm
Ovine teeth	2 (2.0 &1.9)	2 (2.0, 1.9)	2 (2.1 & 1.9)	2 (2.1 & 2.0)	2.2 (2.3 & 2.2)
Bovine teeth	2.2 (2.2 & 2.1)	2.2 (2.4 & 2.2)	2.3 (2.3 & 2.2)	2.3 (2.3 & 2.2)	2.3 (2.4 & 2.2)
Human teeth	2.5 (2.7 & 2.5)	2.5 (2.6 & 2.3)	2.5 (2.7 & 2.1)	2.4 (2.4 & 2.2)	2.3 (2.4 & 2.3)

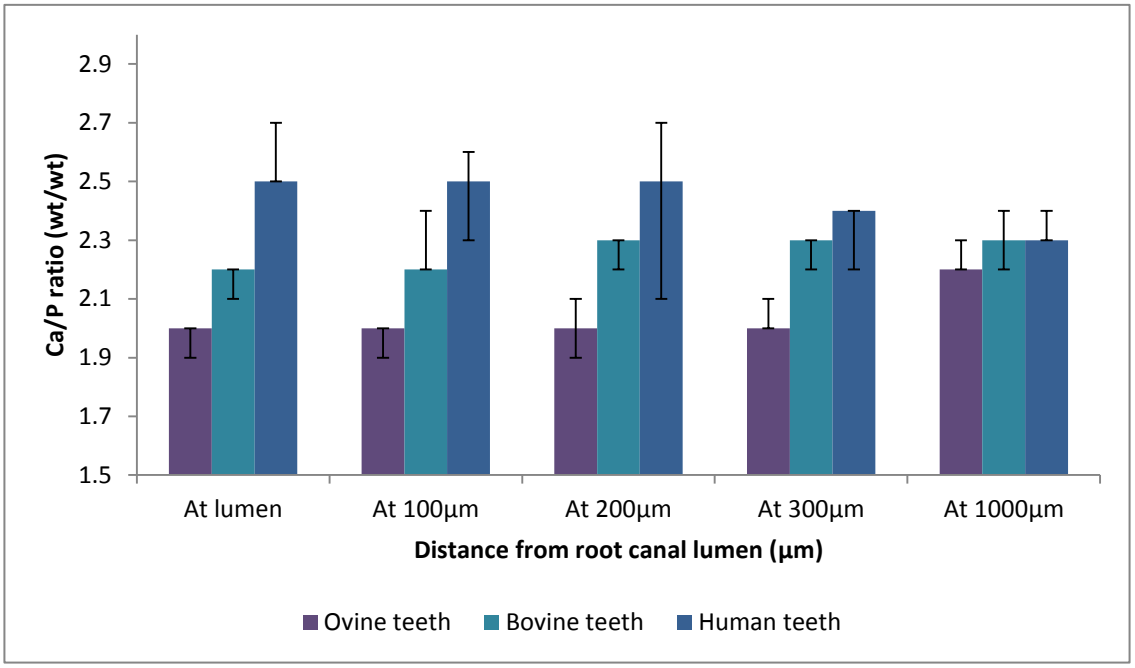

Figure 3.15. Median Ca/P ratios of horizontal cervical dentine sections of ovine, bovine and human dentine at different depths from the root canal lumen. The error bars are the upper and lower quartiles for each group.

3.4.4 Discussion

In studies involving the interaction of dental procedures and materials with dental tissues, it is important to understand the physical properties and chemical composition of the tissue under test. The specimens in this experiment were polished before analysing them with EDAX, to remove the smear layer created by mechanical cutting as this could influence the results. This was recommended in a study which reported that EDAX required polished surfaces, since a saw-generated smear layer may result in different elements characterised by the EDAX (Dogan and Çalt, 2001). However, other elements could be expected to be seen in the spectra involved inside the specimen, and they could come from polishing of the specimen with aluminium oxide or from the storage medium.

It was noted by others that conducting EDAX experiments on dentine specimens could be difficult, due to changes in mineralization rates between different types of dentine and anatomical variations (Hennequin *et al.*, 1994; Hennequin and Douillard, 1995). The current results agreed with this finding, since in each species a non-regular variation in Ca/P ratios was noted between dentine at different depths from the lumen.

Analysing dentine at different distances is useful in the next studies to look at the extent of the effect of the irrigants into distant areas from root canal lumen through dentinal tubules.

Sample preparation and analysis was difficult and resource intensive, and a number of specimens cracked within the low vacuum pressure chamber of the EDAX. This work was, however, considered worthwhile in order to add a further relevant characteristic to the comparison of human and animal teeth

Whilst it was not included in the current work, it may be useful to look at the organic components of dentine, such as collagenous and non-collagenous organic matrix (phosphorylated phosphoproteins), which may account for further variation (Steinfort *et al.*, 1990). Other mineral elements such as Mg, which may replace Ca in areas of dentine and change the Ca/P ratio (Murray, 1936) may also be worthy of consideration in a more detailed comparison of tissues.

The thickness of material samples can influence the spectra of the material or its radiodensity (Fonseca *et al.*, 2004), and care is needed in deciding the optimal specimen

thickness for analysis. No reports could be found on the comparative Ca/P ratios of human, bovine and ovine dentine, and the influence of the details of specimen preparation.

The results of our study showed that differences in the Ca/P ratios between human and bovine teeth were not significant at all depths from the lumen, except at the lumen (0 µm), therefore agreeing with the finding of a study that found the radiodensity of human dentine to be slightly higher than that of bovine dentine (Fonseca *et al.*, 2004). The latter study used dental x-rays to analyse the density of dentine in human and bovine teeth (Fonseca *et al.*, 2004). Such methods may be regarded as qualitative (Chow *et al.*, 1991), rather than directly measuring mineral levels or ratios.

It has previously been shown that the Ca/P ratio in human dentine was approximately 1.67 depending on crystal type, Ca availability, anatomical location and techniques of analysis (Cohen *et al.*, 1992; Marshall G. W., 1993; Hennequin and Douillard, 1995). Another study showed that the Ca/P ratios of enamel and dentine in human, bovine and porcine teeth were 2.17 and 2.13, 2.32 and 2.08, and 2.21 and 1.92 respectively. There was no significant difference between them and these values were approximately similar to the expected value of hydroxyapatite, 2.13 (Falla-Sotelo *et al.*, 2005).

The results of the current study revealed that at the lumen, ovine and bovine teeth had significantly lower Ca/P ratios compared to the human specimens. One explanation could include the fact that cow and sheep teeth may have been younger and less mature than human teeth and may have thicker layer of predentine than human dentine. There has been no previously published study to show the thickness of predentine of ovine and bovine teeth. While human predentine thickness has been reported in the literature to be approximately thick 40.4µm near the apex of the root, where dentinogenesis most active, in the coronal region, where primary dentine has completely formed, it is approximately 14.8µm thick (Couve, 1987).

The variation in Ca/P ratio at different distances from the root canal lumen was investigated to inform later studies on sub-surface tissue changes. This work could be useful in the next studies to assess the possibility of the effect of root canal irrigant such as NaOCl on surface and subsurface change.

3.4.5 Conclusion

Within the limitations of this investigation, the null hypothesis was rejected, since the Ca/P ratios in the cervical dentine of human roots were different from ovine and bovine root dentine at some depths from the lumen.

3.5 **Analysis of dentine stiffness by Atomic Force Microscopy (AFM)**

Dental materials and interventions may alter the mechanical properties of dental tissues. If non-human tissues are to be employed by researchers, it is important to understand how their mechanical properties compare with human tissues. In this study, stiffness analysis was undertaken on horizontal sections of untreated human, ovine and bovine roots, at five different depths from the root canal lumen using AFM.

3.5.1 Aim

To compare the stiffness of human, ovine and bovine root dentine at different depths from the root canal lumen by AFM. This study tested the null hypotheses that stiffness of human root dentine was no different from ovine and bovine root dentine.

3.5.2 Materials and methods

3.5.2.1 Teeth collection and storage

See section 3.2.2 for tooth collection and storage. Fifteen teeth (5 human premolar, 5 ovine incisors and 5 bovine incisors) were used for this study.

3.5.2.2 Specimen preparation

See section 3.2.2 for specimen preparation.

3.5.2.3 Storage of the samples and AFM preparation

See section 3.4.2 (about the storage of the samples and EDAX preparation). Subsequently, these specimens were transferred into 1% chloramine-T at 4˚C for one day before analysis by AFM (JPK Instruments, Nanowizard 3, Berlin, Germany), instead of the storage with the 2% glutaraldehyde fixative.

AFM analysis usually takes 1 day per group, so in this experiment, each group was prepared on different days in order to regulate the duration of sample storage in 1% chloramine-T at 4˚C and keep it constant at each step.

3.5.2.4 Analysis of the of the samples by the AFM

Part of the resin mould of each specimen was cut with a flex diamond disc (Skill*denta, Skillbond®, Skillbond Direct Ltd Dudley House, UK) under water-cooling to allow the specimen to fit within the AFM flow-cell. Only one cervical section was taken from each sample to be analysed by the AFM, making 5 cervical sections were made from each group. Each specimen was fixed on a circular coverslip (diameter approximately 24 mm, thickness approximately 190µm -Agar Scientific, Coverglasses, Round no.1.5, Elektron Technology, UK Ltd, UK) by Araldite (Araldite®, Instant , Switzerland).

The trimmed specimens mounted on circular coverslips were inserted inside the flow-cell (JPK Instruments, Small Volume Fluid Cell ™, Germany) before filling with Dulbecco's Phosphate Buffered Saline (DPBS) (Lonza, BioWhittaker ᴿ, Dulbecco's phosphate buffered saline - 0,0095 M (PO_4) without Ca and Mg, for cell culture, Sterile Filtered, Belgium), pH 7.2 to keep the sample fully hydrated during measurement.

AFM analysis was undertaken in quantitative imaging (QI) mode. The AFM probes were made from non-conductive silicon nitride (Si_3N_4) (Bruker Ca, USA) with 0.35 N/m nominal spring which were calibrated using force spectroscopy to determine their sensitivity and spring constant.

Calibration of probe sensitivity was done against a glass slide using force spectroscopy and the spring constant was then measured using the thermal vibration method. During each analysis, stiffness measurements were made using a probe to scan each area on the specimen with dimension of 50µm x 50µm.The image resolution of the scanned area was 256 x 256. Stiffness was measured from the linear portion of the unloading curve in each location on the 256 x 256 area.

Next, the dentine stiffness was analysed at 0µm, 100µm, 200µm, 300µm and 1000µm from the canal lumen, using the x-direction and y-direction moving scales of the AFM analysing tip after elevation using the z-direction moving stage. Results were analysed using Kruskal-Wallis and Mann-Whitney U tests ($p<0.05$).

3.5.3 Results

Ovine, bovine and human specimens were found to present similar dentine structure, with imaging of dentinal tubules, peritubular and the intertubular dentine (Figure 3.16, Figure 3.18 and Figure 3.20 respectively). In addition, it was observed that the direction of the dentinal tubules was longitudinal, but with differences in size. The diameter of the dentinal tubules in human and bovine teeth was more similar to each other than to ovine. Typical force distance curves are shown in Figure 3.17, Figure 3.19 and Figure 3.21. All show similar patterns of vertical deflection (extended) per distance and vertical deflection (retract) per distance, although there were differences in their values.

The median dentine stiffness and upper and lower quartiles for each group is shown in Table 3.8. The stiffness of human dentine was higher than bovine and ovine at all depths from the root canal lumen; bovine stiffness values were in the middle, while ovine had the lowest values. Both of human and bovine root dentine was significantly stiffer than ovine dentine at all depths from the canal lumen ($p<0.05$). Human root dentine was significantly stiffer than the bovine dentine up to 300µm ($p<0.05$) from the canal lumen. Variation in the median stiffness values for human teeth was higher than for other species at all depths from root canal lumen (Figure 3.22).

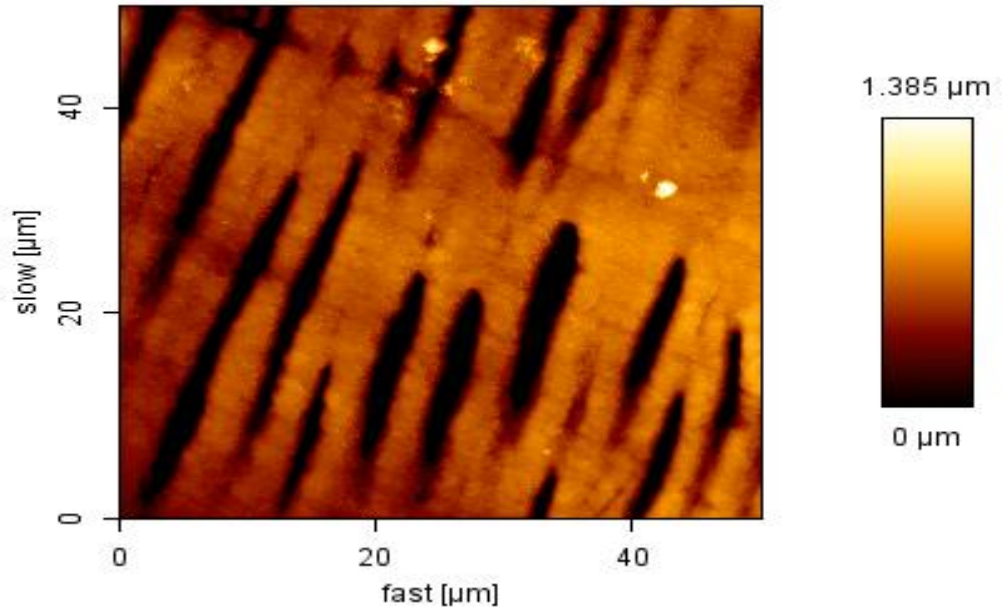

Figure 3.16. AFM image to show the structure of root canal dentine walls in the cervical third of ovine teeth.

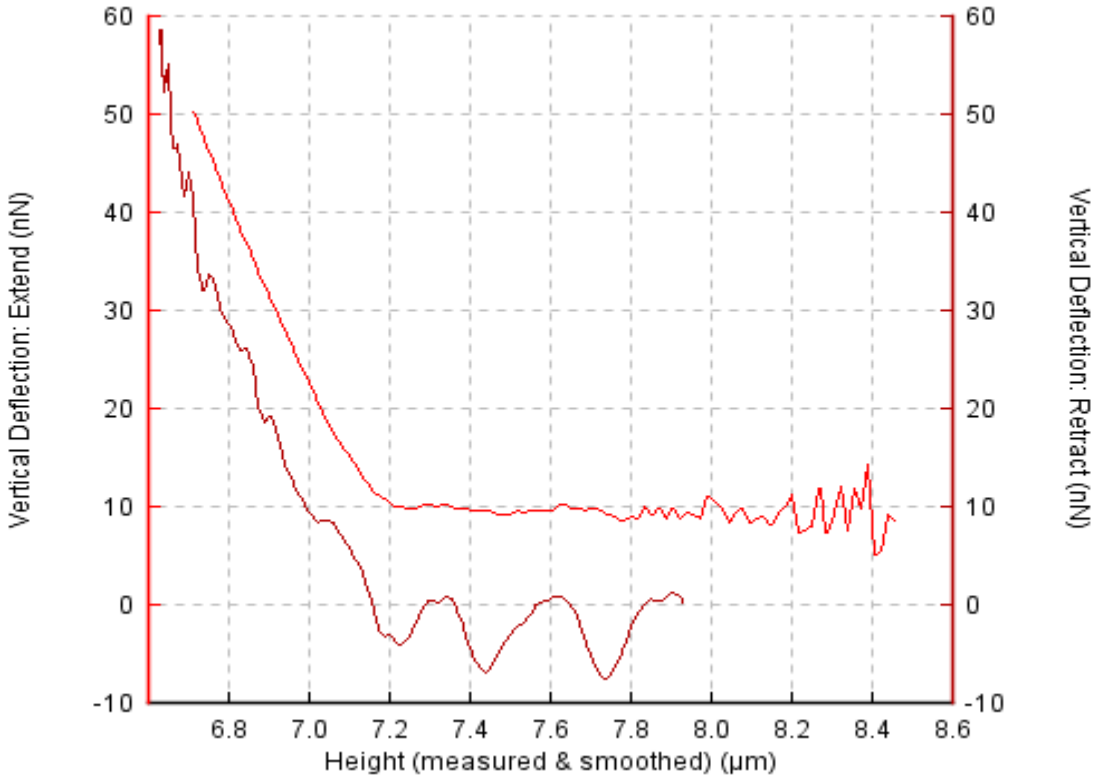

Figure 3.17. Typical force curve of root canal dentine walls in the cervical third of ovine teeth.

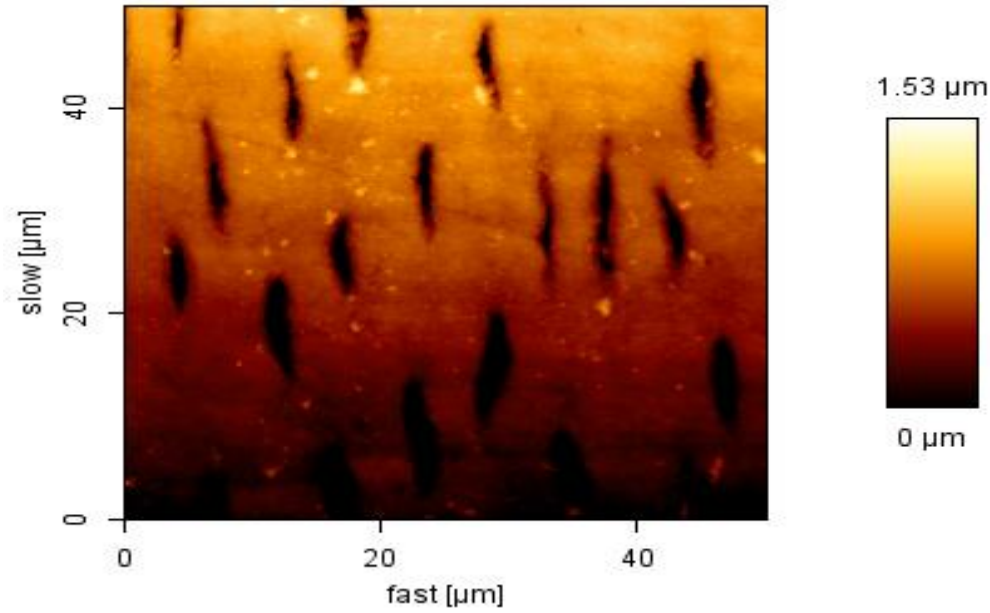

Figure 3.18. AFM image to show the structure of root canal dentine walls in the cervical third of bovine teeth.

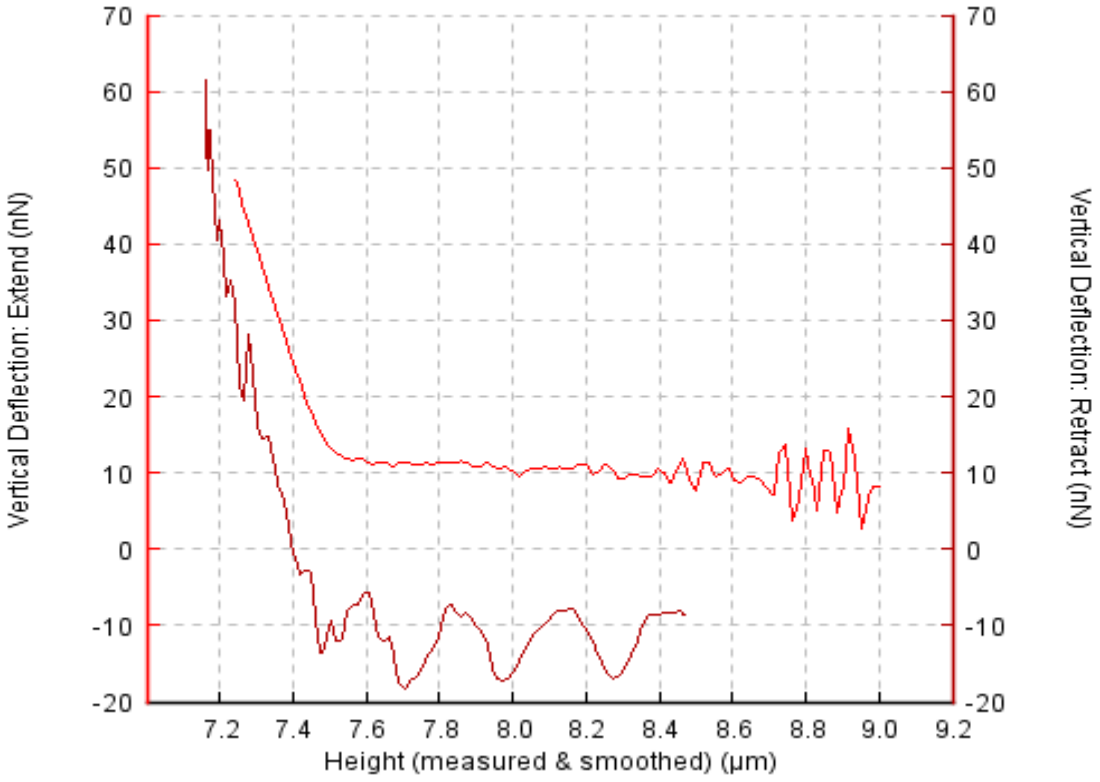

Figure 3.19. Typical force curve of root canal dentine walls in the cervical third of bovine teeth.

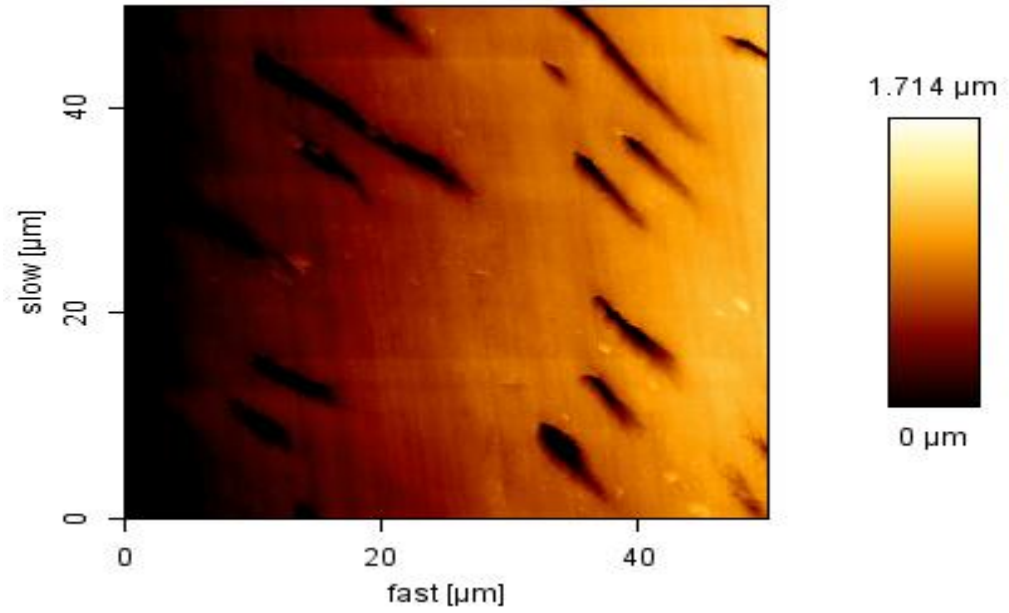

Figure 3.20. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth.

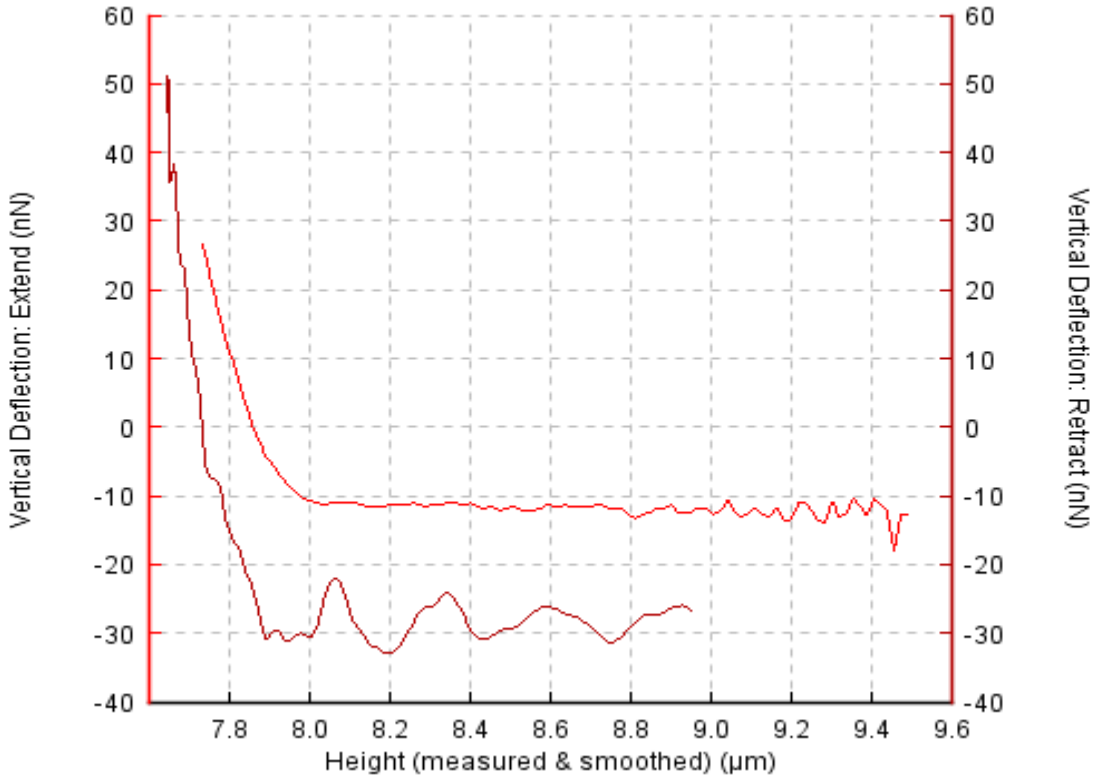

Figure 3.21. Typical force curve of root canal dentine walls in the cervical third of human teeth.

Table 3.8. Median (upper and lower quartiles) of the dentine stiffness of horizontal cervical dentine sections of ovine, bovine and human teeth at, 0µm, 100µm, 200µm, 300µm and 1000µm from root canal lumen.

Group	At 0µm	At 100µm	At 200µm	At 300µm	At 1000µm
Ovine teeth	114 (122 & 99)	114 (123 & 108)	114 (125 & 107)	119 (126 & 108)	118 (127 & 106)
Bovine teeth	150 (154 & 147)	149 (157 & 144)	150 (153 & 144)	152 (156 & 146)	149 (150 & 147)
Human teeth	177 (195 & 167)	177 (211 & 160)	182 (197 & 168)	195 (218 & 169)	180 (219 & 157)

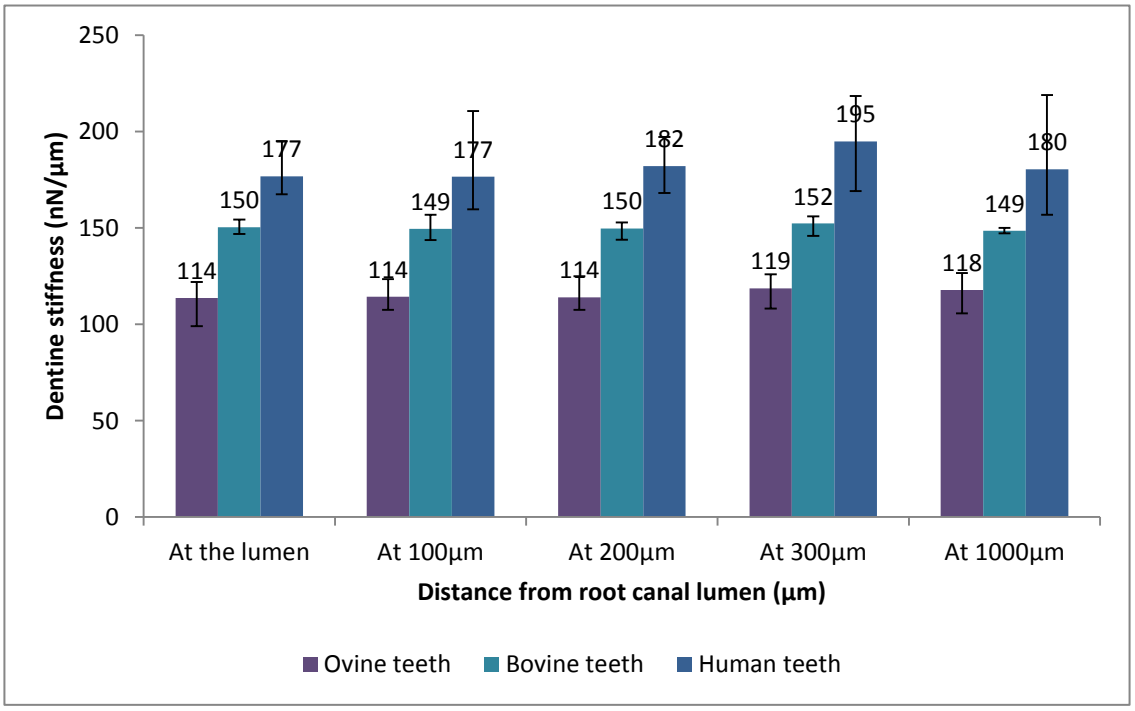

Figure 3.22. Median dentine stiffness (nN/µm) of horizontal cervical dentine sections of ovine, bovine and human dentine at different depths from the root canal lumen. The error bars are the upper and lower quartiles for each group.

3.5.4 Discussion

An understanding of the mechanical properties of dentine is necessary in order to identify the distribution of forces throughout tooth structure and to understand mechanical property changes that could result from restorative or endodontic treatment.

Dentine is a heterogeneous composite material which consists of tubules with a diameter of approximately 0.9 – 2.5 μm surrounded by a highly mineralised peritubular dentine, (approximately 95% mineral phase by volume), embedded within intertubular dentine (approximately 30% mineral phase by volume) (Garberoglio and Brännström, 1976; Marshall G. W., 1993).

The AFM can measure the principal structure of dentine, including its peritubular and intertubular elements (Kinney *et al.*, 1996a; Habelitz *et al.*, 2002b). Such detailed analysis was not possible with a more crude microindenter method, since the latter provided only a composite average value of dentine mechanical properties. In addition, it was found that the measurement of dentine properties such as hardness and elastic properties such as Young's modulus by the microindenter method varied according to the site of measurement between enamel and pulp, depending on the density of the dentinal tubules or differences in dentine structure (Craig *et al.*, 1959; Fusayama and Maeda, 1969; Pashley *et al.*, 1985; Kinney *et al.*, 1996a; Marshall *et al.*, 1997).

Another advantage of the AFM, is that it allows imaging in a hydrated state. In this respect, it is quite different from conventional SEM (Kinney *et al.*, 1996a; Kinney *et al.*, 1996b; Marshall *et al.*, 1997). Dehydration of dentine can result in denaturation of collagen fibres and re-immersion in water may not renature the tissue (El Feninat *et al.*, 1998), so the preservation of a moist environment is critical. For AFM, no staining or surface coating of specimens is required (Meyer *et al.*, 2003; Sanches *et al.*, 2009; Bhushan, 2010). AFM can provide quantitative information and other information about the specimen such as the dimensions of dentinal tubules, roughness, stiffness and hardness.

Key limitations of the AFM are the limited area that can be measured at each analysis, and the time required for each analysis. Any defect on the surface of the sample or any air bubble inside the liquid cell may also affect imaging and make the analysis invalid.

Figure 3.16, Figure 3.18 and Figure 3.20 showed that ovine, bovine and human cervical root dentine may have many features in common, with dentinal tubules, peritubular and intertubular dentine, and with dentinal tubules orientated at right angles to the root canal lumen. Differences in tubular diameter were inferred, however, though these were not formally measured in the current investigations.

There are no published studies that compare the stiffness of human, ovine or bovine teeth although many studies have employed bovine teeth as substitutes for human teeth (Ruse *et al.*, 1990; Nishimura *et al.*, 2008). The results of this study showed that human cervical root dentine was significantly stiffer than ovine cervical dentine at all depths from the root canal lumen, human cervical root dentine was also significantly stiffer than bovine up to 300μm ($p<0.05$) from the canal lumen. Bovine root was significantly stiffer than ovine root dentine at all depths from the root canal lumen. The results of this experiment showed that there was a difference in the detailed structure of dentine between animal and human teeth. Values for the mechanical properties of dentine provided by the literature vary widely and it is unknown whether this is related to differences in the local structure of dentine or variation in the testing methods that were used (Marshall *et al.*, 1997).

3.5.5 Conclusion

Within the limits of this study, the null hypothesis was partly rejected since the stiffness of human cervical root dentine was different from ovine and bovine at some depths from the root canal lumen dentine.

Chapter 4: The removal of saw-generated smear layer from dentine

4.1.1 Introduction

When dentine surfaces are cut, a smear layer is produced, comprising of chips of dentine, remnants of vital or necrotic pulp tissue, microbial components, retained irrigants and other material present within the preparation (McComb and Smith, 1975; Hülsmann *et al.*, 1997; Violich and Chandler, 2010). The smears layer formed by cavity preparation and root canal preparation are not necessarily identical, because of differences in the cutting tools employed, the rinsing solutions applied, differences in the nature of root and coronal dentine, and the presence of both pulp debris and microbial biofilms within root canals (Eick *et al.*, 1970; Violich and Chandler, 2010).

Studies have also shown that steel burs, tungsten carbide, diamond burs and endodontic files result in smear layers of variable thickness, roughness, volume and density (Bramante *et al.*, 2010; Parente *et al.*, 2010). Eick *et al* (1970) found that smear layer composed of particles ranging from less than 0.5µm to 15µm (Eick *et al.*, 1970), and under the SEM, the smear layer has an amorphous, irregular and granular appearance (Brannstrom *et al.*, 1980). Root canal instrumentation results in 1-5 µm thickness smear layer on the dentine walls, while its penetration inside dentinal tubules has been reported to range from 6 to 40µm (Mader *et al.*, 1984).

Smear layers may also be generated in the research laboratory when dentine surfaces are cut and polished with saws and abrasives (Eliyas *et al.*; Clarkson *et al.*, 2011). Laboratory sectioning of teeth is often undertaken in order to visualise the underlying dental tissues, and the smear layers generated may present a barrier to effective visualisation and analysis (Dogan and Çalt, 2001; Kho and Baumgartner, 2006). Different methods of smear layer removal, such as the application of acids, may damage the underlying dentine and overwhelm subtle changes caused by other dentine treatments under investigation (Torabinejad *et al.*, 2003b). Gentler methods are therefore needed. One suggested method is polishing, in which successive grades of abrasive papers and diamond polishing pastes are applied, commencing at #800 grit. After that, size #1200, #2400 and #4000 abrasive papers were used, before final

polishing was done by 3µm and 0.4µm diamond suspensions with corresponding polishing cloths (Ryou *et al.*, 2012).

Studies within this thesis sought to investigate sub-surface dentine changes caused by root canal irrigation with NaOCl and EDTA. Following root canal irrigation, horizontal root sections were prepared with a mechanical saw for analysis by SEM, EDAX and AFM, and it was considered necessary to produce smear-free, and in the case of AFM, flat surfaces for analysis.

The following section describes a series of studies to develop a method of removing laboratory-generated smear layer without damaging the subjacent dentine.

4.2 Mechanical brushing and ultrasonication

4.2.1 Aim

To investigate methods of gently removing saw-generated smear layers from horizontal sections of root dentine during the investigation of sub-surface effects caused by root canal irrigation. This study tested the null hypotheses that combination of mechanical brushing with a soft nylon toothbrush and ultrasonication in distilled water, 100% methanol or 5% SDS had no effect on removal of smear layer from mechanically cut human dentine surface.

4.2.2 Materials and methods

4.2.2.1 Teeth collection and storage

Forty freshly extracted human premolar teeth were collected for this study. See section 1.2.2 for details of tooth collection and storage.

4.2.2.2 Specimen preparation

The teeth were de-coronated with a low speed saw at 5 rpm, under constant cold water irrigation (Testbourne Model 650, South bay Technology (SBT), INC.) to produce uniform root specimens, at similar distances from the CEJ. Their pulp tissue was grossly extirpated with a medium size barbed broach (Maillefer, Ballaigues, Switzerland), and

the root canals were irrigated with 3ml distilled water using a Luer-Loc syringe with a 27-gauge Monoject endodontic needle (Monoject, Gosport, UK).

4.2.2.3 Treatment

Forty horizontal sections of dentine (one per tooth) were prepared from the cervical third of human roots with a circular diamond saw running at 5rpm under constant water irrigation (Testbourne Model 650, South bay Technology (SBT), INC.) and stored in 1% chloramine-T (wt/v) (SIGMA-ALDRICH, UK) at 4˚C until they were needed (Figure 4.1). Sections were divided randomly into 8 groups (n = 5):

Group 1 was not treated and served as a control of smear layer development.

Group 2 was ultrasonicated for 15 minutes in distilled water. This was undertaken by immersing the specimen in distilled water within a vial, and placing the vial in the water bath of an ultrasonic machine (Langford T80, Langford electronics Ltd, Birmingham, UK) which was activated for 15 minutes.

Group 3 was brushed mechanically with a soft nylon toothbrush (Oral-B, Pro-Health Soft Full Head Toothbrush, Boots Pharmacy, UK) for 15 minutes, in the chamber of a mechanical brushing machine (Fracmo, Fractional H.P., Motors Ltd. London, England) whose chamber was filled with distilled water. The mechanical brushing machine speed was 140 rpm.

Group 4 was ultrasonicated for 15 minutes in distilled water, before brushing with a soft nylon toothbrush and distilled water in a mechanical brushing machine for 15minutes, followed by a further episode of ultrasonication for 15 minutes in distilled water as previously described.

Groups 5 and 6 received the same treatment as group 4, but were ultrasonicated and brushed in 100% methanol and 5% SDS (sodium dodecyl sulfate) (SIGMA-ALDRICH, UK) respectively instead of distilled water.

Group 7 was ultrasonicated for 30 minutes in distilled water only.

Group 8 was ultrasonicated for 2 h in distilled water only.

4.2.2.4 Storage of the samples and SEM preparation

All sections were fixed in 2% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3, to preserve canal contents for SEM analysis. Specimens were then dehydrated in ascending concentrations of ethanol (25%, 50%, 75% each for half hour), 100% ethanol for 1 hour, and stored in fresh 100% ethanol before critical point drying (Tsousimis Samori 780 CPD, Tsousimis Research Group., Rockville, Maryland, USA). The samples, were then mounted on aluminium stubs, and gold sputter coated (Polaron E5100 cool sputter coater 12nm, Hertfordshire, England) for SEM analysis (Cambridge S240, Cambridge, England) at 8 kV accelerating voltage.

4.2.2.5 Analysis of the of the samples by the SEM

An overview of whole specimen was first done at low magnification to identify representative areas in each specimen, before capturing representative images at 500x magnification.

SEM images were scored for remaining smear layer using the previously published 5 point Hülsmann scoring system (Hülsmann *et al.*, 1997) (appendix A):

Score 1: No smear layer with open dentinal tubules.

Score 2: Small amount of smear layer and some open dentinal tubules.

Score 3: Homogenous smear layer covering the root canal wall and only few open dentinal tubules.

Score 4: Homogenous smear layer that covers the entire root canal wall and no open dentinal tubules.

Score 5: Heavy and non- homogenous smear layer that covers the entire root canal wall.

Results were analysed using the Kruskal-Wallis and Mann-Whitney U tests ($p<0.05$) in SPSS 19.0 (SPSS Inc, Chicago, IL).

Scoring consistency was assessed by reading images 3 times, with a 2 week gap between each episode of scoring.

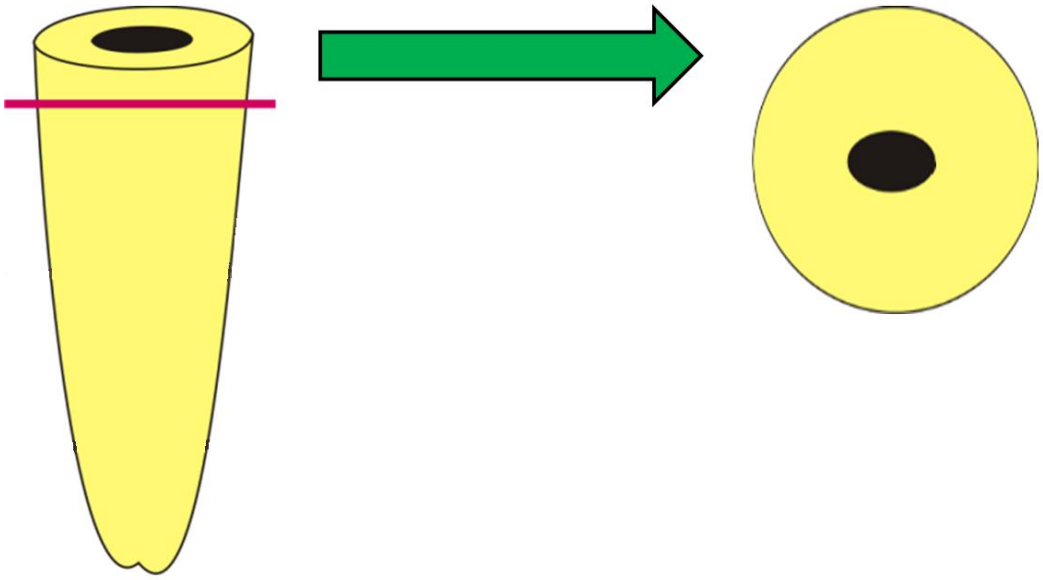

Figure 4.1. This figure shows horizontal root section.

4.2.3 Results

Samples in the control group, Group 1 exhibited complete surface coverage by a homogenous smear layer, with no open dentinal tubules (median score 5) (Figure 4.2). Groups 2, 7 and 8 that were treated by ultrasonication in distilled water for 15 minutes, 30 minutes and 2 h respectively had incomplete removal of smear layer (Figure 4.3 and Figure 4.5). Group 3 that was treated by brushing in distilled water also showed incomplete removal of smear layer (Figure 4.4). The cleanest surfaces were observed in Groups 4, 5 and 6, which were treated by repeated brushing/ultrasonication in distilled water, methanol and SDS respectively (Figure 4.6). Median smear layer scores and maximum/minimum values are shown in Figure 4.7. Groups 4, 5 and 6 had significantly lower scores for remaining smear layer than the control group ($p<0.05$). In addition, the other groups 3, 7 and 8, except group 2, had had significantly lower scores for remaining smear layer than the control group ($p<0.05$).

The trend observed was that specimens treated by repeated brushing and ultrasonication in water, methanol or SDS (Groups 4, 5 and 6) resulted in more effective smear layer removal than those treated by a single episode of brushing or ultrasonication (Group 2, 3, 7 and 8) ($p<0.05$), though variation was highest in the methanol group (Group 5). It was also observed that increasing the period of ultrasonic treatment alone from 15

minutes to 30 minutes resulted in more effective removal of smear layer, but increasing the period of exposure to 2 hours resulted in no further improvement.

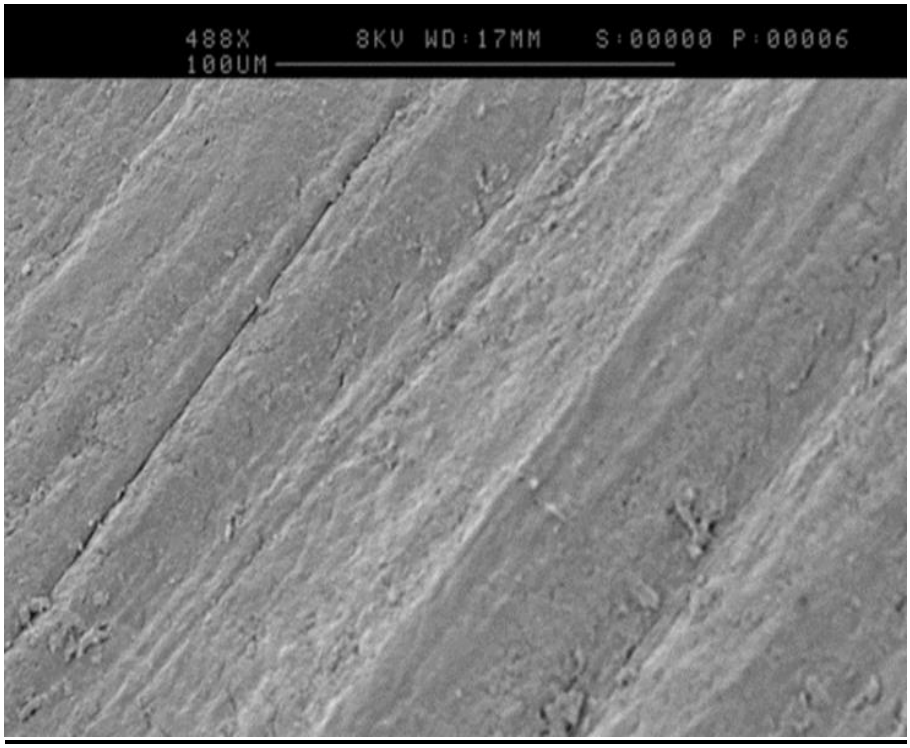

Figure 4.2. SEM image (500x magnification) to show Hülsmann score 5 in Group 1 (control group).

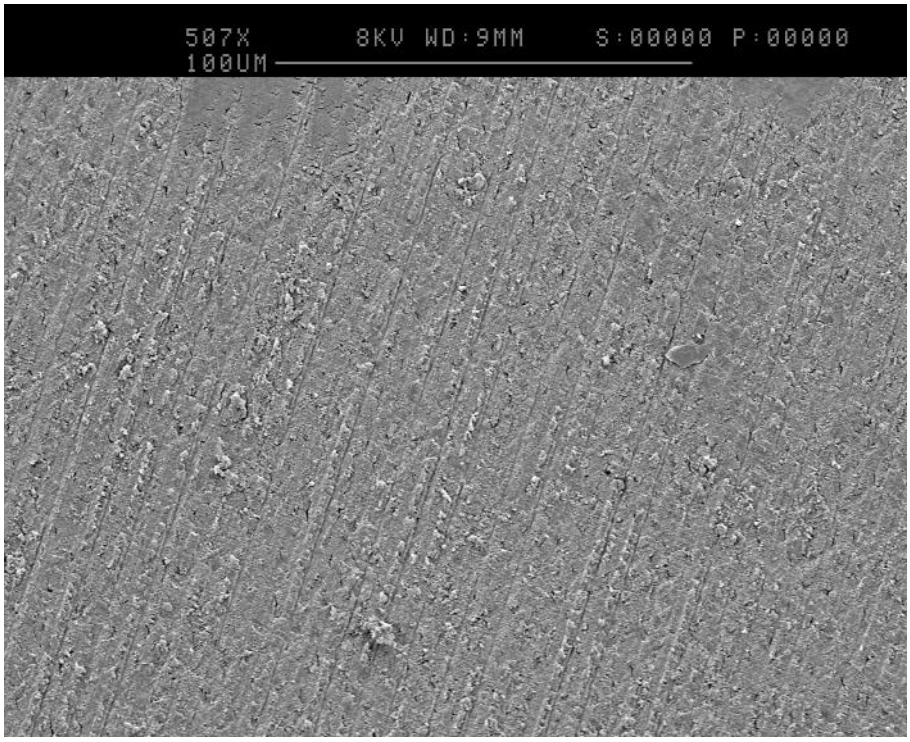

Figure 4.3. SEM image (500x magnification) to show Hülsmann score 4, in Group 2, treated by ultrasonication in distilled water for 15 minutes.

Figure 4.4. SEM image (500x magnification) to show Hülsmann score 3, in Group 3, treated by brushing in distilled water for 15 minutes.

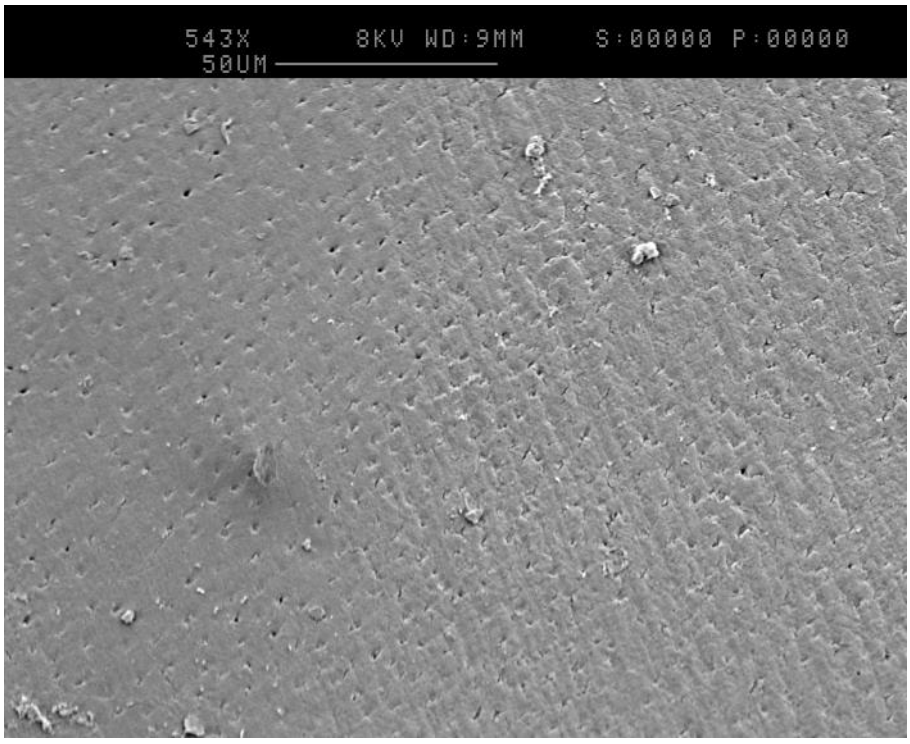

Figure 4.5. SEM image (500x magnification) to show Hülsmann score 2, in Group 7, treated ultrasonication in distilled water for 30 minutes.

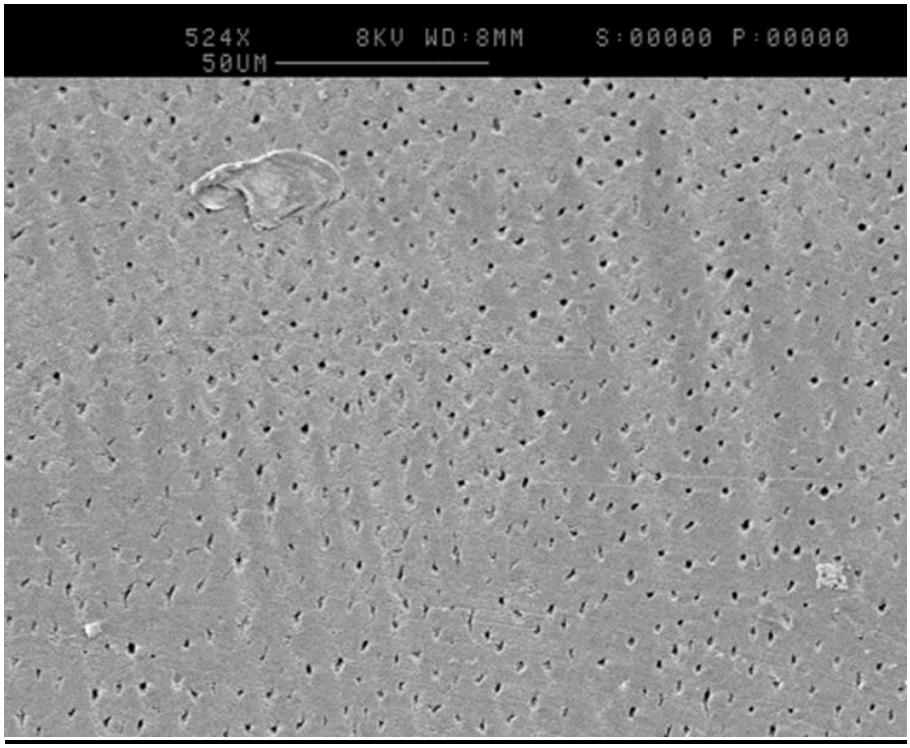

Figure 4.6. SEM image (500x magnification) to show Hülsmann score 1, in Group 4, treated by brushing and ultrasonication in distilled water.

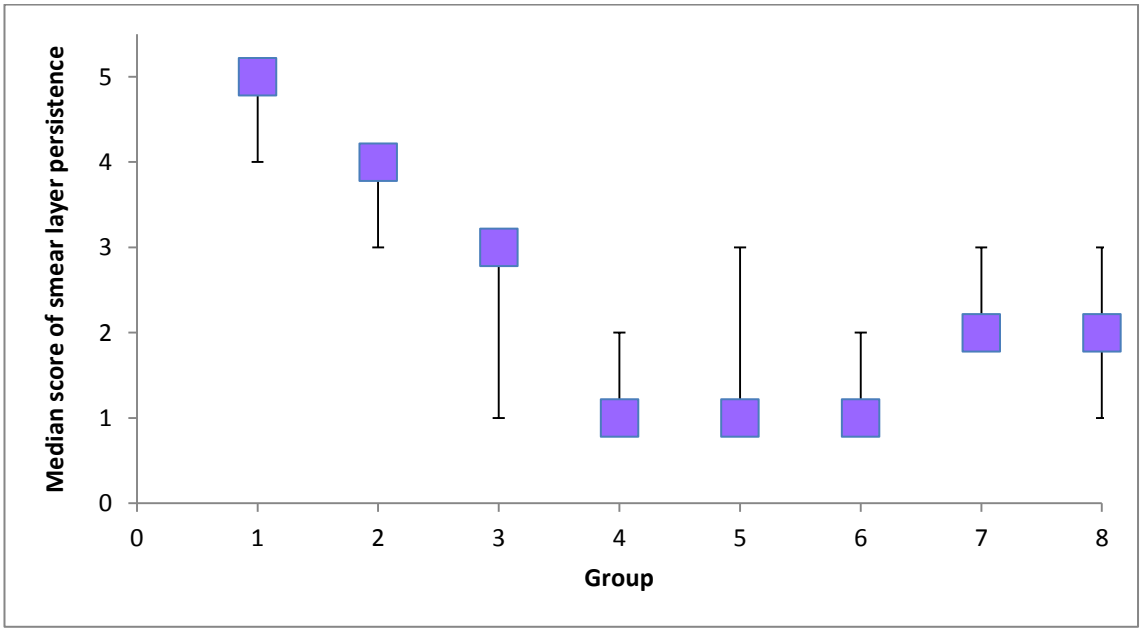

Figure 4.7. Median smear layer scores (Hülsmann *et al*'s. 1997) on the surface of horizontal dentine slices of groups 1-8. The difference was significant between the non-treated group and all of the other groups, except group 2 ($p<0.05$, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the maximum and minimum scores for each group.

4.2.4 Discussion

In pilot studies, attempts were made to remove the smear layer from root sections with different concentrations of citric acid, phosphoric acid, NaOCl, EDTA, and combinations (Table 4.1 and Table 4.2). All of them caused visible changes to dentine surfaces, such as acid erosion (Figure 4.8). In addition, organic solvents such as 100% methanol, 100% ethanol, 100% propanol, 100% acetone and detergents such as 5% SDS in combination with ultrasonication (without the use of mechanical brushing) were found to be ineffective in smear layer removal (Figure 4.9).

The addition of controlled brushing allowed predictable smear layer removal whether the solution was distilled water, 100% methanol or 5% SDS. Macroscopic dentine damage was not apparent. All of these experimental groups (Groups 4, 5 and 6) were regarded as suitable for analysis of the underlying dentine by SEM, EDAX and AFM. However, the surface roughness of the specimens created by the saw was too great to allow analysis of the specimen by the AFM. The need was therefore identified to create both smear-free and smooth surfaces for AFM analysis, and this became the focus of the next element of this investigation (El Feninat *et al.*, 2001b). It was concluded from the current study that repeated brushing and ultrasonication (Groups 4, 5 and 6) resulted in improved smear layer removal, regardless of the liquid medium. Caution was, however, noted since the pH of the distilled water was found to be 6.5, which may theoretically have caused some unidentified demineralisation of the smear layer and the underlying tissue. No systematic investigations were undertaken to verify this.

Table 4.1. A summary of pilot studies to remove saw-generated smear layer from horizontal tooth sections. Smear layer scores were based on the Hülsmann *et al.* (1997) scale.

Material/Technique for smear layer removal from horizontal root sections	**Median score of smear layer persistence**
Water:	
i. Copious irrigation with water during mechanical cutting of the horizontal root slices	5
ii. Ultrasonic cleaning of the slices with horizontal movement of the tip of an ultrasonic scaler close to the section but without contact for 1, 2 & 3 minutes	5, 5, 4
iii. Ultrasonic cleaning by immersion in water in a glass vial and vibration in an ultrasonic bath for 15 minutes	5
Phosphoric acid (32% liquid): section immersed for 1, 2 and 3 minutes	2
Phosphoric acid (40% gel): applied with a micro-brush for 30 sec	1
Diamond paste: Polishing with diamond paste (range of times)	3
Prophylactic paste: 30 sec on a prophylaxis brush	3
NaOCl alone: Immersion in 2.86% NaOCl with ultrasonic bath treatment for 15 minutes	1
EDTA alone: Immersion in 17% EDTA with ultrasonic bath treatment for 15 minutes	1
Combinations of NaOCl and EDTA:	
i. Immersion in 17% EDTA with ultrasonic bath treatment for 5 minutes, followed by the same in 2.86% NaOCl, and then 17% EDTA	1
ii. Immersion in1 2.65% NaOCl with ultrasonic bath treatment for 5 minutes, followed by the same in 17% EDTA, and then, 2.86% NaOCl	1

Table 4.2. It is a continuation of the previous table. It shows a summary of pilot studies to remove saw-generated smear layer from horizontal tooth sections. Smear layer scores were based on the Hülsmann *et al*'s. (1997) scale.

Material/Technique for smear layer removal from horizontal root sections	**Median score of smear layer persistence**
Citric acid: i. Immersion in 6% citric acid with ultrasonic bath treatment for 15 minutes ii. Immersion in 32% citric acid with ultrasonic bath treatment for 15 minutes iii. Immersion in 40% citric acid with ultrasonic bath treatment for 15 minutes	 1 1 1
Methanol: Immersion in 100% methanol with ultrasonication in a water bath for 15 minutes	1
Ethanol: Immersion in 100% ethanol with ultrasonication in a water bath for 15 minutes	3
Propanol: Immersion in 100% propanol with ultrasonication in a water bath for 15 minutes	3
Acetone: Immersion in 100% acetone with ultrasonication in a water bath for 15 minutes	2.5
SDS: Immersion in 5% SDS with ultrasonication in a water bath for 15 minutes	2

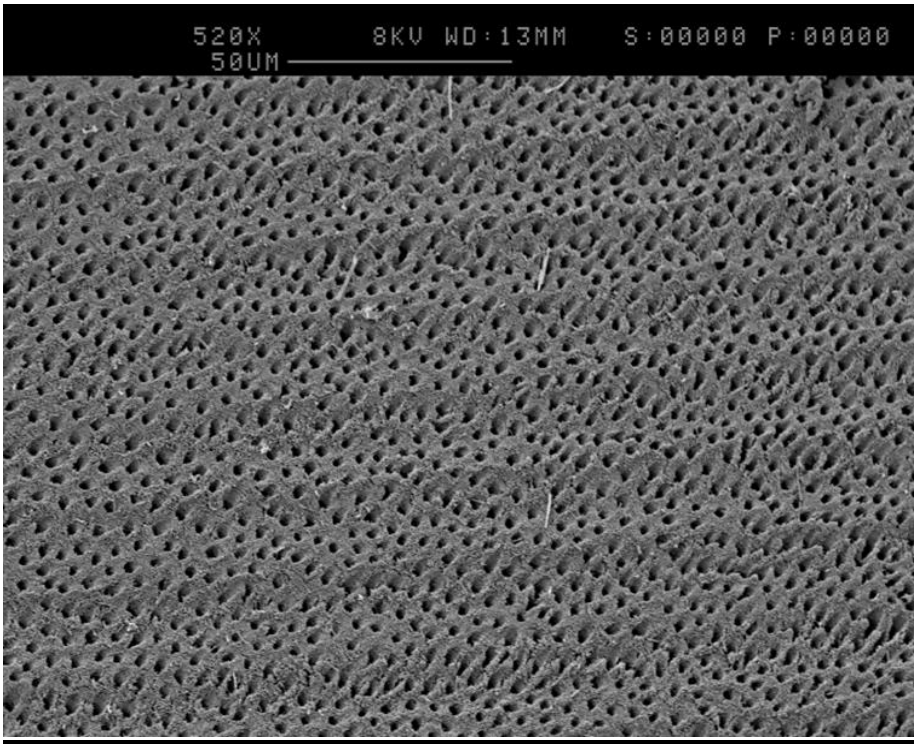

Figure 4.8. SEM image (500x magnification) to show image from pilot study with 6% citric acid treatment, showing smear layer removal but with dentine erosion (Hülsmann score 1).

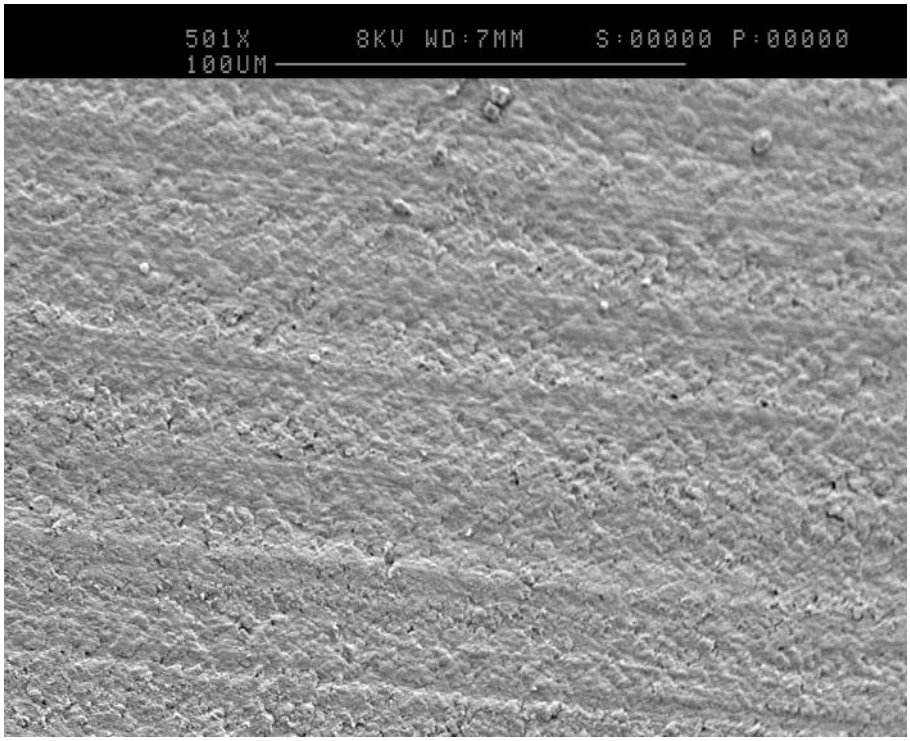

Figure 4.9. SEM image (500x magnification) to show image from pilot study with ultrasonication with 5% SDS without mechanical brushing (Hülsmann score 4).

4.2.5 Conclusions

The results of this investigation rejected the null hypothesis and found that combination of controlled mechanical brushing and ultrasonication of specimens in distilled water, 100% methanol or 5% SDS resulted in significant smear layer reduction compared with the control.

4.3 **Macro and micro polishing**

4.3.1 Aim

The aim of this study was to remove smear layer from mechanically cut human dentine slices using macro and micro polishing.

This study tested the null hypotheses that macro and micro polishing would have no effect on removal of smear layer from mechanically cut human dentine surfaces.

4.3.2 Materials and methods

4.3.2.1 Teeth collection and storage

Ten freshly extracted human premolar teeth were collected for this study. See section 3.2.2 for details of tooth collection and storage.

4.3.2.2 Specimen preparation

See section 3.2.2 for details of specimen preparation.

4.3.2.3 Treatment

Ten horizontal sections of dentine were prepared from the cervical third of human roots (one per tooth). See section 3.2.2 for details of their preparation. The sections were divided randomly into 2 groups (n = 5):

Group 1 was not treated and served as a control of smear layer development.

Group 2 was polished using macro and micro polish as follows:

First, specimens were sequentially polished with grades P600, P800, P1000 and P1200 abrasive papers abrasive papers (Norton) with universal polisher (Metaserv), followed by aluminium oxide, 1µm, 0.3µm and 0.05µm (BUEHLER® USA) with rotary polishing machine (Metaserv Rotary Pregrinder), see section 3.4.2 (the storage of samples and EDAX preparation):

4.3.2.4 Storage of the samples and SEM preparation

See section 3.2.2 for details of samples and SEM preparation.

4.3.2.5 Analysis of the of the samples by the SEM

See section 4.2.2 for details of the analysis of the samples by the SEM. The results were analysed using the Mann-Whitney U test ($p<0.05$) in SPSS 19.0 (SPSS Inc, Chicago, IL).

4.3.3 Results

Median smear layer scores and maximum and minimum values are summarised in Figure 4.12. Samples in the control group (Group 1) exhibited complete surface coverage by a non-homogenous smear layer, with no open dentinal tubules (median score 5) (Figure 4.10). Specimens in Group 2 had no smear layer on the dentine surface (median score 1) (Figure 4.11). The difference between Group 2 and the control Group was statistically significant ($p<0.05$).

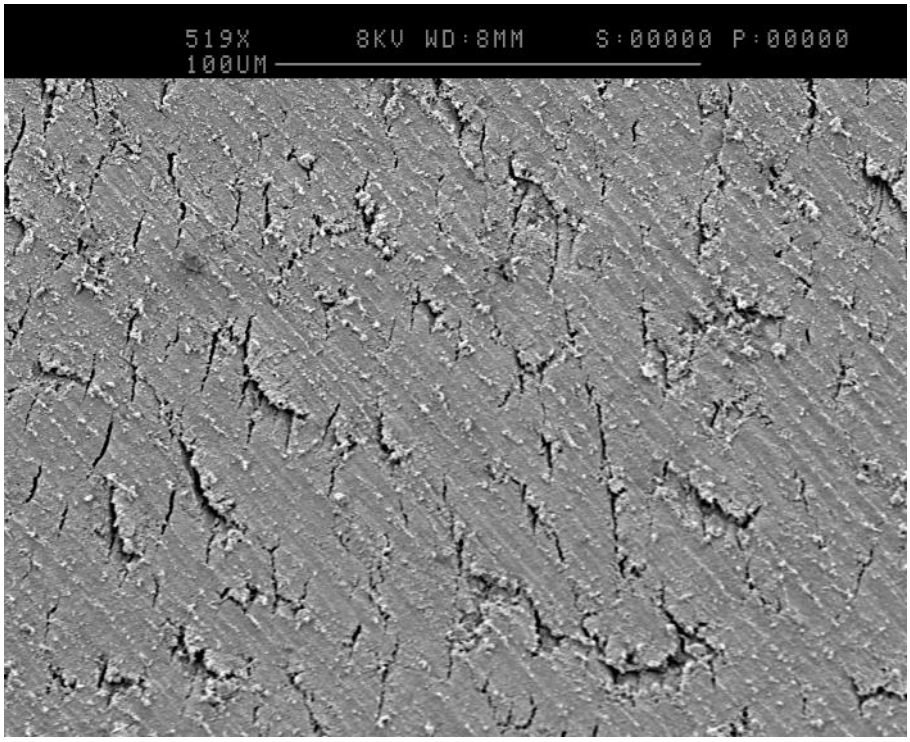

Figure 4.10. SEM image (500x magnification) to show the control group (Group 1) with complete smear layer (Hülsmann score 5).

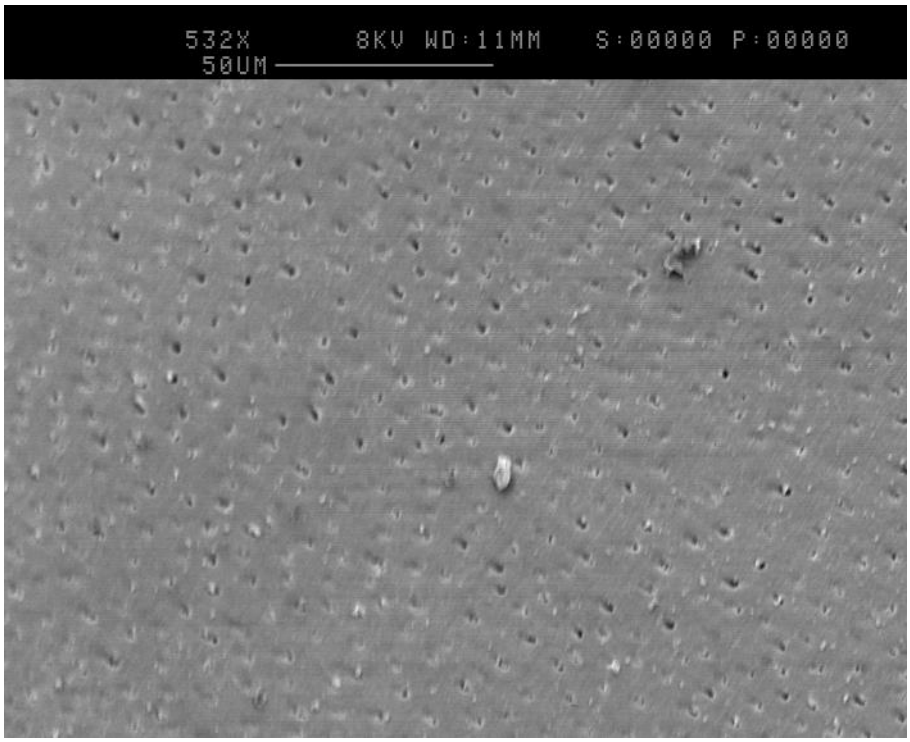

Figure 4.11. SEM image (500x magnification) to show a representative Group 2 specimen after polishing with sand papers (600, 800, 1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1).

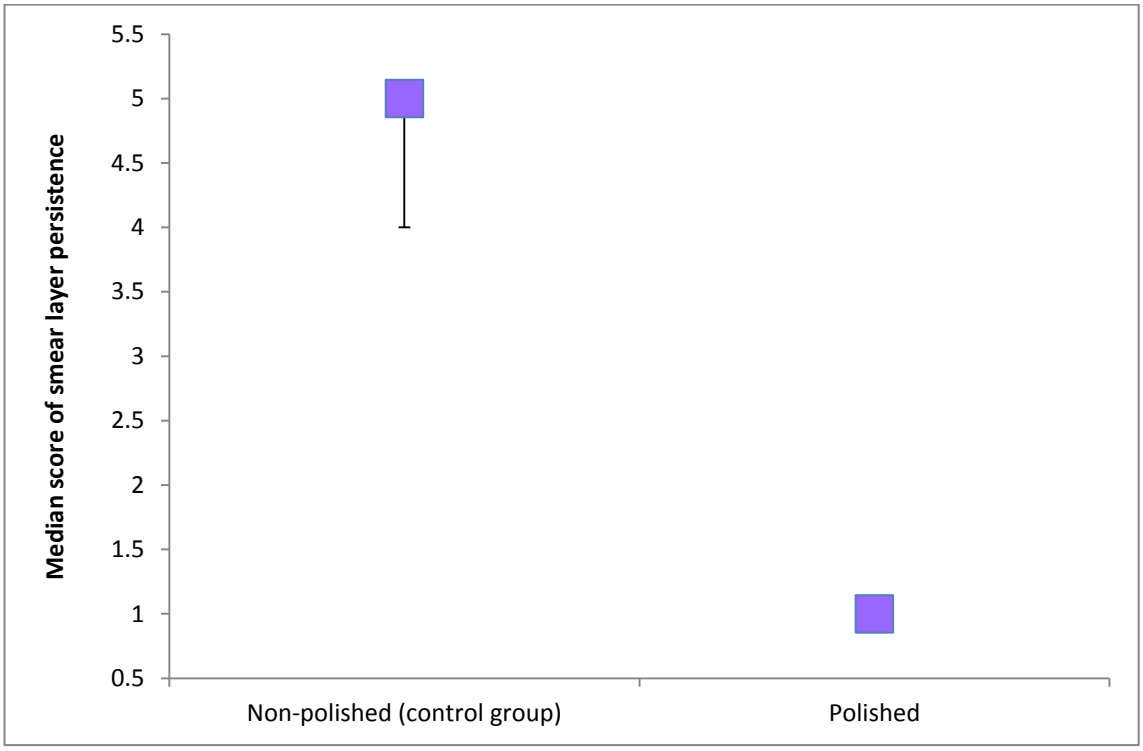

Figure 4.12. Median score of smear layer persistence on the surface of horizontal dentine slices of groups 1 and 2. The difference was significant ($p<0.05$, Kruskal-Wallis test). Smear layer scores used the Hülsmann *et al*'s. (1997) scale. The error bars are the maximum and minimum scores for each group.

4.3.4 Discussion

The results of this study showed that macro and micro polishing of dentine removed the smear layer from dentine surfaces. The results of this study were supported by other studies that adopted the method of macro and micro polishing to prepare specimens in order to measure the microhardness of horizontal dentine slices (Pimenta *et al.*, 2012; Tartari *et al.*, 2013), to observe demineralisation (De-Deus *et al.*, 2006; De-Deus *et al.*, 2008), to analyse the Ca/P or the relative mineral to collagen ratios of dentine structure (Dogan and Çalt, 2001; Ryou *et al.*, 2011), and to measure the nanohardness and modulus of elasticity by AFM (Cheron *et al.*, 2011).

Another study polished horizontal dentine slices with a fine grain grinding stone before the macro and micro polishing (Dayal *et al.*, 2005), and another study polished horizontal dentine slices only with macro polishing (Baron *et al.*, 2013). Yet other studies added ultrasonication as a third step to macro and micro polishing (Chng *et al.*, 2005; Forner *et al.*, 2009). The current study confirmed the ability of sequential macro and micro polishing to remove laboratory-generated smear layers. Although no formal investigations were undertaken to assess the smoothness of the samples in comparison with those managed in the previous investigation by brushing and ultrasonication, it was the observation of the operator that the surfaces appeared smoother, and suitable for AFM analysis. This was confirmed by subjecting specimens prepared in this way to AFM analysis without tip breakage. In an effort to reduce polishing steps and minimise the risks of physical damage to dentine, the next study attempted to remove smear layer and create smooth surfaces by micro polishing with aluminium oxide alone.

4.3.5 Conclusion

The results of this investigation rejected the null hypothesis and found that that macro and micro polishing were able to remove smear layer from the surface of mechanically cut horizontal slices of root dentine.

4.4 Micro polishing

4.4.1 Aim

This study aimed to remove smear layer from mechanically cut human dentine surfaces by micro polishing alone.

This study tested the null hypotheses that micro polishing alone had no effect on removal smear layer from mechanically cut horizontal sections of human root dentine.

4.4.2 Materials and methods

4.4.2.1 Teeth collection and storage

Thirty freshly extracted human premolar teeth were collected for this study. See section 3.2.2 for details of tooth collection and storage.

4.4.2.2 Specimen preparation

See section 3.2.2 for details of specimen preparation.

4.4.2.3 Treatment

Thirty horizontal sections of dentine were prepared from the cervical third of human roots; see section 3.2.2 for their preparation. The sections were divided randomly into 6 groups (n = 5):

Group 1 was not treated and served as a control of smear layer development.

Group 2 was sequentially polished with aluminium oxide, 1µm, 0.3µm and 0.05µm and a rotary micro polishing machine (Metaserv Rotary Pregrinder).

Group 3 was sequentially polished with P1200 abrasive papers (Norton) and a universal macro polisher (Metaserv), followed by aluminium oxide, 1µm, 0.3µm and 0.05µm and a rotary micro polishing machine (Metaserv Rotary Pregrinder).

Group 4 was sequentially polished with P1000 and P1200 abrasive papers, followed by aluminium oxide, 1µm, 0.3µm and 0.05µm.

Group 5 was sequentially polished with P800, P1000 and P1200 abrasive papers, followed by aluminium oxide, 1µm, 0.3µm and 0.05µm.

Group 6 was sequentially polished with P600, P800, P1000 and P1200 abrasive papers, followed by aluminium oxide, 1µm, 0.3µm and 0.05µm, see section 3.4.2 (the storage of the samples and EDAX preparation).

4.4.2.4 Storage of the samples and SEM preparation

See section 3.2.2 for details of samples and SEM preparation.

4.4.2.5 Analysis of the of the samples by the SEM

See section 4.2.2 for details of the analysis of the samples by the SEM. The results were analysed using the Kruskal-Wallis test ($p<0.05$) in SPSS 19.0 (SPSS Inc, Chicago, IL).

4.4.3 Results

Figure 4.19 shows median smear layer scores and maximum and minimum values for all groups. Samples in the control group, Group 1 exhibited complete surface coverage by a homogenous smear layer, with no open dentinal tubules (median score 5) (Figure 4.13). All of the experimental groups 2-6 were free from smear layer (median score 1) (Figure 4.14, Figure 4.15, Figure 4.16, Figure 4.17 and Figure 4.18 respectively). The differences between experimental and control groups were statistically significant ($p<0.05$).

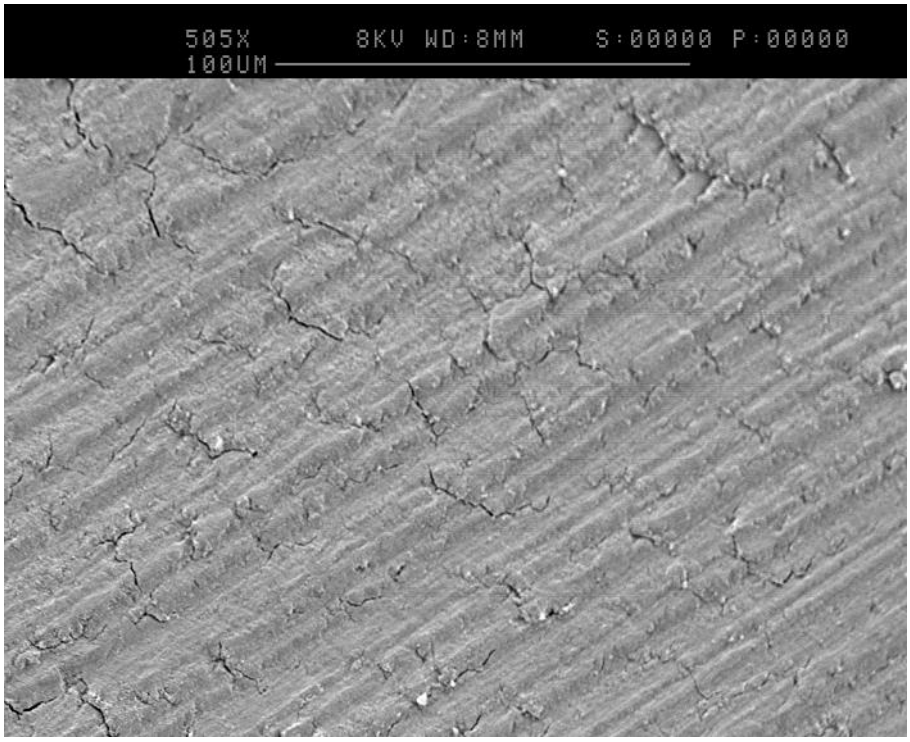

Figure 4.13. SEM image (500x magnification) to show the control group (Group 1) with complete smear layer (Hülsmann score 5).

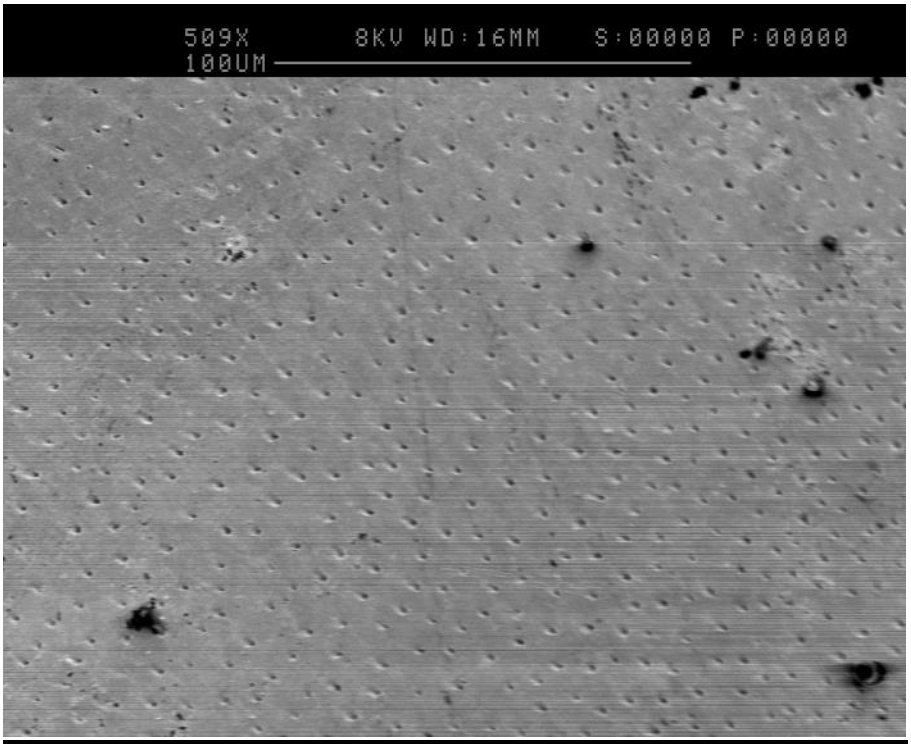

Figure 4.14. SEM image (500x magnification) to show representative Group 2 specimen after polishing with only aluminium oxide (Hülsmann score 1).

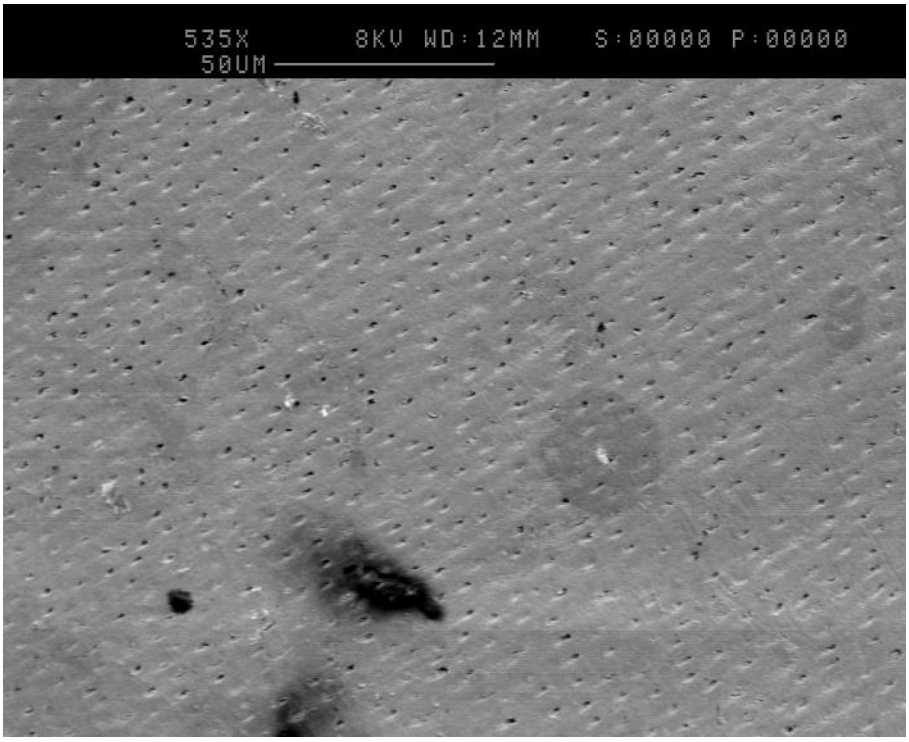

Figure 4.15. SEM image (500x magnification) to show representative Group 3 specimen after polishing with sand papers (1200µm), and then with aluminium oxide (Hülsmann score 1).

Figure 4.16. SEM image (500x magnification) to show representative Group 4 specimen after polishing with sand papers (1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1).

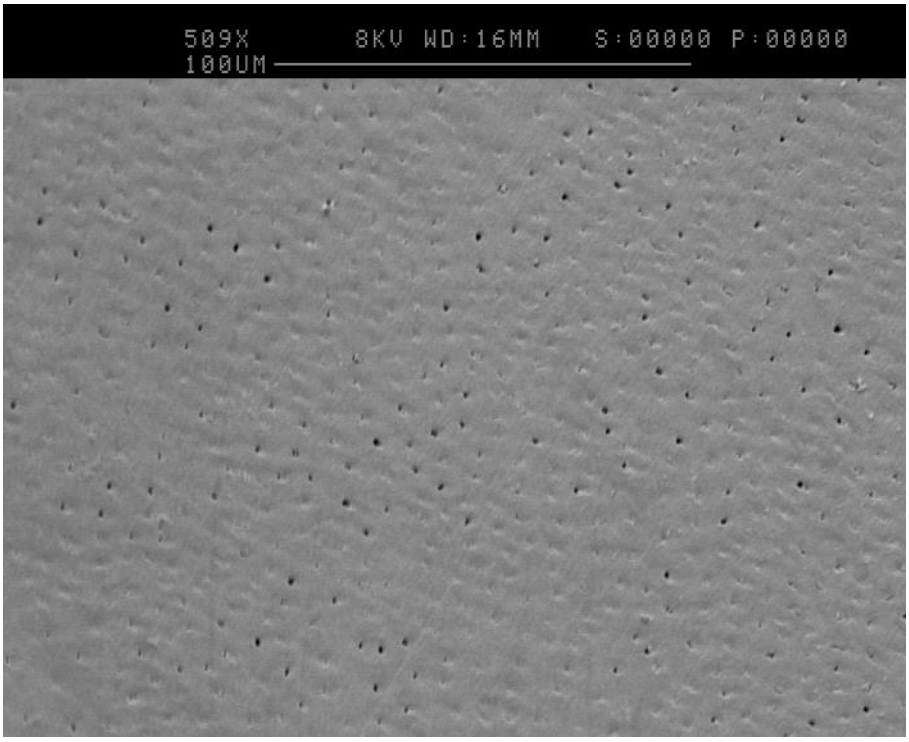

Figure 4.17. SEM image (500x magnification) to show representative Group 5 specimen after polishing with sand papers (800, 1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1).

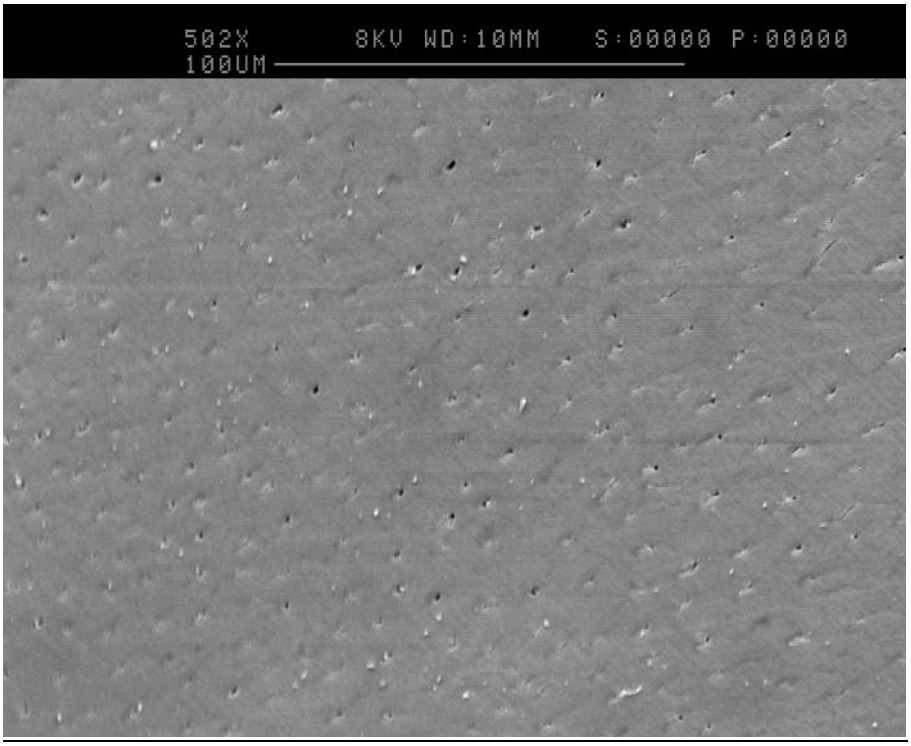

Figure 4.18. SEM image (500x magnification) to show representative Group 6 specimen after polishing with sand papers (600, 800, 1000 and 1200µm), and then with aluminium oxide (Hülsmann score 1).

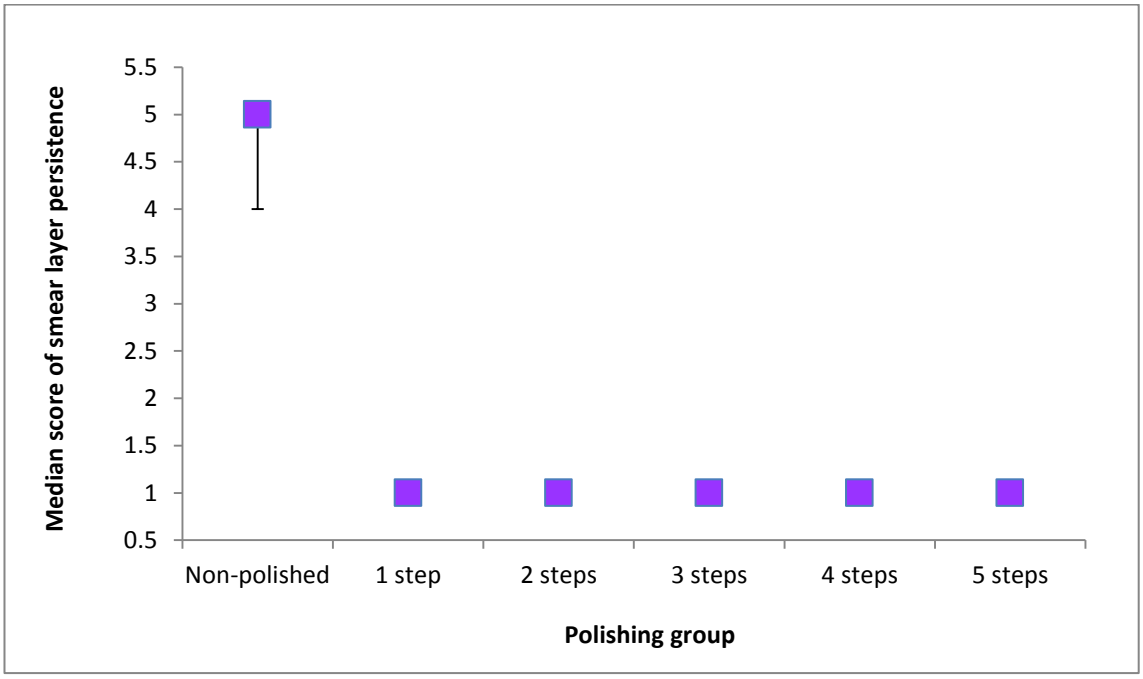

Figure 4.19. Median smear layer scores (Hülsmann *et al.*, 1997) for control (1) and experimental (2-6) Groups, following micro polishing. The difference between Group 1 and Groups 2-6 was significant ($p<0.05$, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the maximum and minimum scores for each group.

4.4.4 Discussion

The aim of this particular study was to remove smear layer and produce smooth surfaces by micro-polishing only. The method adopted was confirmed to remove saw-generated smear layer, and confirmed the findings of Marshall *et al* (2000) and Kinney *et al.* (1996) (Kinney *et al.*, 1996a). Marshal *et al* (2000) (Fong *et al.*, 2000) added the second step of ultrasonication after micro-polishing, and it was concluded from the current study that this did not appear to be necessary for smear layer elimination, see the AFM images in section 6.2 (Effect of NaOCl on human root dentine surface). The effects of post-polishing ultrasonication on other aspects of cleaning, such as the elimination of traces of polishing materials was not investigated. It should, however, be noted that the water employed for ultrasonication of specimens by Fong *et al.* (2000) was pH 6, and therefore had the potential to demineralise the dentine surfaces.

Fong (Fong *et al.*, 2000) also described the inclusion of 0.05µm aluminium powder to reduce specimen roughness, but it was necessary to keep the aluminium slurry at pH 3 in order to prevent particle agglomeration. This again presents a real risk of specimen demineralisation.

There was once again no systematic measurement of sample roughness following micro-polishing, but the testing of specimens within the AFM resulted in unacceptable risk of tip failure. This method therefore failed to meet both agendas, and was not adopted for the preparation of specimens for AFM analysis.

4.4.5 Conclusion

The results of this investigation rejected the null hypothesis and found that that smear layers generated during the sectioning of dental root specimens can be removed by using micro polishing of specimens, without need for macro polishing. This technique did not, however, result in specimens of sufficient smoothness for safe analysis by AFM.

Chapter 5: Removal of pulp debris and predentine from the walls of root canals

5.1.1 Introduction

In the literature, many studies have analysed the cleanliness of root canal walls by scoring debris and smear layer after various instrumentation and irrigation regimes (Hülsmann *et al.*, 1997; Hülsmann *et al.*, 2005). Debris is composed of pulp remnants and particles that attach to root canal walls in regions that may or may not have been touched by instruments. Debris is different from smear layer, since the smear layer is a surface film formed on dentine walls after instrumentation and consists of dentine particles, pulp remnants, retained irrigant and/or bacterial components (American Association of Endodontists Glossary, 1999). Smear layer can be seen only in instrumented root canals (McComb and Smith, 1975; Mader *et al.*, 1984).

It is necessary to remove pulpal tissue from root canals as part of the process of cleaning and shaping, since this may provide substrate for the metabolism of microorganisms (Distel *et al.*, 2002; Peters, 2004). Areas of root canal systems that have been untouched by instruments may also be covered with predentine, and whilst this may not provide nutrition for microorganisms, it is uncertain what impact its retention may have on the outcome of clinical endodontic treatments.

Removing residual pulp debris from root canals is largely reliant on the actions of irrigant solutions which may dissolve organic matter and create strong currents to carry dissolved and undissolved material away from the canal lumen and walls (Moser and Heuer, 1982; Chow, 1983; Sedgley *et al.*, 2005; Boutsioukis *et al.*, 2009; Tay *et al.*, 2010) (Shen *et al.*, 2012).

For optimal effectiveness, the irrigant should come in direct contact with the whole of the root canal surface, and it is recognised that this may be the most challenging in the apical third (Shen *et al.*, 2012). Factors that impact on the effectiveness of irrigants such as NaOCl include concentration, exposure time, temperature and volume of exchange, in addition to the method of delivery and the generation of shearing forces against the canal walls (Hand *et al.*, 1978). The tubular structure of dentine may also be important,

with microorganisms penetrating to depths of 200 µm or more from the root canal lumen (Haapasalo and Ørstavik, 1987; Love and Jenkinson, 2002; ElAyouti *et al.*, 2008; Paqué *et al.*, 2009a). Tubular penetration by disinfectant irrigants may again be influenced by factors including the concentration, temperature and exposure time of the irrigant, and the patency of the dentinal tubules (Hand *et al.*, 1978; Zou *et al.*, 2010).

Despite efforts to optimise root canal irrigation (Shen *et al.*, 2012), the ideal root canal irrigant and irrigation regime has yet to be defined (Cunningham and Balekjian, 1980; Cunningham and Joseph, 1980; Abou-Rass and Oglesby, 1981; Cotter *et al.*, 1985; Kamburis *et al.*, 2003; Sirtes *et al.*, 2005; Giardino *et al.*, 2006; Zehnder, 2006; Christensen *et al.*, 2008; Haapasalo *et al.*, 2010). NaOCl, with its antimicrobial and tissue-dissolving actions is currently the closest to ideal (Shen *et al.*, 2012).

The studies described in this chapter attempt to advance understanding by exploring the ability of NaOCl to remove pulpal debris and predentine from the walls of root canals, whilst doing the minimum damage to the underlying dentine.

5.2 Continuous irrigation to remove pulp debris and predentine from ovine root canal and its effect on the Ca/P ratios of root dentine

This study was undertaken to assess the ability of high-concentration NaOCl solutions to remove pulp debris and predentine from the walls of ovine root canals, whilst monitoring the effects on Ca/P ratios at the root canal lumen and at increasing depths within the root canal wall.

This study tested the null hypotheses that alteration of the concentration (5% and 10%), application time (0, 5 and 10 minutes) and temperature (25˚C and 60˚C) of NaOCl solutions would have no effects on the ability to remove pulp debris and predentine from the walls ovine root canals. In addition, this study tested the null hypothesis that the same variables would have no effect on the Ca/P ratio of ovine root dentine at different depths from the root canal lumen.

5.2.1 Materials and method

5.2.1.1 Teeth collection and storage

One hundred and sixty ovine mandibular incisor teeth were collected for this study. See section 3.2.2 for details of tooth collection and storage.

5.2.1.2 Specimen preparation

See section 3.2.2 for details of specimen preparation.

5.2.1.3 Treatment

Roots were divided randomly into four control groups, (n=10 per group; total control teeth 40), and twelve experimental groups (n=10 per group; total experimental teeth 120). At the start of the experiment, the apices of the roots were sealed with soft red wax (Metrodent Ltd, Huddersfield, UK) to simulate a closed-ended canal system, Figure 5.1.

A cylindrical space was created inside a mould of polystyrene based clear resin (Bondaglass, Bondaglass-Voss Ltd.), in a 30 mm cylindrical sample mould

(BUEHLER® USA). This cylindrical space was used to fix the root during irrigation, Figure 5.1.

Two concentrations of NaOCl (5% and 10%) were freshly prepared from a 10% stock solution of NaOCl (Sigma-Aldrich, UK), by dilution with distilled water. Firstly, 1 L of distilled water was added to 1 L of 10% NaOCl stock solution (1 L 10% NaOCl: 1 L distilled water), before confirming the concentration by iodometric titration.

Temperature control of NaOCl solutions (25˚C and 60˚C) was achieved by placing them in closed beakers, immersed in temperature-regulated water baths.

Control Groups (Groups 1-4):

G1 (non-treated):

In this group, the roots received no further treatment after gross pulp extirpation with a barbed broach, and were immersed in fixative (2% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3) to preserve canal contents for SEM analysis.

G2, G3 and G4:

In these groups, 3 mL distilled water was applied to root canals via a Luer-Loc syringe with a 27-gauge Monoject endodontic needle (Monoject, Gosport, UK), before inserting an ultrasonic file size 15 (Maillefer, Ballaigues, Switzerland) to within 1 mm of the root apex and activating with a piezoelectric ultrasonic unit (Piezon Master, EMS, Le Sentier, Switzerland) for 30 seconds at power level three; seeking at all times to avoid contact of the activated file with the canal walls (Lee *et al.,* 2004; van der Sluis *et al.*, 2005).

The distilled water was then left inside the canal for 0 minutes before a final flush with distilled water. This treatment was conducted once in case of the Group 2 (0 minute treatment), twice for Group 3 (5 minute treatment) and 3 times for Group 4 (10 minute treatment), see Table 5.1.

Experimental groups (Groups 5 to 16; see Table 5.1):

The same steps which were used in the control (distilled water) groups (2, 3 and 4) were repeated using NaOCl solutions of different concentrations and temperatures in experimental Groups 5, 6 and 7 (5% at 25˚C); Groups 8, 9 and 10 (5% at 60˚C); Groups11, 12 and 13 (10% at 25˚C); and Groups 14, 15 and 16 (10% at 60˚C).

Finally, immediately after treatment, all canals were flushed with 3mL distilled water via a Luer-Loc syringe with a 27-gauge Monoject endodontic needle (Monoject, Gosport, UK), inserted to within 1mm of root-end. The apical seals of soft red wax were then removed, before fixing roots by immersion in 2% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3, prior to SEM or EDAX analysis.

5.2.1.4 Storage of the samples and SEM and EDAX preparation

Half of the specimens (n=5 for each group) were removed from the fixative and prepared for the SEM (see section 3.2.2 for SEM preparation).

The other half of the specimens (n=5 for each group) were removed from the fixative and prepared for EDAX. Horizontal sections were then prepared from the cervical third of each root, see section 3.4.2 for details of storage of the samples and EDAX preparation.

5.2.1.5 Analysis of the of the samples by the SEM and the EDAX

Extensive pilot studies revealed that the widely used Hülsmann *et al.* (1997) scoring system was unsuitable for scoring pulpal debris and predentine on the walls of the uninstrumented root canals included in this study.

A new scoring system was therefore developed as follows (appendix B):

Score 1: Cellular debris and intact predentine layer observed. No evidence of exposed calcospherites.

Score 2: No cellular debris, but intact layer of predentine. No evidence of exposed calcospherites.

Score 3: Predentine partly removed. Evidence of exposed calcospherites.

Score 4: Complete removal of predentine. No evidence of dentine demineralisation.

Score 5: Complete removal of predentine. Evidence of dentine demineralisation.

An overview of the whole specimen was first done at low magnification to identify representative areas in each third of the root, before capturing images at higher magnification. Representative SEM images (500x magnification) were then captured of canal walls in the apical, middle and coronal third, and images were scored by a single

operator. The scoring was done after coding of the SEM images, so the observer examined the images in blind manner. Repeat scoring was undertaken to ensure intra-observer reliability. Analysis was undertaken in SPSS 19.00 (SPSS Inc, Chicago, IL) to compare the scores, using the chi square test ($p<0.05$).

See section 3.4.2 for details of analysis of the samples by the EDAX. The analysis of Ca/P ratios was undertaken in SPSS using the Kruskal-Wallis and Mann-Whitney U tests ($p<0.05$).

Figure 5.1. Cylindrical mould with a tooth mounted in soft red wax to hold it in position and create an apically closed system for irrigation.

Table 5.1. Summary of treatment regimes for ovine roots.

Group	Treatment	Concentration	Temperature (°C)	Time (min)
1	No-treatment	N/A	N/A	N/A
2	H_2O	N/A	N/A	0
3	H_2O	N/A	N/A	5
4	H_2O	N/A	N/A	10
5	NaOCl	5%	25	0
6	NaOCl	5%	25	5
7	NaOCl	5%	25	10
8	NaOCl	5%	60	0
9	NaOCl	5%	60	5
10	NaOCl	5%	60	10
11	NaOCl	10%	25	0
12	NaOCl	10%	25	5
13	NaOCl	10%	25	10
14	NaOCl	10%	60	0
15	NaOCl	10%	60	5
16	NaOCl	10%	60	10

5.2.2 Results

Untreated control teeth (Group 1) had pulp debris and predentine scores of 1. Similar results were found for all of the groups treated with distilled water (Groups 2-4).

In the experimental groups 5-16, pulp debris and predentine was either partially removed, with some exposure of calcospherites (score 3), or the pulp debris and predentine was completely removed (score 4), Figure 5.3, Figure 5.4 and Figure 5.5. Some images also showed damage to the root canal walls, probably caused by the ultrasonically energised file (Figure 5.5). No attempt was, however, made to assess formally the frequency or severity of canal wall damage caused by ultrasonic instruments.

Figures 5.6-5.17 show that all treatment regimes involving NaOCl resulted in significantly lower debris and predentine scores than regimes involving distilled water. Figure 5.6, Figure 5.7 and Figure 5.8 show that there was a significant difference between the groups treated with 5% NaOCl at 25°C for 0, 5 and 10 minutes (Groups 5, 6 and 7) and the control groups 1-4, at the cervical, middle and apical thirds. Figure 5.9, Figure 5.10 and Figure 5.11 show that there was a significant difference between the groups treated with 5% NaOCl at 60°C for 0, 5 and 10 minutes (Groups 8, 9 and 10) and the control groups 1-4, at the cervical, middle and apical thirds.

Figure 5.12, Figure 5.13 and Figure 5.14 show that there was a significant difference between the groups treated 10% NaOCl at 25°C for 0, 5 and 10 minutes (Groups 11, 12 and 13) and the control groups 1-4, at the cervical, middle and apical thirds. Figure 5.15, Figure 5.16 and Figure 5.17 show that there was a significant difference between the groups treated with 10% NaOCl at 60°C for 0, 5 and 10 minutes (Groups 14, 15 and 16) and the control groups 1-4, at the cervical, middle and apical thirds.

Figure 5.18 shows that NaOCl treatments were more effective than untreated control and water treatments at all levels (coronal, middle and apical). Within the NaOCl treatment groups, increasing the concentration of the solution and increasing the time of application had a greater effect than increasing the temperature.

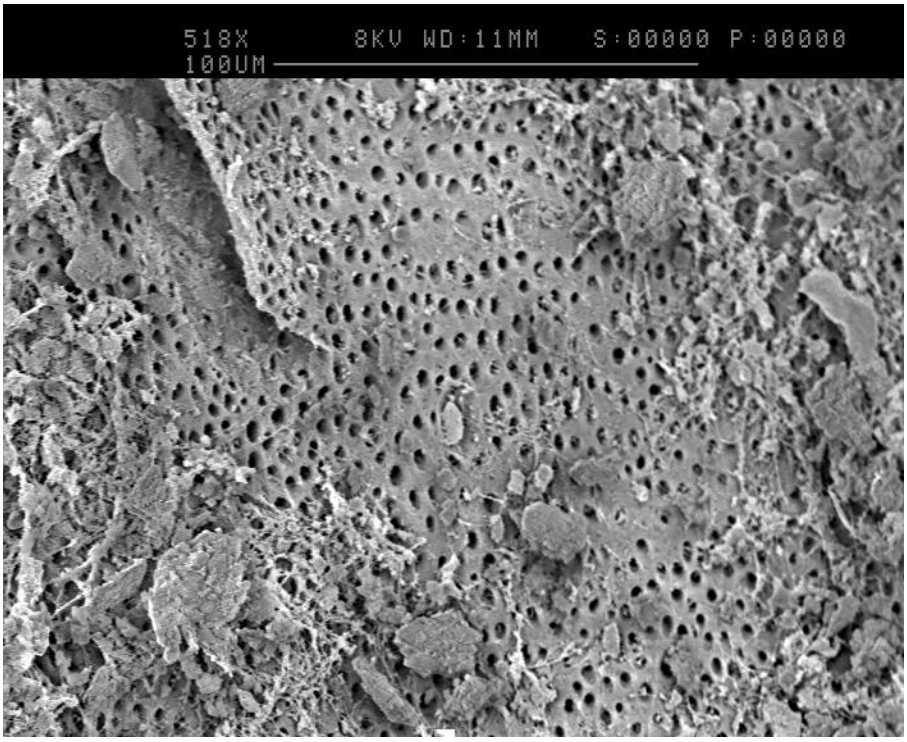

Figure 5.2. SEM image (500x magnification) of the ovine root canal wall in the cervical third to show debris and predentine Score 1.

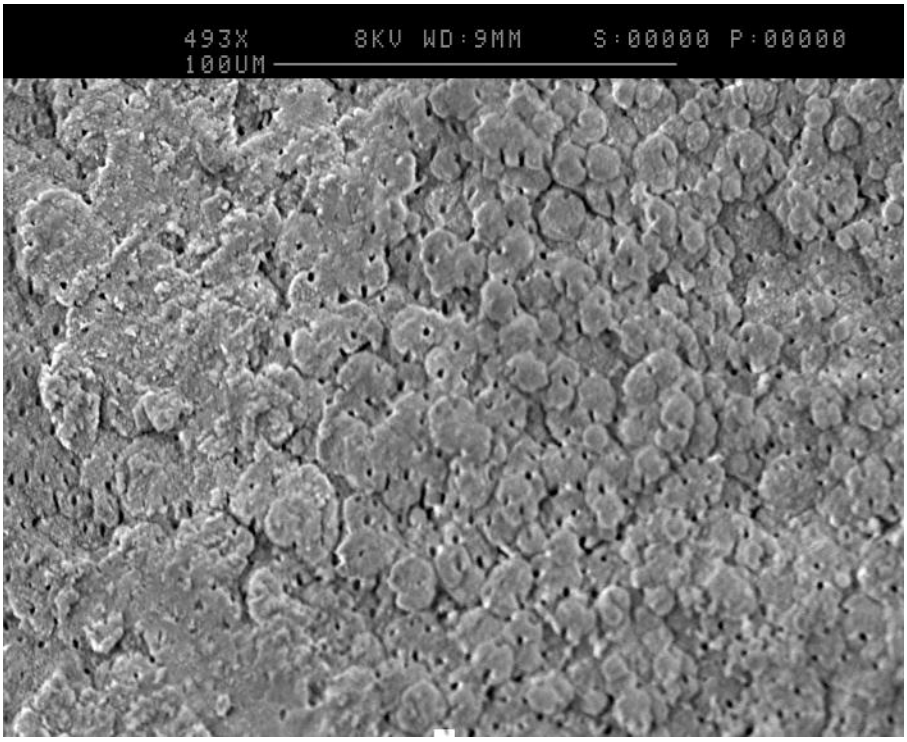

Figure 5.3. SEM image (500x magnification) of the root canal wall in the cervical third after dynamic continuous replenishment with 5% NaOCl at 25˚C for 5 minutes (Group 6), with a debris and predentine score of 3.

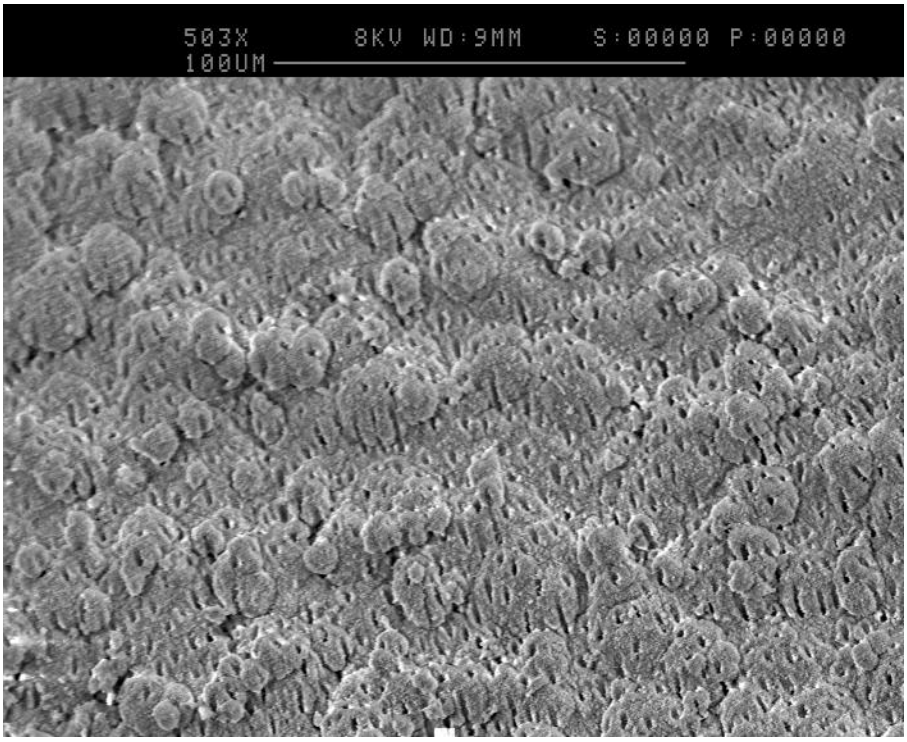

Figure 5.4. SEM image (500x magnification) of the root canal wall in the cervical third after dynamic continuous replenishment with 5% NaOCl at 25˚C for 10 minutes (Group 7), with debris and predentine score of 4.

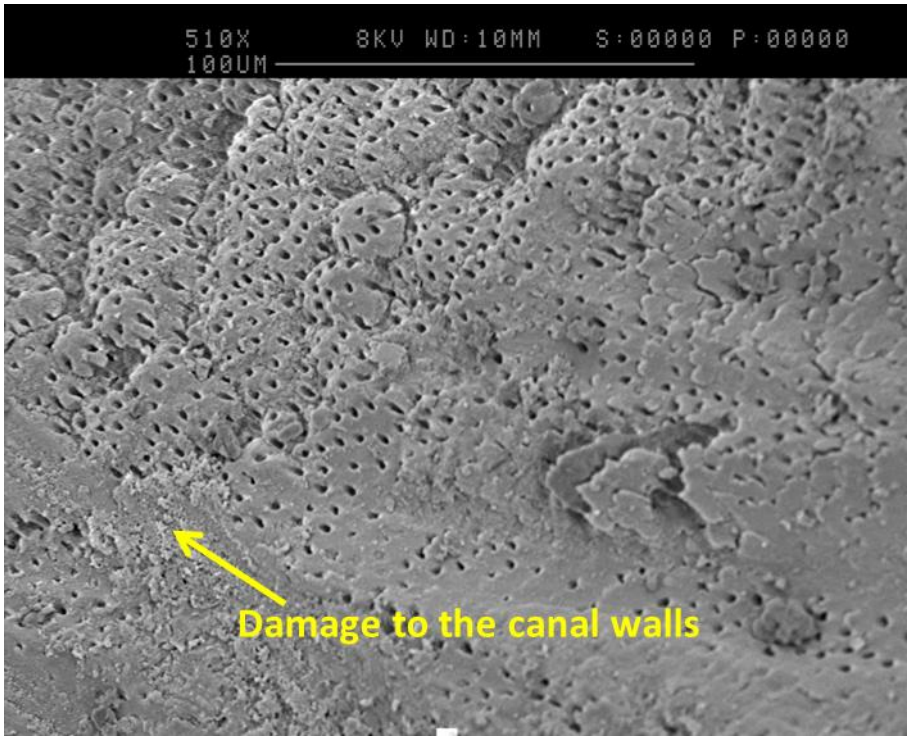

Figure 5.5. SEM image (500x magnification) of the root canal wall in the cervical third after dynamic continuous replenishment with 10% NaOCl at 25˚C for 5 minutes (Group 9), with debris and predentine score of 4. Damage to the canal walls was noted, probably from contact with the ultrasonic file.

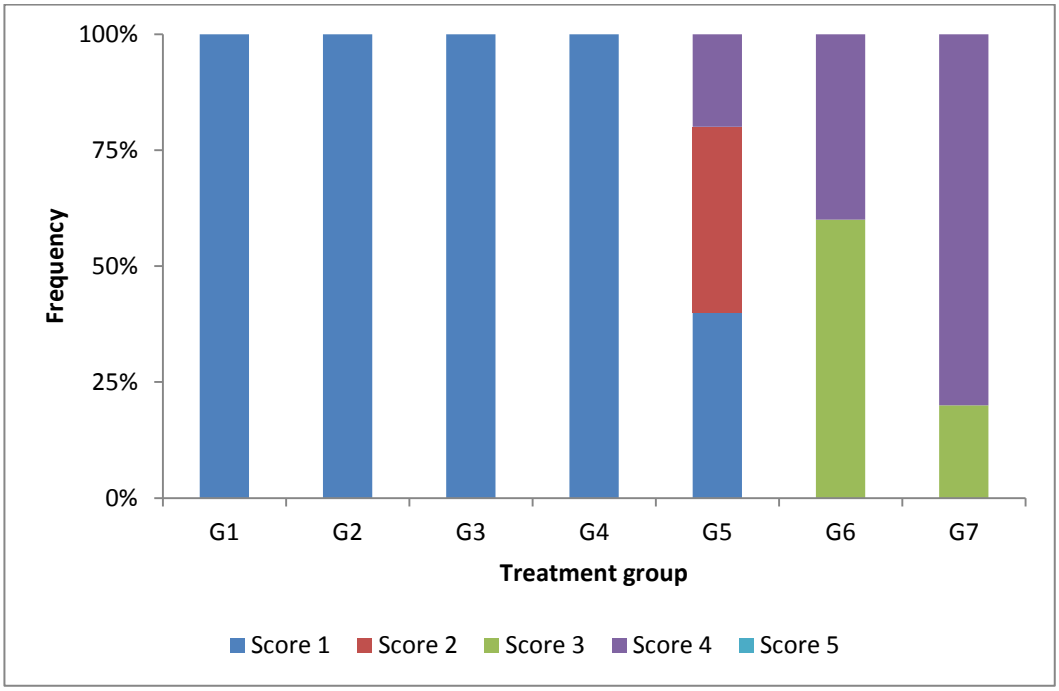

Figure 5.6. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 25°C for 0, 5 and 10 minutes. The difference between all experimental groups and the controls was significant ($p<0.05$, chi square test).

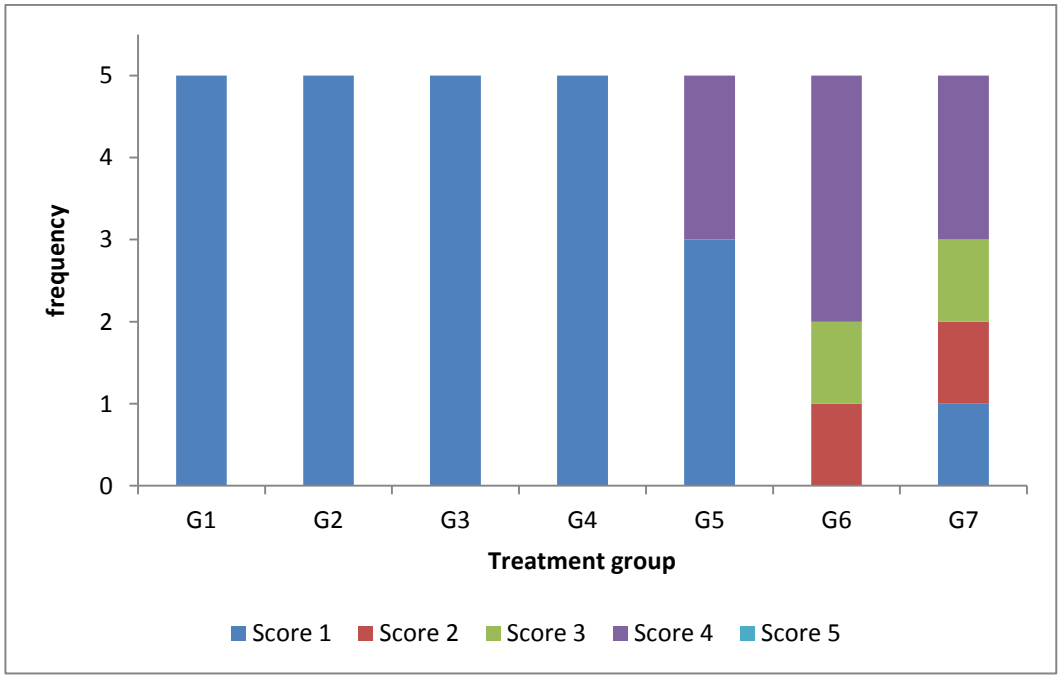

Figure 5.7. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 25°C for 0, 5 and 10 minutes. The difference between all of the experimental groups and the controls was significant ($p<0.05$, chi square test).

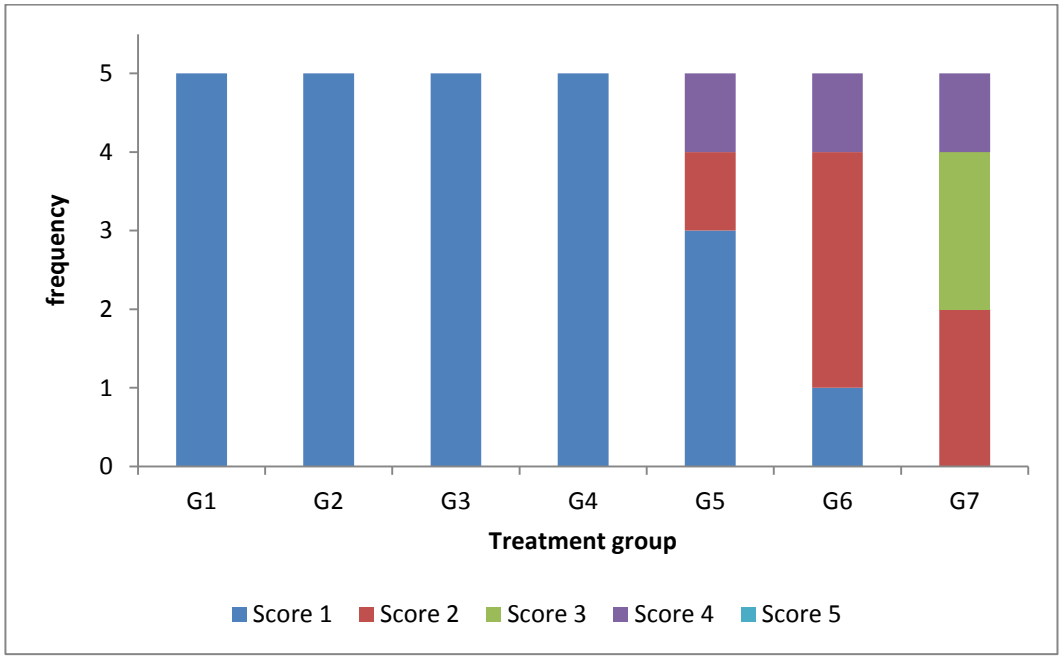

Figure 5.8. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 25°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

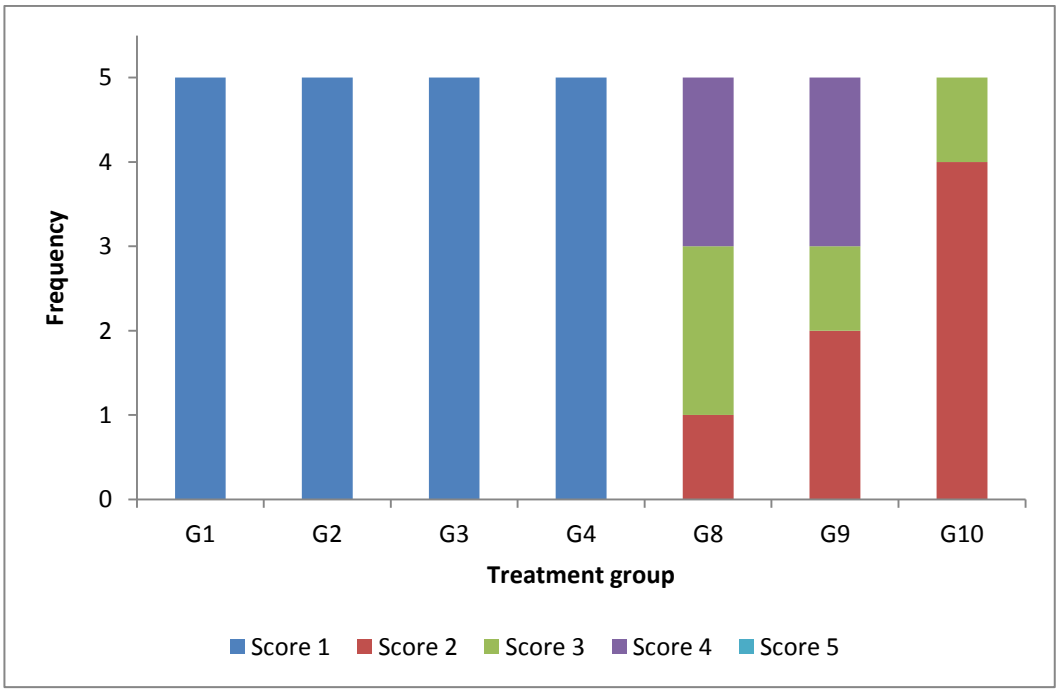

Figure 5.9. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled for water 0, 5 and 10 minutes and irrigation with 5% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

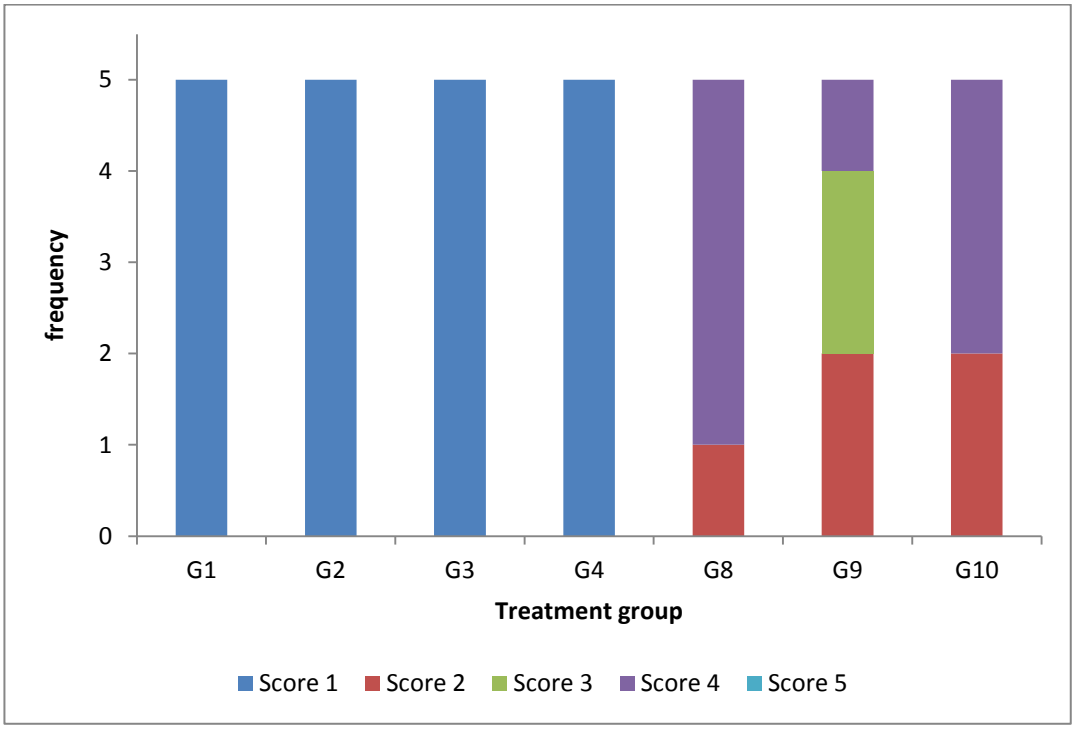

Figure 5.10. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 5% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

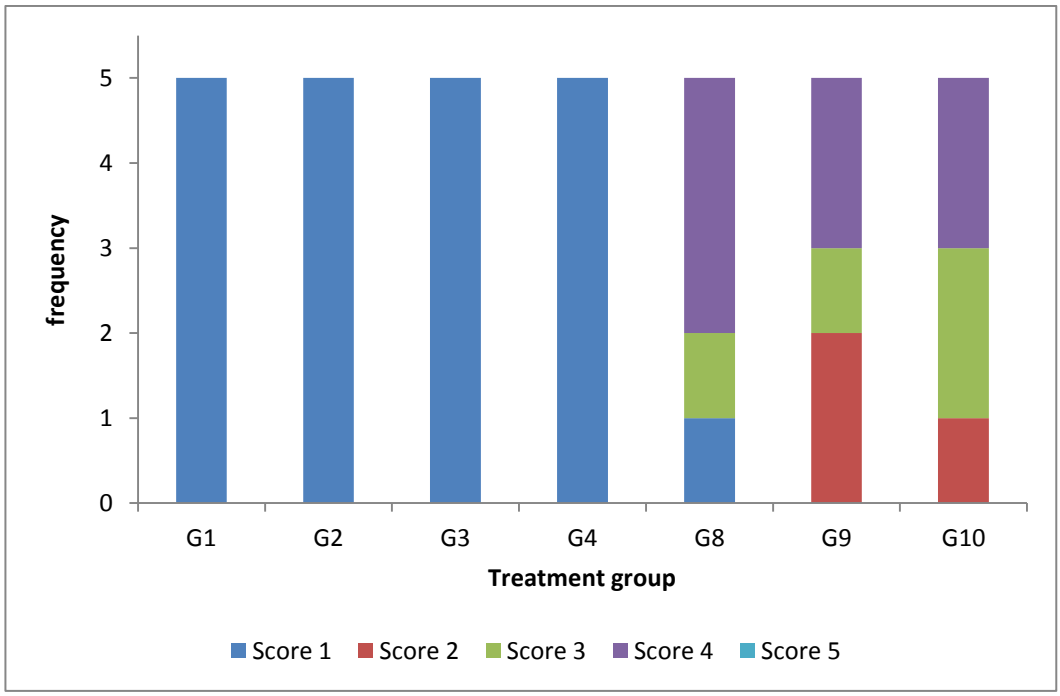

Figure 5.11. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0 minutes, 5 minutes and 10 minutes and irrigation with 5% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

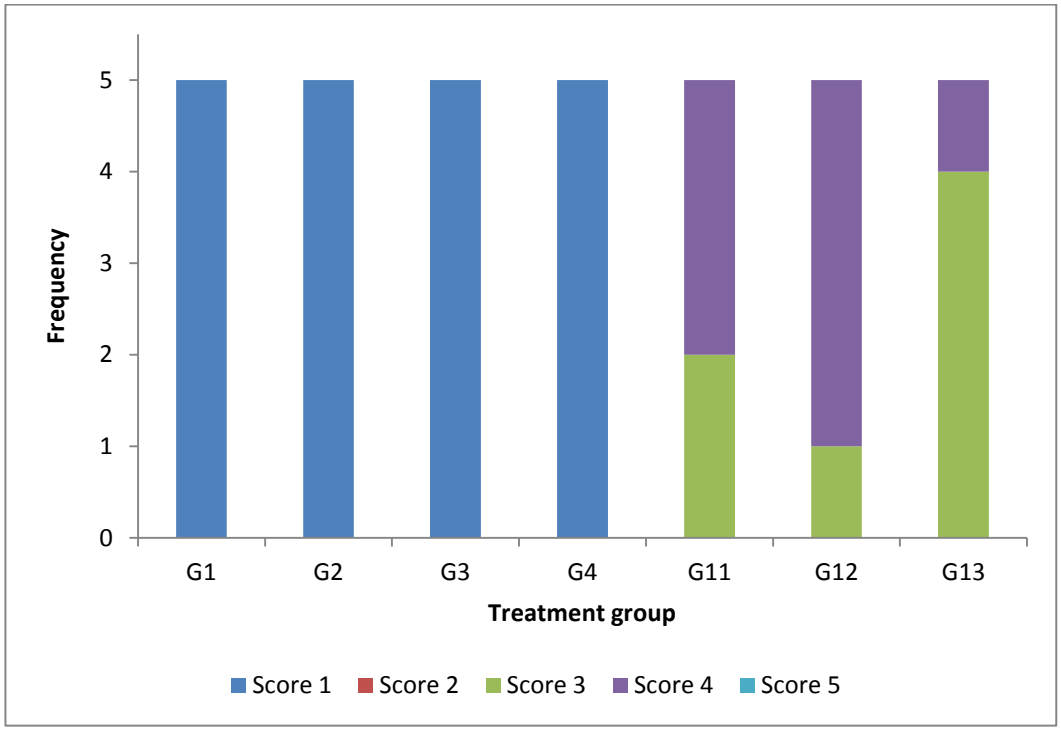

Figure 5.12. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 25°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

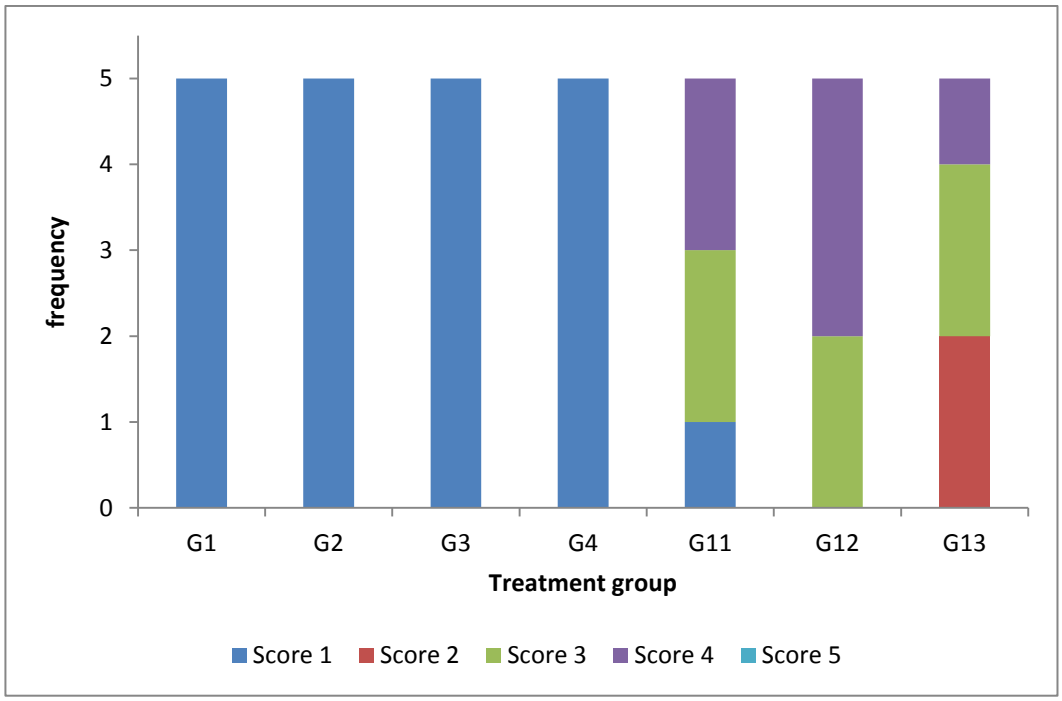

Figure 5.13. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 25°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

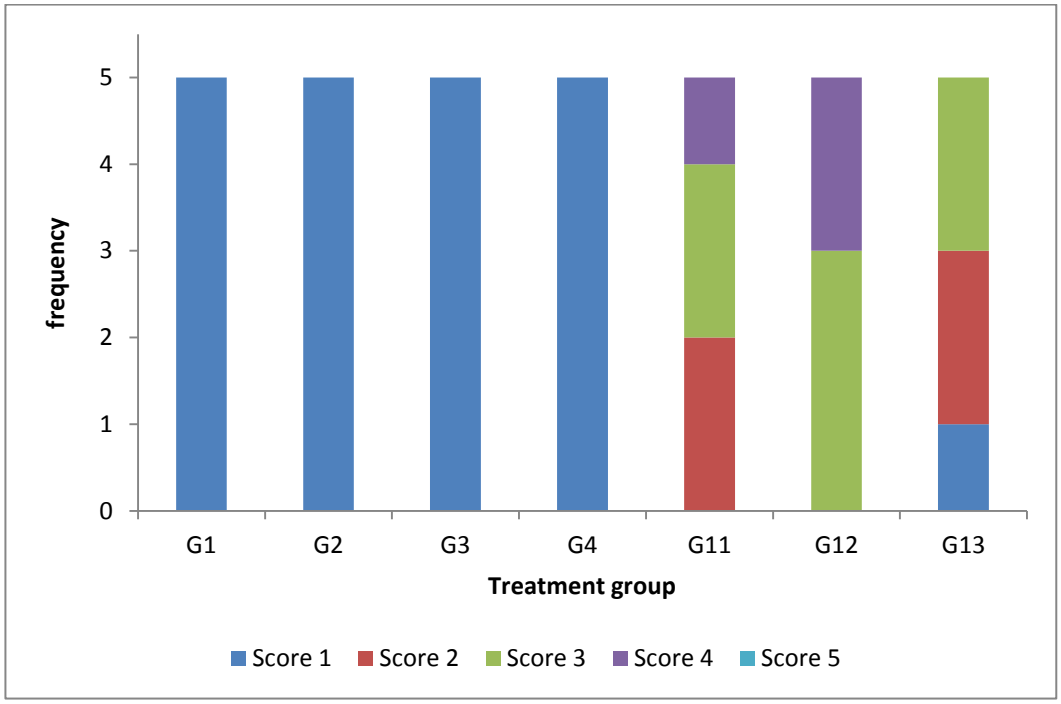

Figure 5.14. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 25°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

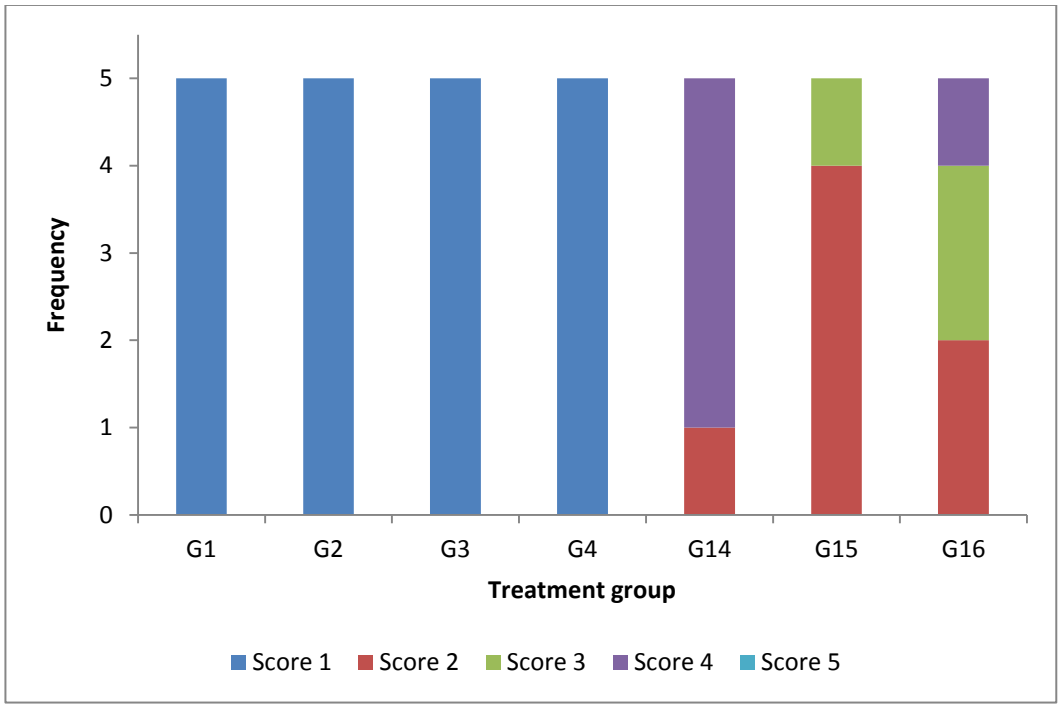

Figure 5.15. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

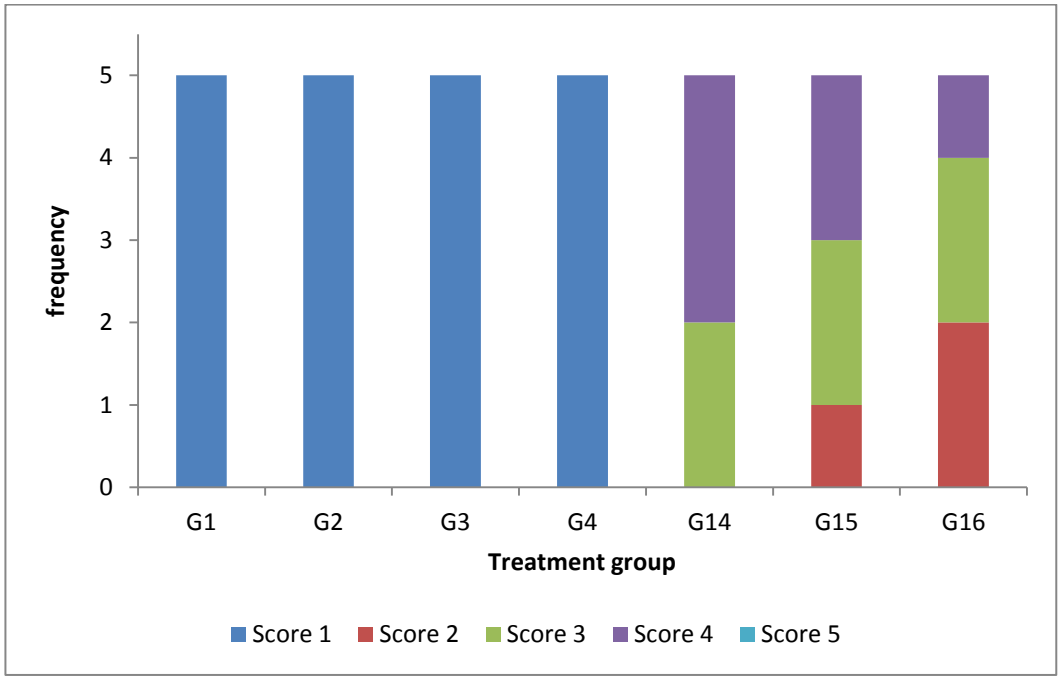

Figure 5.16. Pulp debris and predentine scores from the middle thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

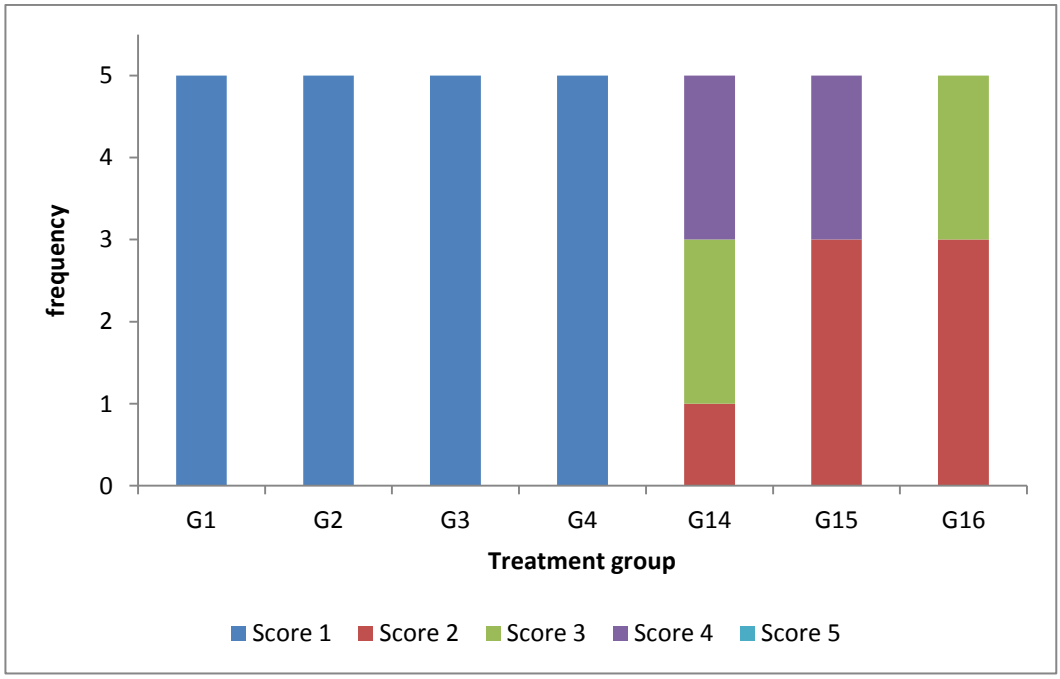

Figure 5.17. Pulp debris and predentine scores from the apical thirds of ovine root canals after no treatment (control), irrigation with distilled water for 0, 5 and 10 minutes and irrigation with 10% NaOCl at 60°C for 0, 5 and 10 minutes. The difference was significant between experimental groups and the control groups ($p<0.05$, chi square test).

Chi-Square Tests

Place on the root		Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Cervical	Pearson Chi-Square	119.945[a]	45	.000	.[b]		
	Likelihood Ratio	127.020	45	.000	.000		
	Fisher's Exact Test	84.213			.000		
	Linear-by-Linear Association	27.665[c]	1	.000	.000	.000	.000
	N of Valid Cases	80					
Middle	Pearson Chi-Square	93.133[d]	45	.000	.[b]		
	Likelihood Ratio	109.913	45	.000	.000		
	Fisher's Exact Test	72.462			.000		
	Linear-by-Linear Association	27.599[e]	1	.000	.000	.000	.000
	N of Valid Cases	80					
Apical	Pearson Chi-Square	89.566[f]	45	.000	.[b]		
	Likelihood Ratio	107.145	45	.000	.000		
	Fisher's Exact Test	70.612			.000		
	Linear-by-Linear Association	22.817[g]	1	.000	.000	.000	.000
	N of Valid Cases	80					

a. 64 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

b. Cannot be computed because there is insufficient memory.

c. The standardized statistic is 5.260.

d. 64 cells (100.0%) have expected count less than 5. The minimum expected count is .75.

e. The standardized statistic is 5.253.

f. 64 cells (100.0%) have expected count less than 5. The minimum expected count is 1.13.

g. The standardized statistic is 4.777.

Figure 5.18. Chi square analysis to identify significant differences in pulp debris and predentine scores for ovine root canals in their cervical, middle and apical thirds after no-treatment and irrigation with distilled water and irrigation with 5% NaOCl at 25°C, 5% NaOCl at 60°C, 10% NaOCl at 25°C, 10% NaOCl at 60°C, for 0 minutes, 5 minutes and 10 minutes respectively. NaOCl treatments were significantly more effective than untreated control and water treatments at all levels (cervical, middle and apical thirds) ($p<0.05$, chi square test).

Table 5.2 and Table 5.3, show the median scores (upper and lower quartiles) of Ca/P ratios for the untreated control (Group 1), water treated controls (Groups 1-4) and NaOCl treated experimental Groups (5-16). These ratios were from the cervical third of

the root at five different depths from the lumen of the root canal, 0µm, 100µm, 200µm, 300µm and 500µm.

Figure 5.19, Figure 5.20, Figure 5.21, Figure 5.22 and Figure 5.23 show the Ca/P ratio of the control Groups 1-4 and the experimental Groups 5-16 at all depths from the lumen of the root canal at the cervical third. In the non-treated group Ca/P ratios were similar at the lumen and deeper areas, with the exception of 1000µm, where the ratio was higher.

Treatment with distilled water (Groups 2, 3 and 4) resulted in no significant change of Ca/P ratio in comparison with the non-treated group (Group 1) ($p>0.05$), except at a depth of 100µm in Group 4 and at 300µm in Groups 2 and 4.

There was no significant difference between the groups treated with 5% NaOCl at 25°C for 0 minutes, 5 minutes and 10 minutes (Groups 5, 6 and 7) and the non-treated control group (Group1) ($p>0.05$), except at the lumen after 5 minutes treatment ($p<0.05$).

There was no significant difference between the group treated with 5% NaOCl at 60°C for 0 minutes 5 minutes and 10 minutes (Groups 8, 9 and 10) and the untreated control group (Group1) ($p>0.05$), except at 100µm and 200µm after 0 minutes treatment, at the lumen and at 300µm after 5 minutes treatments and at the lumen, at 100µm, at 200µm and at 300µm following 10 minutes treatment.

Furthermore, there was no significant difference between the groups treated with 10% NaOCl at 25°C for 0 minutes, 5 minutes and 10 minutes (Groups 11, 12 and 13) and the untreated control group (Group1) at all points ($p>0.05$), except at the lumen and at 200µm with 0 minutes treatment ($p<0.05$).

Again, there was no significant difference between the groups treated with 10% NaOCl at 60°C for 0 minutes, 5 minutes and 10 minutes (Groups 14, 15 and 16) and the untreated control group (Group1) at all points ($p>0.05$), except at 100µm after 0 minutes treatment, at the lumen after 5 minutes treatment and at the lumen and at 100µm following 10 minutes treatment ($p<0.05$).

Table 5.2. Median (upper and lower quartiles) of the Ca/P ratio of the cervical third of root dentine in control and experimental groups at different depths, and the significant (a) and non-significant (b) differences ($p<0.05$), between the non-treated control (Group 1) and the other Groups (2-16).

Treatment group	At 0µm	At 100 µm	At 200 µm	At 300µm	At 1000µm
Group 1: No-treatment	**1.95 (1.98 & 1.89)**	**2.0 (2.00 & 194)**	**2.0 (2.10 & 1.94)**	**2.0 (2.1 & 1.99)**	**2.2 (2.30 & 2.16)**
Group 2: Distilled H_2O 0 minutes	**2.0 (2.24 & 1.95)b**	**2.0 (2.10 & 1.99)b**	**2.1 (2.10 & 2.00)b**	**2.1 (2.10 & 2.00)a**	**2.2 (2.22 & 2.1)b**
Group 3: Distilled H_2O 5 minutes	**2.0 (2.66 & 1.90)b**	**2.1 (2.21 & 2.00)b**	**2.0 (2.10 & 1.99)b**	**2.0 (2.10 & 2.00)b**	**2.3 (2.30 & 2.19)b**
Group 4: Distilled H_2O 10 minutes	**1.94 (2.00 & 1.82)b**	**2.1 (2.66 & 2.10)a**	**2.0 (2.30 & 1.96)b**	**2.1 (2.20 & 2.10)a**	**2.2 (2.30 & 2.16)b**
Group 5: 5% NaOCl 25˚C 0 minutes	**1.9 (1.99 & 1.89)b**	**2.0 (2.10 & 1.87)b**	**2.0 (2.10 & 1.99)b**	**2.0 (2.12 & 1.93)b**	**2.2 (2.32 & 1.13)b**
Group 6: 5% NaOCl 25˚C 5 minutes	**2.1 (2.10 & 2.00)a**	**2.1 (2.10 & 1.98)b**	**2.0 (2.100 & 1.98)b**	**2.1 (2.24 & 2.10)b**	**2.2 (2.30 & 2.14)b**
Group 7: 5% NaOCl 25˚C 10 minutes	**1.92 (2.10 & 1.86)b**	**2.0 (2.17 & 1.99)b**	**2.0 (2.11 & 1.99)b**	**2.0 (2.16 & 1.97)b**	**2.2 (2.28 & 2.13)b**
Group 8: 5% NaOCl 60˚C 0 minutes	**2.0 (2.11 & 1.86)b**	**2.1 (2.16 & 2.00)a**	**2.1 (2.25 & 2.10)a**	**2.2 (2.22 & 2.10)b**	**2.2 (2.23 & 1.9)b**

Table 5.3. It is a continuation of the previous table. It shows median (upper and lower quartiles) of the Ca/P ratio of root dentine at different depths and the significance (a) and non-significance (b) differences ($p<0.05$), between the non-treated group (group 1) and the other groups 2-16.

Treatment group	At 0µm	At 100 µm	At 200 µm	At 300µm	At 1000µm
Group 9: 5% NaOCl 60˚C 5 minutes	**2.2 (2.32 & 2.00)a**	**2.0 (2.15 & 1.97)b**	**2.2 (2.18 & 2.10)b**	**2.1 (2.27 & 2.00)a**	**2.3 (2.33 & 2.22)b**
Group 10: 5% NaOCl 60˚C 10 minutes	**2.1 (2.26 & 2.00)a**	**2.1 (2.33 & 2.10)a**	**2.2 (2.20 & 2.00)a**	**2.2 (2.27 & 2.11)a**	**2.2 (2.24 & 2.10)b**
Group 11: 10% NaOCl 25˚C 0 minutes	**2.1 (2.33 & 1.97)a**	**2.1 (2.25 & 1.99)b**	**2.1 (2.24 & 2.10)a**	**2.2 (2.26 & 2.00)b**	**2.1 (2.26 & 2.10)b**
Group 12: 10% NaOCl 25˚C 5 minutes	**1.9 (2.100 & 1.85)b**	**2.1 (2.12 & 1.95)**	**2.2 (2.10 & 1.99)b**	**2.0 (2.00 & 1.93)b**	**2.1 (2.28 & 2.11)b**
Group 13: 10% NaOCl 25˚C 10 minutes	**1.94 (1.99 & 1.89)b**	**2.0 (2.10 & 1.97)b**	**2.0 (2.10 & 1.94)b**	**2.1 (2.15 & 2.00)b**	**2.1 (2.22 & 2.00)b**
Group 14: 10% NaOCl 60˚C 0 minutes	**2.0 (2.21 & 1.96)b**	**2.1 (2.27 & 2.00)a**	**2.2 (2.34 & 2.00)b**	**2.1 (2.20 & 2.00)b**	**2.2 (2.28 & 2.10)b**
Group 15: 10% NaOCl 60˚C 5 minutes	**2.1 (2.16 & 1.99)a**	**2.1 (2.13 & 1.94)b**	**2.0 (2.10 & 1.94)b**	**2.1 (2.13 & 2.00)b**	**2.1 (2.24 & 2.10)b**
Group 16: 10% NaOCl 60˚C 10 minutes	**2.2 (2.27 & 1.16)a**	**2.1 (2.32 & 2.00)a**	**2.2 (2.29 & 2.00)b**	**2.2 (2.21 & 2.00)b**	**2.2 (2.20 & 2.10)b**

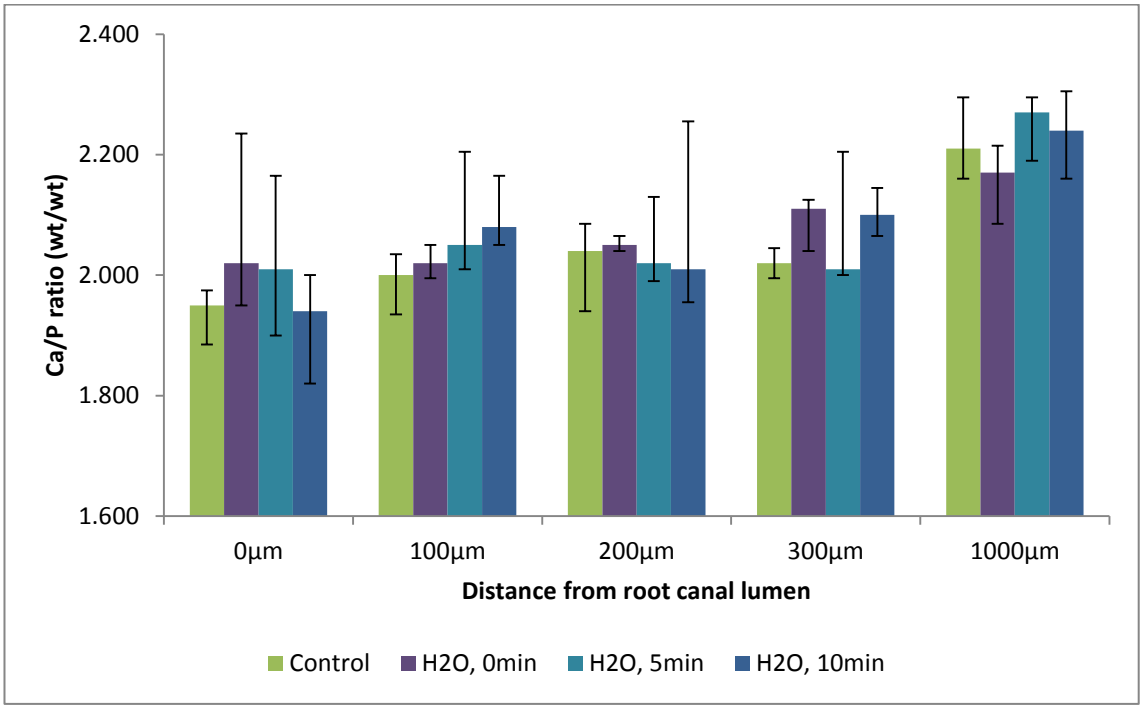

Figure 5.19. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no treatment and irrigation with distilled water for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group.

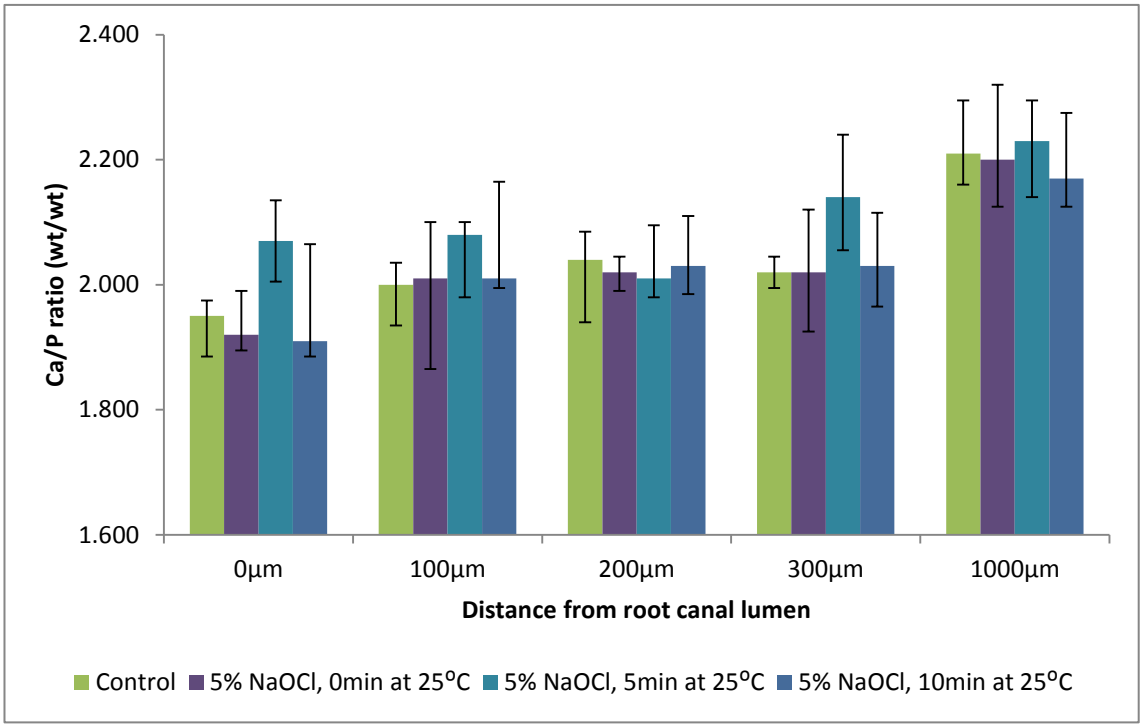

Figure 5.20. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 5% NaOCl at 25°C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group.

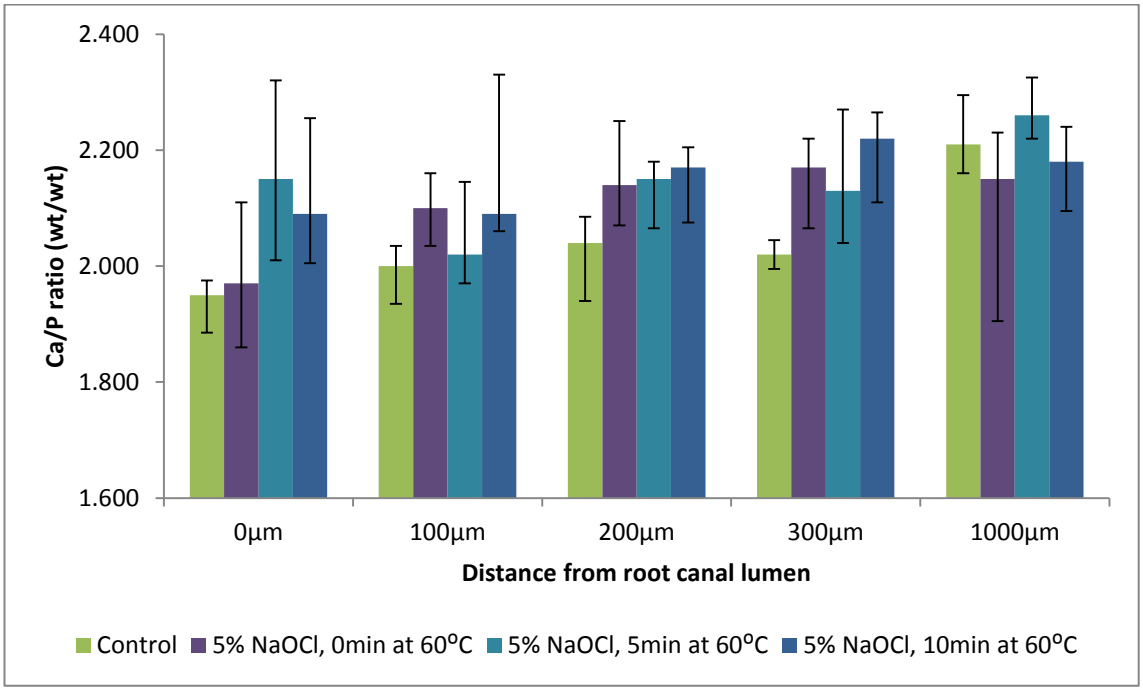

Figure 5.21. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 5% NaOCl at 60°C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group.

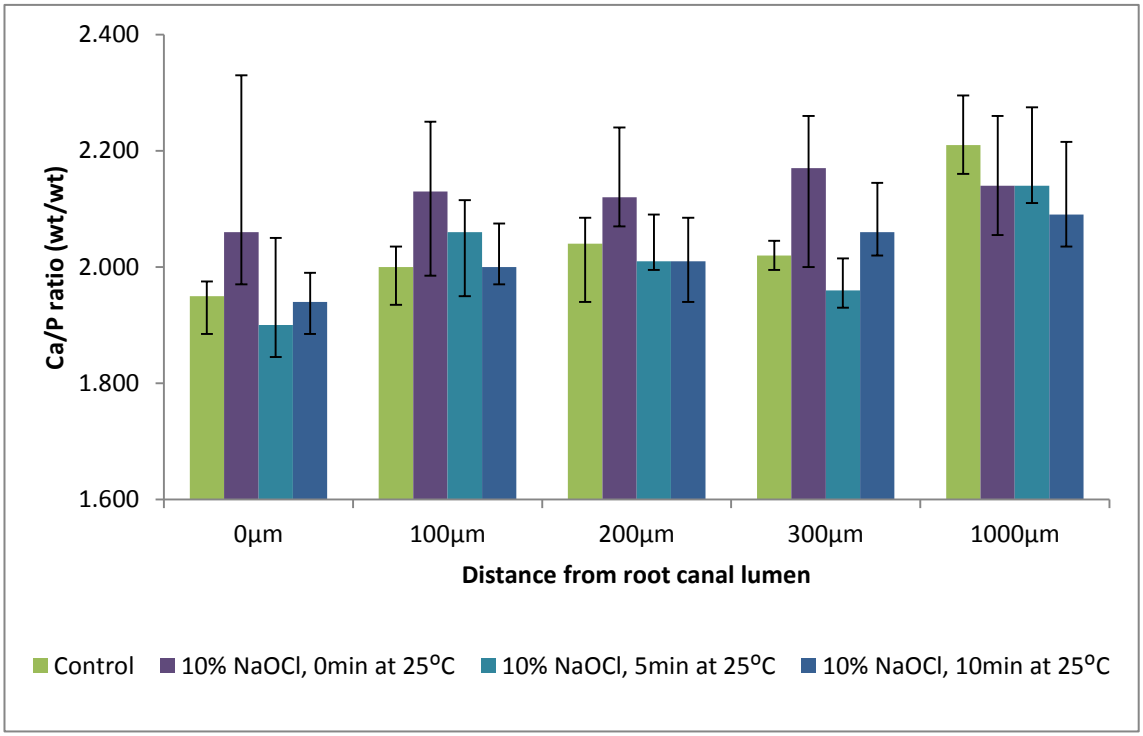

Figure 5.22. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 10% NaOCl at 25°C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group.

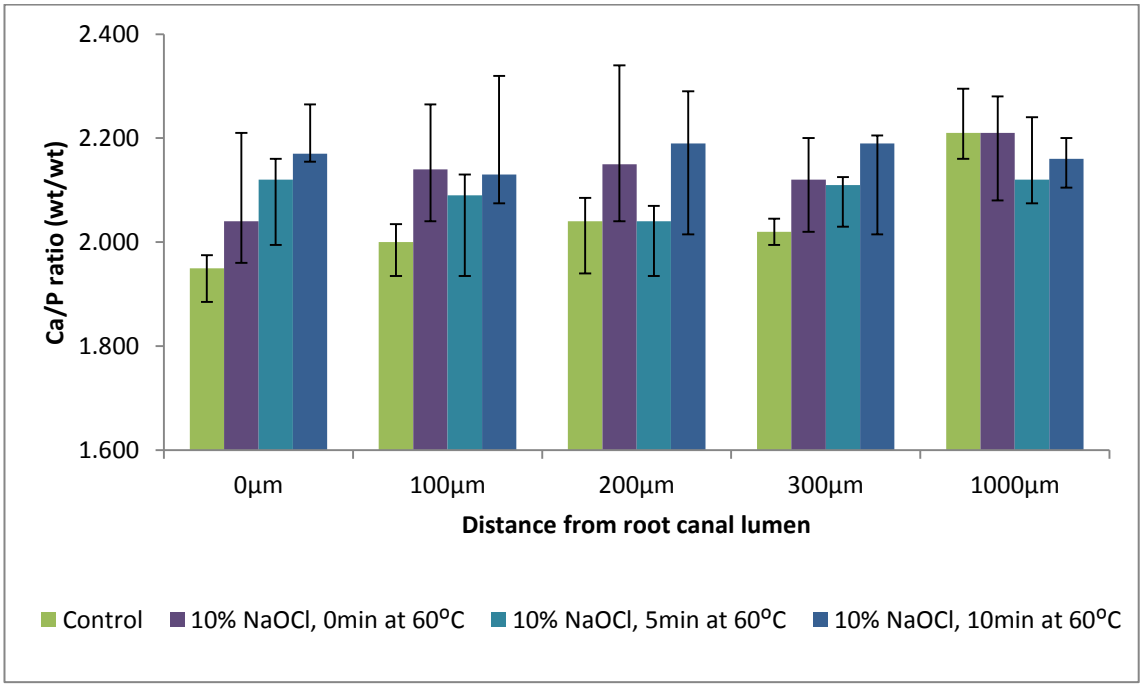

Figure 5.23. Median Ca/P ratio at different depths (0-1000µm) from the lumen of horizontal sections of cervical ovine root dentine, after no-treatment and irrigation with 10% NaOCl at 60 °C for 0 minutes, 5 minutes and 10 minutes. The error bars are upper and lower quartiles for each group.

5.2.3 Discussion

The Hülsmann *et al.* (1997) scoring system (Hülsmann *et al.*, 1997) was found in pilot studies to be unsuitable for use in the current work. Important differences between the specimens assessed by Hülsmann *et al.* (1997) and those in the current work are that the current investigations did not mechanically instrument canals, meaning that cellular debris and predentine was abundant and smear layer was absent. The current work was also interested to identify signs of dentine demineralisation caused by irrigant regimes. A new scoring system was therefore developed, allowing residual pulp debris and predentine, and the development of dentine damage to be scored, the scale was not, however, subjected to systematic and rigorous evaluation of its validity in a range of settings, and in the hands of a range of assessors. There was equally no systematic evaluation of inter and intra-examiner reliability and consistency. These considerations should be addressed in future work.

High concentration NaOCl studies were examined in the current study, since pilot studies had shown that solutions of approximately 0.5-2.5% concentration had very limited effects in terms of pulp debris and predentine removal. Other aspects of these

control studies may also have been relevant, including the observation that in canals filled brim-full with irrigant, ultrasonic activation rapidly empties the canals of solution. Others have reported previously that 2.6% NaOCl had limited pulp-dissolving capacity, whilst 5.25% NaOCl at 60˚C was the most effective (Abou-Rass and Oglesby, 1981). The biological costs were not, however, explored. In the present pilot studies, the heating of 2.86% NaOCl made no significant difference to its efficacy.

The current work was limited to the effects of plain NaOCl solutions. Some reports have noted that the addition of surfactants can enhance the ability of NaOCl to dissolve pulp tissue and enhance its penetration of root canals (Cameron, 1986a; Clarkson *et al.*, 2006). Others have reported no such improvement (Clarkson *et al.*, 2012; Jungbluth *et al.*, 2012). The apices of the roots in this experiment were sealed with soft red wax to simulate a closed-ended canal system, which more closely simulates the clinical environment, where fluid dynamics in the apical third may be compromised compared to open systems (Baumgartner and Mader, 1987; O'Connell *et al.*, 2000; Albrecht *et al.*, 2004).

Other aspects of this research model that are less faithful to clinical practice include filling canals brim-full with solution, rather than flushing, and the results should therefore be interpreted with caution, since the effects of fluid exchange and shearing forces resulting from fluid flow cannot be accounted for.

In this experiment, the smallest size of the ultrasonic file available, size 15, was used, since others have found that small ultrasonic files produced higher streaming velocities within the irrigant and greater acoustic streaming than size 25 (Ahmad *et al.*, 1987b).

The intensity of acoustic microstreaming is directly related to the streaming velocity of the irrigant, while it is inversely related to the surface area of the file touching the root canal wall or radius of the ultrasonic file according to Equation 5.1:

Equation 5.1 $$v = f * amp^2 / r$$

In this equation v is the streaming velocity of the irrigant, f is 2π times the driving frequency of the ultrasonic file, amp is the displace amplitude of the ultrasonic file and r is the radius of the ultrasonic file (van der Sluis *et al.*, 2007).

According to this equation, all other factors being equal, the smaller the radius of the ultrasonic file, the higher the acoustic microstreaming. A higher driving frequency of the ultrasonic file also results in higher amplitude of the file oscillation, which results in higher streaming velocity of the irrigant. This could result in higher shear stresses along the root canal walls and consequently in more efficient removal from root canal walls of debris and bacteria (van der Sluis *et al.*, 2007). The shear stress is directly proportional to the kinematic viscosity of the irrigant (n) and the streaming velocity of the irrigant, while it is inversely related to the boundary layer thickness (t) by (Ahmad *et al.*, 1988) Equation 5.2:

$$\text{Equation 5.2 shear stress} = \mathrm{n} * v/\mathrm{t}$$

An additional factor which could result from the ultrasonically activated irrigation is stable and unstable cavitation (sonluminescence) which creates new bubbles inside the irrigant or expansion of the already present bubbles, then their contraction and then their rupture inside the irrigant (the process is called nuclei in a liquid) (Roy *et al.*, 1994; Brenner *et al.*, 2002).

The displacement amplitude of the ultrasonic file tip is at its maximum value at the tip of the file (Ahmad *et al.*, 1987a). Consequently, ultrasonic file oscillation at the apical part of the root canal could be expected to result in more powerful acoustic microstreaming, higher shear stresses along the root canal walls and in cleaner root canal walls in the apical third. The opposite was actually observed, in which the frequency of clean canals in the middle and cervical thirds was much higher than the apical third. The current investigations did not explore fluid dynamics. An important observation to be made once again is that vibration of the ultrasonic file resulted in rapid emptying of solution. It would, paradoxically expected that this would leave residual fluid in the apical third of the root and allow enhanced chemical action in this region.

Although, ultrasonic (or sonic) activation of irrigant results in acoustic microstreaming and cavitation, physically they do not happen when the ultrasonic (or sonic) tip enters places free of irrigant such as the apical vapour lock area (Schoeffel). The small diameter of the ovine root canal could limit the transverse oscillation of the ultrasonic file, and the small size of the ovine root canal could also limit the minimum amount of

the irrigant which could be required to dissolve all of the debris inside the root canal. The disadvantage of using natural root canals in comparison with simulated root canals is that the natural root canals have no standardised anatomy, shape or internal dimension unlike simulated root canals. The use of an ovine tooth model did, however, allow some standardisation of root size.

Sonic devices are believed to produce less shearing stresses during activated irrigation than ultrasonic devices (Ahmad *et al.*, 1987b), though the relative cleaning ability remains contentious (Stamos *et al.*, 1987; Jensen *et al.*, 1999; Sabins *et al.*, 2003). The current study made no attempt to explore the relative merits of sonic and ultrasonic activation, or the effects of different parameters such as file size and activation power on the efficacy and risks of activated irrigation.

Pilot studies were also undertaken as part of the current work to examine qualitatively the pulp-dissolving effects of NaOCl on bovine tissue. Differences were noted in the time required for pulp dissolution by 0.5%, 2.5%, 5% and 10% NaOCl solutions. Anecdotally, the 0.5% solution achieved its outcome more slowly than 2.5-10% solutions, reinforcing the decision to explore 5% and 10% solutions in the current work. These solutions were also speculated to be capable of causing more damage to root dentine than lower concentrations, and suggesting that if significant change was not observed after treatment with 5% and 10% solutions, similar work on more dilute solutions would not be justified.

Others have reported that NaOCl solutions of less than 1% had no dissolving ability on vital pulp tissue and little on necrotic pulp tissue. But after 5 minutes, approximately 90% of the necrotic pulp was dissolved by 1, 3 and 5% NaOCl solutions (Gordon *et al.*, 1981). Some studies have shown that NaOCl was more effective in cleaning wider than narrow areas of root canals, since the fluid dynamics and volume of NaOCl in wider areas may be more favourable (Senia *et al.*, 1971; Ram, 1977; Wu and Wesselink, 1995; Shuping *et al.*, 2000). Again, no systematic evaluations of this sort were undertaken in the current research, though some inter-species differences between human and ovine teeth reported in chapter 3 may be worthy of further investigation.

In the current work, irrigant solutions were applied to root canals by hand, whilst some studies describe the use of more controlled, mechanical methods (Boutsioukis *et al.*,

2007a; Desai and Himel, 2009; Park *et al.*, 2013). Efforts were, however, made by the single operator to standardise actions.

The results indicate that 5% and 10% NaOCl solutions cleaned pulp debris and removed predentine from the walls of ovine root canals more effectively than water. It is not possible to identify if the improved results compared to the pilot studies on weaker NaOCl solutions were due to the increased concentration of NaOCl, the method and volume of delivery, or a combination of all of these. The complex relationships of variables such as concentration and flow have been reported previously (Stojicic *et al.*, 2010).

Notch-ended needles were selected in this study, since a study analysed the effectiveness of 4 designs of needle tips (notched-end, side-vented with closed-end, side vented with open-end and bevelled needle) on irrigant flow pattern, fluid velocity and apical wall pressure and found notched-ended and bevelled needles promoted deep irrigant exchange (Shen *et al.*, 2010).

Size 27 G needles were selected for this experiment, allowing penetration within 1 mm of the ovine tooth apices without any binding or interference from the root canal walls. In addition, a study found that 27 G needles resulted in higher flow rate of irrigant, longer duration of irrigant delivery and lower intra-barrel pressure than smaller diameter 30 G needles (Boutsioukis *et al.*, 2007a).

Although in passive ultrasonic irrigation (PUI) preventing the ultrasonic file from touching canal walls and allowing free transverse oscillation to promote acoustic streaming may promote debris removal (Ahmad *et al.*, 1987a), avoiding damaging wall contact is difficult (Boutsioukis *et al.*, 2013b), leading to replacement of the term ‘passive ultrasonic irrigation’ with ‘ultrasonically activated irrigation’. In the current work, canal wall damage was noted on numerous occasions (Figure 5.5). Further studies are warranted within the ovine tooth model to quantify the frequency and nature of canal wall damage caused by ultrasonically energised files.

The Ca/P ratios of horizontal sections of root canal dentine were not affected by irrigation of the root canal with NaOCl, though a number of exceptions were observed. Comparison between the non-treated and distilled water-treated revealed few differences in Ca/P ratios in the canal walls at varying depths from the lumen, though

again, some significant differences were noted in areas at 300µm depth from the lumen in the 0 and 10 minutes treatment.

According to these results, Ca/P ratios in the walls of dental roots following canal irrigation with NaOCl (5% and 10% ; 25˚C and 60˚C; and for 0 minutes, 5 minutes and 10 minutes exposure) showed some significant increase ($p>0.05$) and some non-significant increase in comparison with the non-treated group ($p>0.05$). This finding is surprising, since others have shown that NaOCl causes dissolution of organic part of dentine and increases the Ca/P ratio of dentine as a consequence (Dogan and Çalt, 2001). Explanations are difficult, but may represent differences in the experimental models.

5.2.4 Conclusion

According to the results of this experiment, the null hypothesis was refuted and it was shown that the NaOCl at concentrations of 5% and 10%, at 25˚C and 60˚C, and for 0 minutes, 5 minutes and 10 minutes exposure times removed debris from root canals of ovine teeth, and also removed predentine.

The null hypothesis in connection with Ca/P ratios was supported, since NaOCl treatments had no significant influence on recorded values.

Although it was not recorded in any systematic manner, the observation of canal wall damage, presumably caused by ultrasonic file contact, prompted further studies to investigate whether similar cleaning outcomes could be achieved following activation of NaOCl solutions by potentially less damaging methods.

5.3 **The substitution of ultrasonic agitation of NaOCl irrigant with other methods**

To assess the possibility of removing pulp debris and predentine from the walls of ovine root canals with 5% NaOCl solution by the following methods:

- Brim-full, static application
- Continuous replenishment by needle and syringe
- Manual dynamic agitation with a gutta-percha point

This study tested the null hypotheses that the method of delivery and activation of 5% NaOCl has no influence on root canal wall cleanliness and surface damage in an ovine tooth model.

5.3.1 Materials and methods

5.3.1.1 Teeth collection and storage

Twenty de-coronated ovine incisor teeth were used in this study. See section 3.2.2 for teeth collection and storage.

5.3.1.2 Specimen preparation

See section 3.2.2 for details of specimen preparation.

5.3.1.3 Treatment

The de-coronated roots were divided randomly into one control and three experimental groups (n=5) and their apices were sealed with soft red wax to create a closed system.

Treatments were as follows Table 5.4:

Group 1 (non-treated control):

In this group, the roots received no further treatment after gross pulp extirpation with a barbed broach and canal contents were fixed by immersion in 2% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3 prior to SEM analysis.

Group 2 (5% NaOCl, static brim-full):

At the start of the experiment (0 minutes), the canals were slowly filled brim-full with 5% NaOCl, delivered through a 27 gauge needle, connected to a 3mL Luer-Loc syringe, with the needle tip positioned 1-2 mm from the root end (Lee *et al.*, 2004; van der Sluis *et al.*, 2005; Boutsioukis *et al.*, 2009; Boutsioukis *et al.*, 2010a). After 5 minutes, a new needle was inserted and the canal contents aspirated through the irrigating needle, before filling the canal brim-full once again using a fresh volume of 5% NaOCl. This was followed immediately by a final rinse with 3 mL distilled water, delivered at a flow rate of 0.26 mL sec^{-1} (Boutsioukis *et al.*, 2007a; Boutsioukis *et al.*, 2010b). Finally, the

apical seal of soft red wax was removed from the root apices and the roots were immersed in fixative (2% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3) to preserve canal contents for SEM analysis.

Group 3 (5% NaOCl delivered continuously):

At the start of the experiment (0 minutes), 3 mL of the irrigant was delivered into the canal, via a 27 gauge needle and syringe as described in Group 2, the exception here being that the irrigant was delivered continuously at a flow rate of 0.26 mL sec^{-1}, rather than simply filling canals statically brim-full (Boutsioukis *et al.*, 2007a; Boutsioukis *et al.*, 2010b). The needle position was held in a static position throughout the irrigation process. After irrigation, the canals were left brim-full with irrigant for 5 minutes with no further agitation. After 5 minutes, a new needle was inserted and the canal contents aspirated through the irrigating needle and the process repeated with a fresh volume of 5% NaOCl, before aspirating canal contents as before. The canal then was immediately flushed with 3mL distilled water as described in Group 2. Finally, the apical seal of soft red wax was removed and roots fixed as previously described in Group 2.

Group 4 (5% NaOCl with manual dynamic irrigation with gutta-percha point):

At the start of the experiment (0 minutes), 3 mL of the irrigant was delivered into the canal, via a 27 gauge needle and syringe as described in Group 2. Following this, the irrigant was agitated in the canals by manually pumping an ISO gutta-percha cone size 25 (Maillefer, Ballaigues, Switzerland) up and down, extending to the length of the canal, with an amplitude of 3 mm, at a rate of 100 strokes $minute^{-1}$ for 1 minute (McGill, 2008; Parente *et al.*, 2010). After agitation, the canals were left brim-full with irrigant for 5 minutes with no further agitation. After 5 minutes, a new needle was inserted and the canal contents aspirated through the irrigating as before. The process was repeated with a fresh volume of 5% NaOCl, and then the canal contents were again aspirated through the irrigating needle. Immediately following this, the canal was flushed with 3mL distilled water as described in Group 2, before removing the apical wax seal and fixing as previously described in Group 2. A summary of the treatment regimes is shown in Table 5.4.

Table 5.4. Summary of treatment regimens of ovine roots.

Group	Treatment	Concentration	Temperature (˚C)	Time (minutes)
1	Non-treated	N/A	N/A	N/A
2	NaOCl (brim-full)	5%	25	5
3	NaOCl (continuous)	5%	25	5
4	NaOCl (manual dynamic)	5%	25	5

5.3.1.4 Storage of the samples and SEM preparation

See section 3.2.2.

5.3.1.5 Analysis of the of the samples by the SEM

See section 5.2.1 for details of analysis of the samples by the SEM and scoring of pulp debris and predentine. Analysis was undertaken in SPSS 19.00 (SPSS Inc, Chicago, IL) to compare the scores, using the chi square test ($p<0.05$).

5.3.2 Results

No evidence of canal wall damage was noted on general inspection of specimens from any of the Groups (1-4). All of these experimental treatments with NaOCl (Groups 2, 3 and 4) resulted in better cleaning of the root canal than the non-treated control group (Figure 5.25, Figure 5.26 and Figure 5.24 respectively). Figure 5.27, Figure 5.28 and Figure 5.29, show that Group 2 (with static brim-full irrigation) was the least effective in pulp debris and predentine removal, whilst Group 4 (with manual dynamic irrigation) was the most effective. Cleaning was least effective in the apical canal third (Figure 5.27 and Figure 5.28), except in Group 4 (Figure 5.29). There was a significant difference between the experimental groups 2, 3 and 4 and the non-treated control group ($p<0.05$), Figure 5.30.

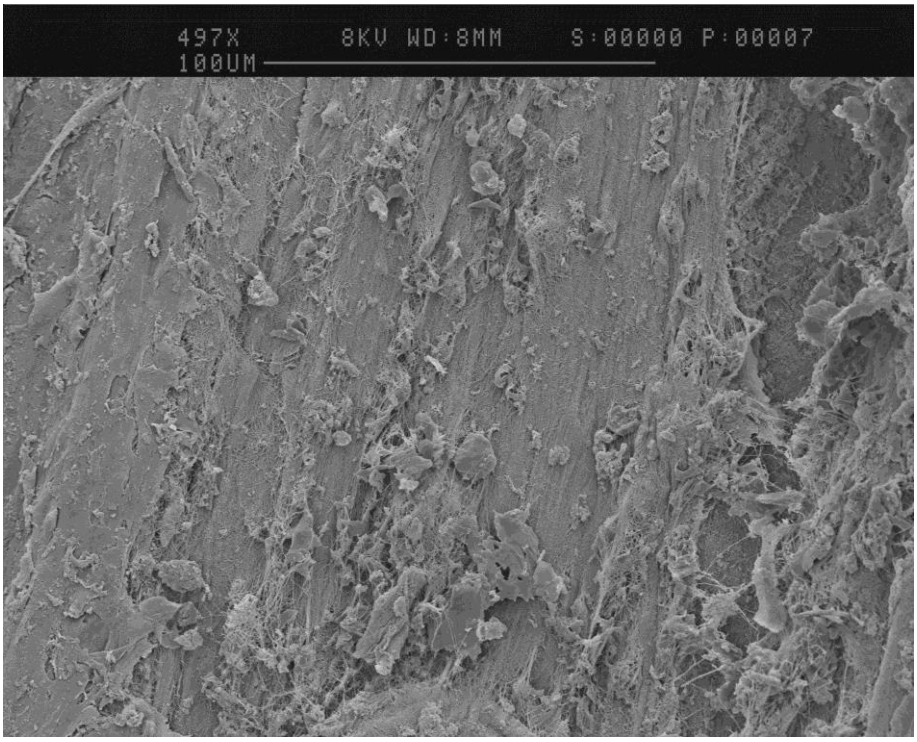

Figure 5.24. SEM image (500x magnification) of an ovine root canal wall in the cervical third without treatment (Group 1, untreated control), showing full coverage with debris and predentine (score 1).

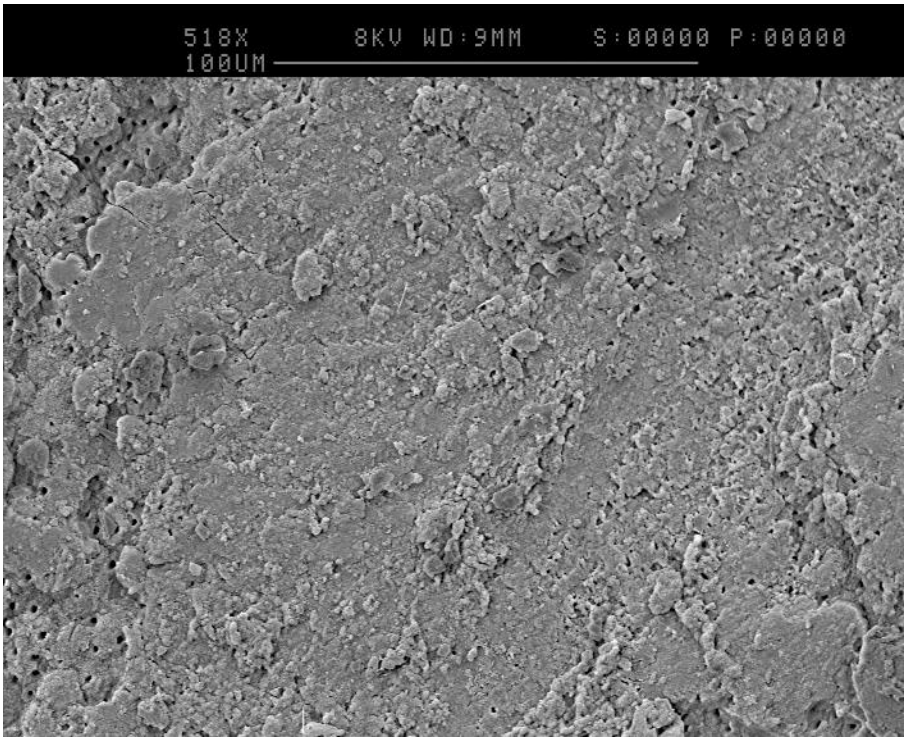

Figure 5.25. SEM image (500x magnification) of the ovine root canal wall in the cervical third from Group 2 to show debris and predentine score of 2.

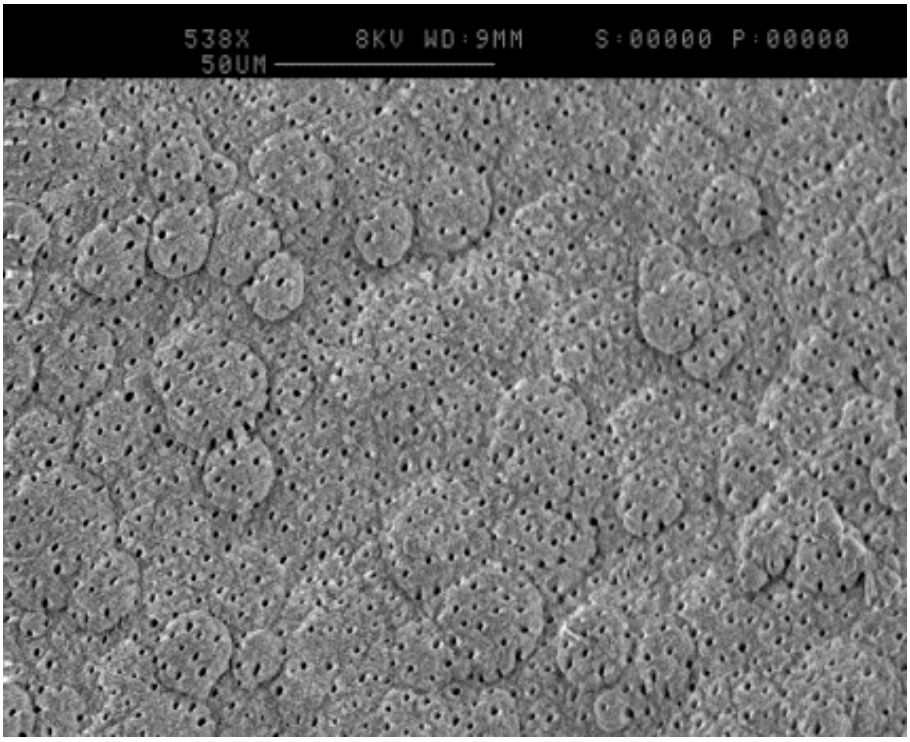

Figure 5.26. SEM image (500x magnification) of the ovine root canal wall at the cervical third from Group 3 to show debris and predentine score of 4.

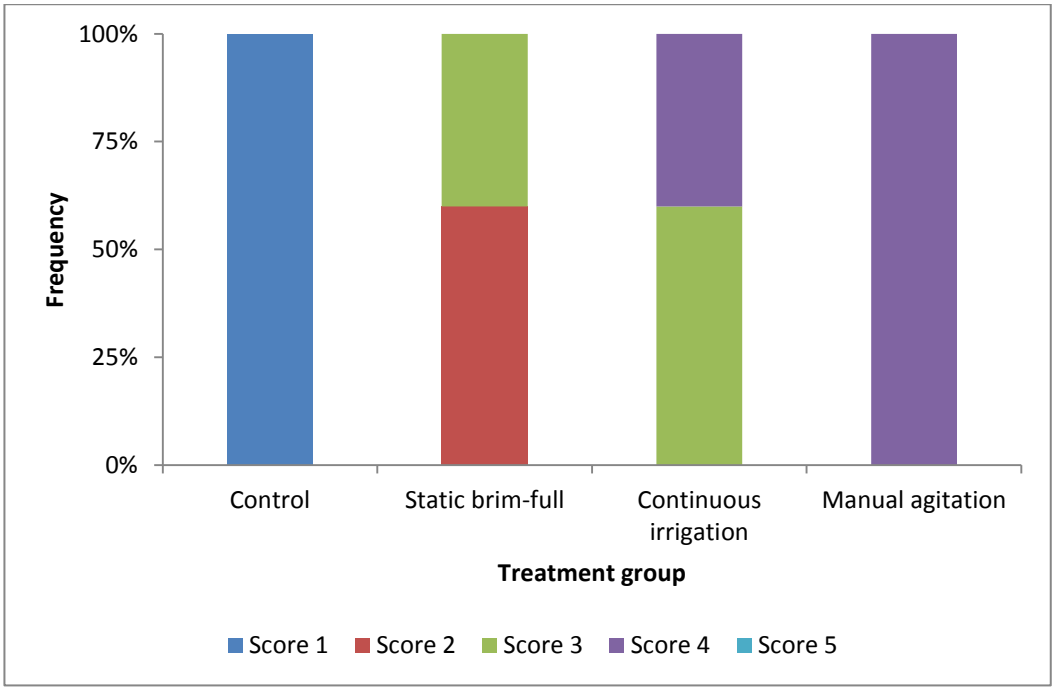

Figure 5.27. Pulp debris and predentine scores from the cervical thirds of ovine root canals after no-treatment, static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25°C for 5 minutes. The difference was significant between the treated Groups 2, 3 and 4 and the non-treated Group 1. In addition, the difference was significant between the treatment groups 2, 3 and 4 ($p<0.05$, chi square test).

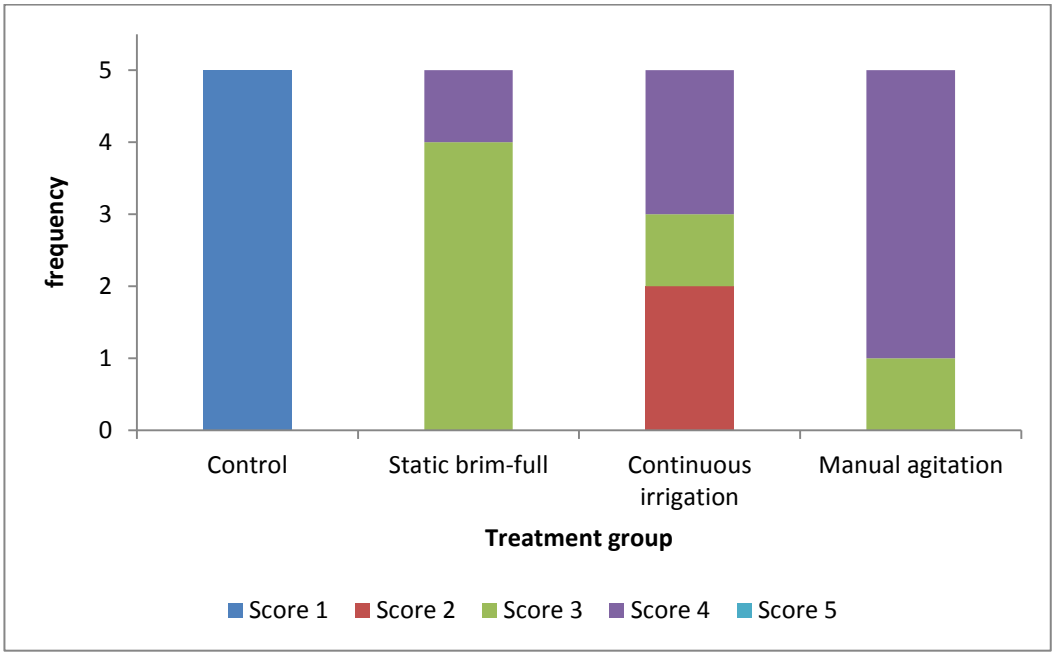

Figure 5.28. Pulp debris and predentine scores from the middle thirds of ovine root canals after no-treatment, static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25°C for 5 minutes. The difference was significant between the treated groups 2, 3 and 4 and the non-treated group 1. The difference was not significant between the treatment groups 2, 3 and 4 ($p<0.05$, chi square test).

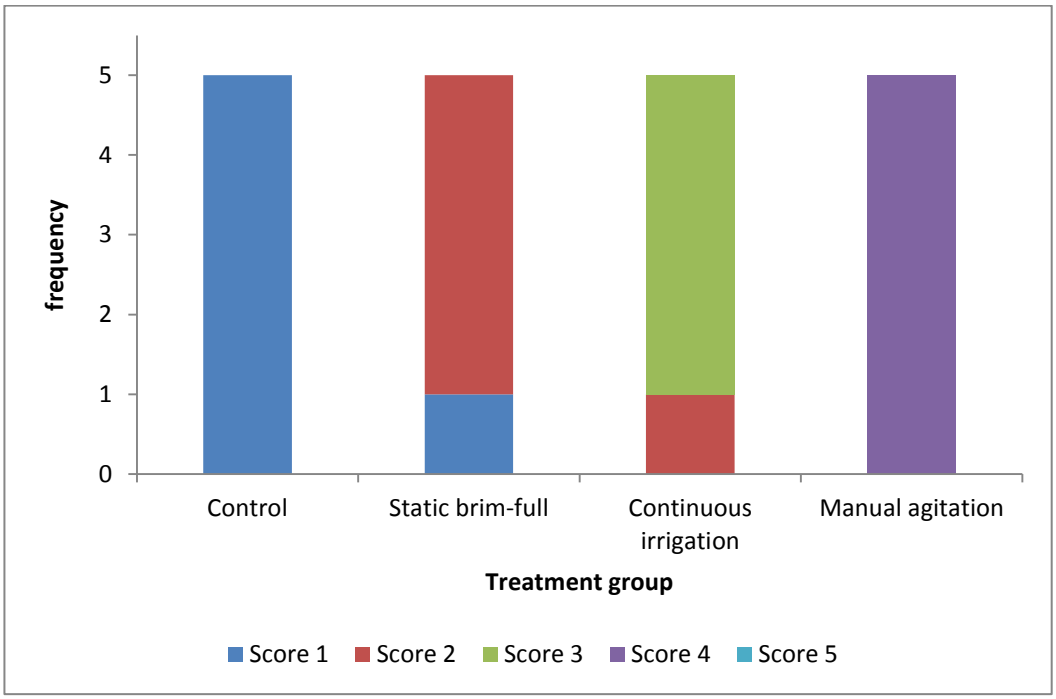

Figure 5.29. Pulp debris and predentine scores from the apical thirds of ovine root canals after no-treatment, static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25°C for 5 minutes. The difference was significant between the treated groups 2, 3 and 4 and the non-treated group 1. In addition, the difference was significant between the treatment groups 2, 3 and 4 ($p<0.05$, chi square test).

Chi-Square Tests

Third		Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Cervical	Pearson Chi-Square	38.971[a]	9	.000	.000		
	Likelihood Ratio	40.346	9	.000	.000		
	Fisher's Exact Test	25.969			.000		
	Linear-by-Linear Association	16.844[b]	1	.000	.000	.000	.000
	N of Valid Cases	20					
Middle	Pearson Chi-Square	32.000[c]	9	.000	.000		
	Likelihood Ratio	31.661	9	.000	.000		
	Fisher's Exact Test	21.222			.000		
	Linear-by-Linear Association	11.510[d]	1	.001	.000	.000	.000
	N of Valid Cases	20					
Apical	Pearson Chi-Square	46.933[e]	9	.000	.000		
	Likelihood Ratio	45.041	9	.000	.000		
	Fisher's Exact Test	29.460			.000		
	Linear-by-Linear Association	17.724[f]	1	.000	.000	.000	.000
	N of Valid Cases	20					

a. 16 cells (100.0%) have expected count less than 5. The minimum expected count is .75.

b. The standardized statistic is 4.104.

c. 16 cells (100.0%) have expected count less than 5. The minimum expected count is .50.

d. The standardized statistic is 3.393.

e. 16 cells (100.0%) have expected count less than 5. The minimum expected count is 1.00.

f. The standardized statistic is 4.210.

Figure 5.30. Chi square test to investigate significant differences in debris and predentine scores in the cervical, middle and apical thirds of ovine roots after static brim-full irrigation with 5% NaOCl, continuous irrigation with 5% NaOCl and manual dynamic irrigation with 5% NaOCl at 25˚C for 5 minutes. The difference was significant between the treated groups 2, 3 and 4 and the non-treated group 1. At the cervical third, the difference between groups 2 and 3 was not significant, between groups 2 and 4 was significant, and between groups 3 and 4 was significant. At the middle third, the difference between groups 2 and 3, groups 2 and 4 and groups 3 and 4 was not significant. At the apical third, the difference between groups 2 and 3, between groups 2 and 4 and between groups 3 and 4 was significant ($p<0.05$, chi square test).

5.3.3 Discussion

This study showed that syringe irrigation of ovine root canals with 5% NaOCl, supplemented by manual dynamic irrigation with a gutta-percha cone cleaned canals and removed predentine effectively and without apparent damage to the root canal walls. Others have shown similar cleaning effectiveness for 5% NaOCl, even without supplementary activation (Rosenfeld *et al.*, 1978).

Various flow rates have been described in the literature on the rate of irrigant delivery with Parente *et al.* (2010) describing 0.083 mL sec^{-1} (Parente *et al.*, 2010),while a higher rate of 0.26 mL sec^{-1} was adopted in the current study as described by (Boutsioukis *et al.*, 2010b). The reason for this was because it has been recognised that the replacement of irrigant can be limited to 1-1.5 mm beyond the needle tip of conventional endodontic irrigating syringes, and a higher flow rate was considered more likely to encourage deep exchange and turbulence (Chow, 1983; Sedgley *et al.*, 2005; Boutsioukis *et al.*, 2009; Gao *et al.*, 2009).

Reports of manual dynamic irrigation are also variable with some recommending of 5 mm (McGill, 2008) and others 2–3 mm amplitude strokes as effective methods in terms of canal cleanliness (Huang *et al.*, 2008; McGill, 2008). Details of the optimal gutta-percha cone dimensions are also unclear. This is a complex area of fluid dynamics and beyond the scope of the current work.

The entrapment of liquid by bubbles in closed root canal systems (vapour lock) is a well recognised confounding factor in root canal irrigation (Bankoff, 1958; Gu *et al.*, 2009). The penetration ability of the liquid into these areas depends on the contact angle, surface tension, wettability of the liquid and depth and size of these closed-end areas (Pesse *et al.*, 2005). The findings of the current and other studies suggest that vapour lock effects can be overcome in closed root canal systems by simple manual dynamic agitation (Huang *et al.*, 2008; Stojicic *et al.*, 2010).

In the current experiment, the size of the gutta-percha cone used for manual agitation of the NaOCl was the same for all samples (ISO size 25) in order to reduce number variables which could influence the results, in spite of the suspected variation in the size of ovine root canals. The data showed that whether or not the gutta-percha cone was well fitted to the size of the root canal or loose, this action assisted cleaning in all thirds

of the root canal especially in the apical third of the root canal, possibly by overcoming vapour lock effects (Senia *et al.*, 1971; Huang *et al.*, 2008; McGill, 2008; Gu *et al.*, 2009), and without apparent physical damage to canal walls Figure 5.26. The significant difference between the manual dynamic irrigation and the other 2 methods (static brim-full and continuous irrigation) at the apical third of the root canal could be a possible reason that the manual dynamic was more effective, Figure 5.30.

Parente *et al.* (2010) instrumented root canals with open and closed-ended apices and supplemented NaOCl and EDTA irrigation with 300 strokes of manual agitation with a gutta-percha point. This resulted in more effective cleaning of root canals with open than with closed-ended apices (Parente *et al.*, 2010). M^{C}Gill *et al* (2008), in a broadly similar investigation to Parente *et al.* (2010) found that manual dynamic irrigation was more efficient than the static irrigation and irrigation with automated dynamic irrigation with RinsEndo (McGill, 2008). These authors used a larger volume of irrigant and higher amplitudes of gutta-percha point strokes than were used in the current investigation. Working with a stained collagen removal model, Huang *et al* (2008) also achieved more effective cleaning with manual dynamic irrigation with gutta-percha point in comparison with the static irrigation (Huang *et al.*, 2008). Details of irrigant concentration and volume, gutta-percha point size and stroke frequency are apparent in all of these studies, but the overall message on the apparent benefits of manual dynamic irrigation are common to all.

The current study involved no mechanical instrumentation of the root canals and treated them only with irrigants. The capacity of 5% NaOCl solutions to remove pulp debris and predentine and to expose calcospherites was confirmed and echoed the findings of Haapasalo *et al* (2012).

5.3.4 Conclusions

Within the constraints of the current study, 5% NaOCl solutions were able to remove pulp debris and predentine from the uninstrumented root canal walls of immature ovine teeth. These effects were significantly enhanced by manual dynamic irrigation with an ISO size 25 gutta-percha cone, and without the canal-wall damage noted after ultrasonic activation. The null hypotheses were thus refuted.

For the final experiment in this section, the findings of the current work will be translated into an investigation on human teeth.

5.4 **Debris removal from human root canals**

The aim of this study was to assess the ability of different concentrations of NaOCl (2.5%, 5% and 10%) to dissolve pulp debris and predentine from the walls of human root canals. This study tested the null hypotheses that 2.5%, 5% and 10% NaOCl applied for 5 minutes would have no effect on pulp debris and predentine removal compared with untreated controls.

5.4.1 Materials and Methods

5.4.1.1 Tooth collection and storage

Twenty-five freshly extracted human premolar teeth were collected and stored in 1% chloramine-T (SIGMA-ALDRICH, UK) at 4˚C until they were needed. Periodontal tissues were removed with a no 22 scalpel blade (Swann Morton, Sheffield, England).

5.4.1.2 Specimen preparation

See section 3.2.2 for specimen preparation.

5.4.1.3 Treatment

Three concentrations of NaOCl, (2.5%, 5% and 10%) were prepared at room temperature for this study (see section 5.2.1 for preparation of 5% and 10% NaOCl). The 2.5% NaOCl was prepared by adding 1 L of distilled water to 1 L of 5% NaOCl (1 L 5% NaOCl: 1 L distilled water), and then the concentration was confirmed by iodometric titration. The pulp of the roots was not fixed, and the roots divided randomly into two control groups (n=5) and three experimental groups (n=5). The roots in the control group (Group 1) served as untreated controls, Group 2 were treated with normal saline, Groups 3-5 were treated with NaOCl at 2.5%, 5% and 10% respectively (Table 5.5).

Group 1 (untreated control):

In this group, the roots received no further treatment after gross pulp extirpation with a barbed broach and irrigation with 3 mL distilled water. They were fixed by immersion in 2% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3 to preserve canal contents for SEM analysis.

Group 2 (normal saline):

The steps of treatment for this group were the same steps were used in the G4 (manual dynamic irrigation with a gutta-perch point) in the previous experiment in section 5.3.1, except the irrigant used here was normal saline.

Group 3 (2.5% NaOCl):

The steps of treatment for this group were the same as for Group 4 in the previous experiment in section 5.3.1, with the exception that a 2.5% NaOCl solution was employed here.

Group 4 (5% NaOCl):

The steps of treatment for this group were again the same as for Group 4 in the previous experiment in section 5.3.1, with the exception that a 5% NaOCl solution was employed here.

Group 5 (10% NaOCl):

The steps of treatment for this group were again the same as for Group 4 in the previous experiment in section 5.3.1, with the exception that a 10% NaOCl solution was employed here.

Table 5.5. Summary of treatment regimes for human roots.

Group	Treatment	Concentration	Temperature (˚C)	Time (minutes)
1	**Non-treated**	**N/A**	**N/A**	**N/A**
2	**Normal saline**	**N/A**	**25**	**5**
3	**NaOCl**	**2.5%**	**25**	**5**
4	**NaOCl**	**5%**	**25**	**5**
5	**NaOCl**	**10%**	**25**	**5**

5.4.1.4 Storage of the samples and SEM preparation

See section 3.2.2 for storage of the samples and SEM preparation.

5.4.1.5 Analysis of the of the samples by the SEM

The reliability of the pulp debris and predentine scoring of SEM images of human root canal for groups 1-5 was done depending on second and third examiners, after training them how to use this scoring system. So, the SEM images were coded and they were analysed by the second and third examiners, and then the results were decoded. First examiner reliability was done between the results of the first examiner and the results of the second examiner using Kappa test in SPSS 19.00 (SPSS Inc, Chicago, IL). This test was also used to assess second examiner reliability between the results of the first and the third examiners.

See section 5.2.1 for details of analysis of the samples by the SEM and scoring of pulp debris and predentine. Analysis was undertaken in SPSS 19.00 (SPSS Inc, Chicago, IL) to compare the scores, using the chi square test ($p<0.05$).

5.4.2 Results

Figure 5.31 and Figure 5.32, show Kappa scores for inter-examiner reliability with the new scoring system. Figure 5.31 shows that there was a significant agreement between the first examiner and the second examiner at 0.717, 0.615 and 0.682 levels for the cervical, middle and apical thirds respectively. Figure 5.32 also shows that there was a significant agreement between the first examiner and the third examiner 0.619, 0.709 and 0.564 levels for the cervical, middle and apical thirds respectively.

Canal wall coverage with pulp debris and predentine was noted in the untreated control group (Group1, score 1) (Figure 5.33). Similar observations were noted for saline treated canals (Group 2).

In all groups treated with NaOCl (Group 3-5, treated with NaOCl 2.5-10% respectively), predentine was removed and globular dentine was observed. In the groups treated with 5% and 10% NaOCl (Groups 4 and 5) no cellular debris was observed (Figure 5.35). Some of the samples in the group treated with the 2.5% NaOCl showed

partial removal of predentine (Figure 5.34), but no cellular debris. Some specimens in this group showed cellular debris and predentine in the apical third (score 1).

All of the root canal debris and predentine was removed from the cervical thirds of human root canals treated with 2.5%, 5%, and 10% NaOCl (Groups 3-5 respectively); all scoring 4 Figure 5.36. All of the root canal debris and predentine was removed from the middle third of all canals treated with 5% and 10% NaOCl (Groups 4 and 5 respectively); all scoring 4. In the middle third of canals treated with 2.5% NaOCl (Group 3), 40% of canals scored 4, 40% scored 3, and 20% scored 2, Figure 5.37.

In the apical third, all canals treated with 5% and 10% NaOCl (Groups 4 and 5) scored 4. In the canals treated with 2.5% NaOCl, 20% scored 4, 40% scored 3, 20% scored 2 and 20% scored 1 Figure 5.38. The lowest concentration studied that was able to predictably remove pulp debris and predentine from all thirds of the canal was 5% with manual dynamic irrigation. There was a statistical significant difference between all at the cervical, middle and apical thirds (Figure 5.39).

Symmetric Measures

Place on root			Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Cervical	Measure of Agreement	Kappa	.717	.111	5.055	.000
	N of Valid Cases		25			
Middle	Measure of Agreement	Kappa	.615	.117	4.523	.000
	N of Valid Cases		25			
Apical	Measure of Agreement	Kappa	.682	.112	4.871	.000
	N of Valid Cases		25			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Figure 5.31. The agreement between the first and the second examiners, which shows the reliability of the first examiner (Kappa score) for pulp debris and predentine scoring of SEM images of human root canal after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes.

Symmetric Measures

Place on root			Value	Asymp. Std. Error[a]	Approx. T[b]	Approx. Sig.
Cervical	Measure of Agreement	Kappa	.619	.116	4.786	.000
	N of Valid Cases		25			
Middle	Measure of Agreement	Kappa	.709	.106	5.768	.000
	N of Valid Cases		25			
Apical	Measure of Agreement	Kappa	.564	.107	5.230	.000
	N of Valid Cases		25			

a. Not assuming the null hypothesis.

b. Using the asymptotic standard error assuming the null hypothesis.

Figure 5.32. The agreement between the first and the third examiners shows the reliability of the first examiner (Kappa score) for pulp debris and predentine scoring of SEM images of human root canal after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes.

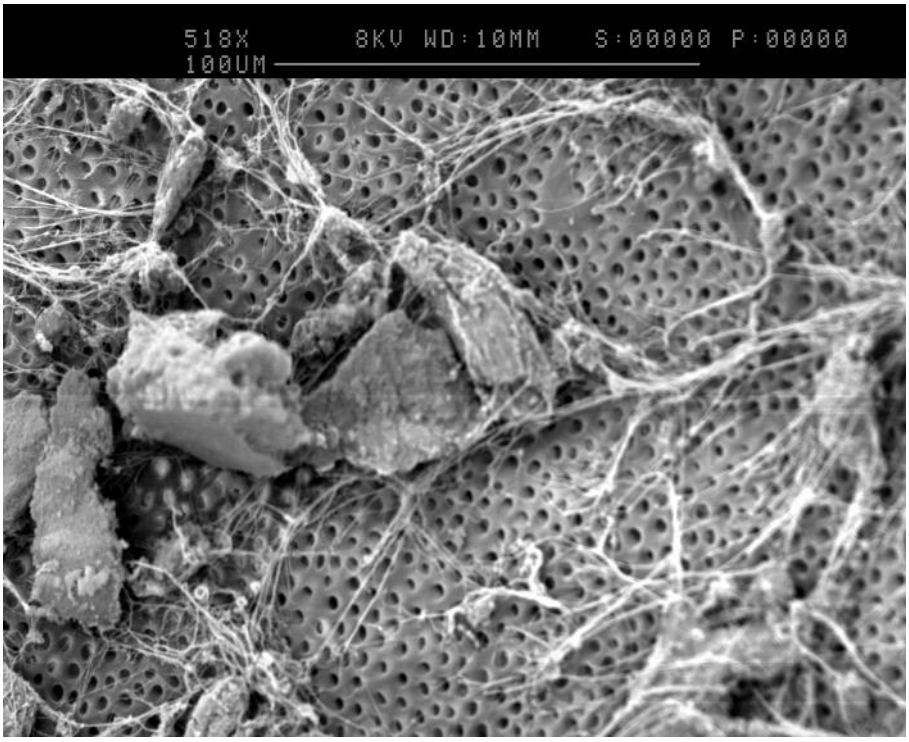

Figure 5.33. SEM image (500x magnification) of the human root canal wall in the cervical third of an untreated control specimen (Group 1), with a debris and predentine score of 1.

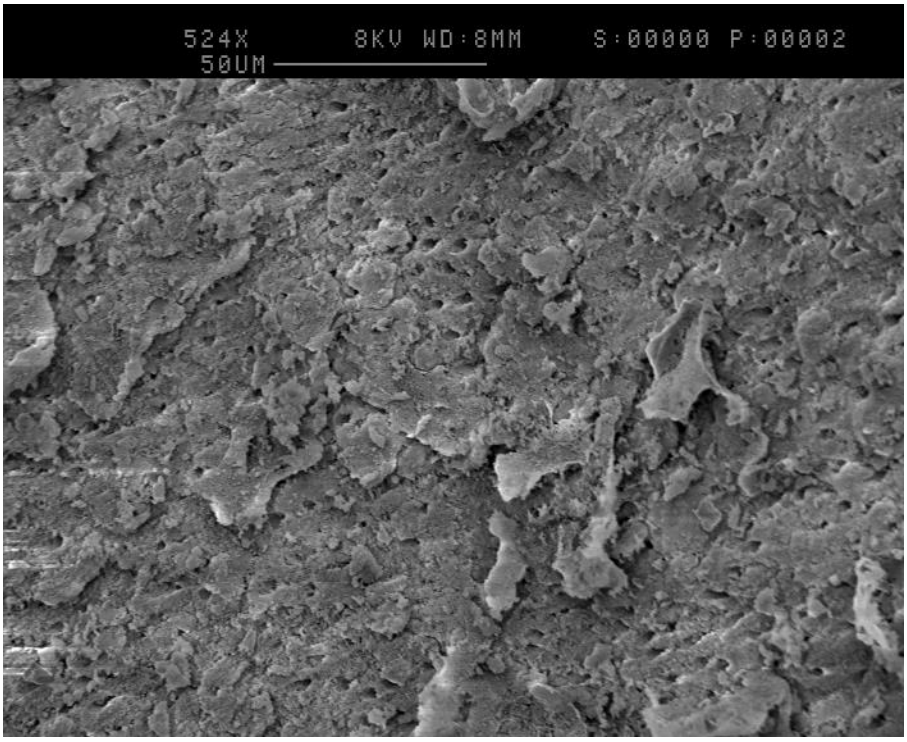

Figure 5.34. SEM image (500x magnification) of the human root canal wall in the middle third of a specimen from Group 3 (NaOCl 2.5%), with a debris and predentine score of 3.

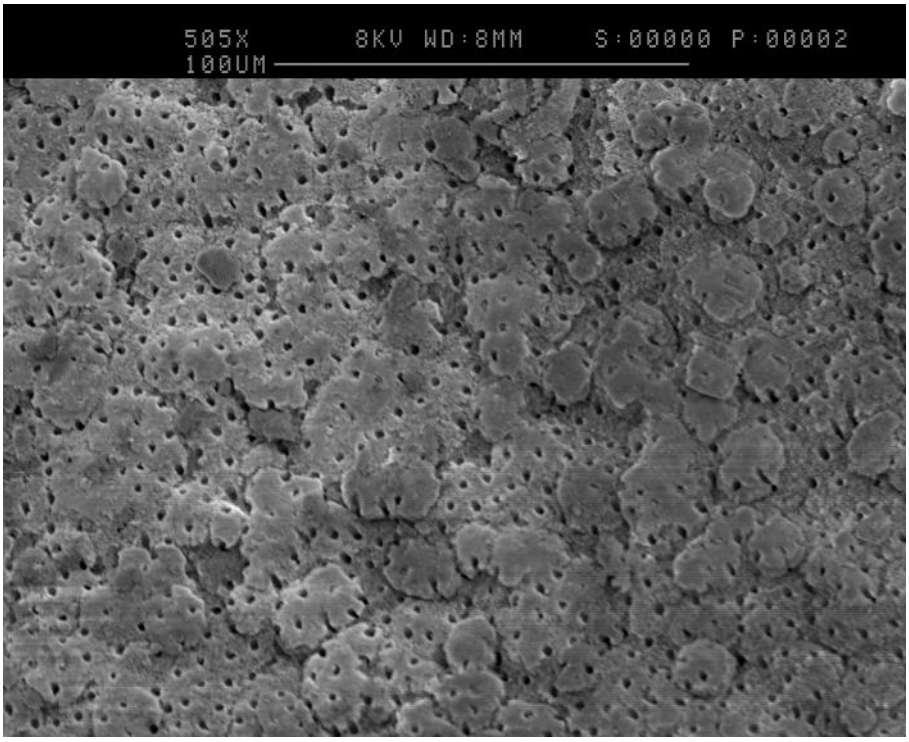

Figure 5.35. SEM image (500x magnification) of the human root canal wall in the cervical third of a specimen from Group 4 (5% NaOCl), with debris and predentine score 4.

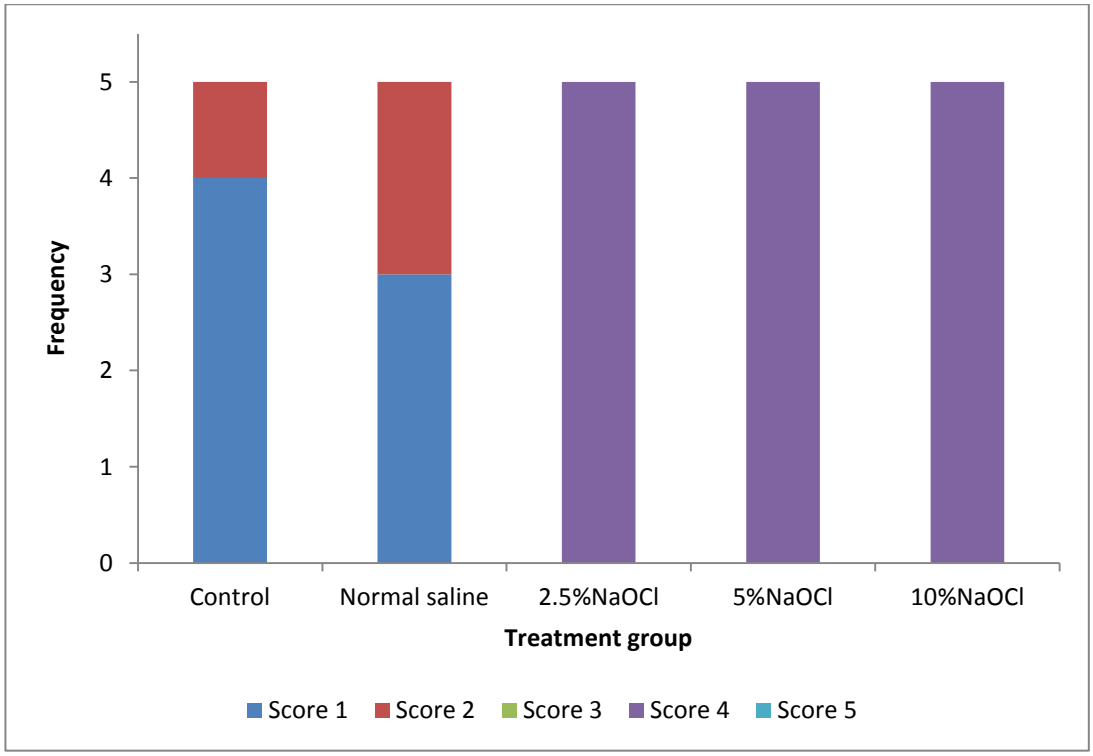

Figure 5.36. Pulp debris and predentine scores for the cervical thirds of human root canals after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25°C for 5 minutes. The difference was significant between the experimental groups 3, 4 and 5 and groups 1 and 2 ($p<0.05$, chi square test).

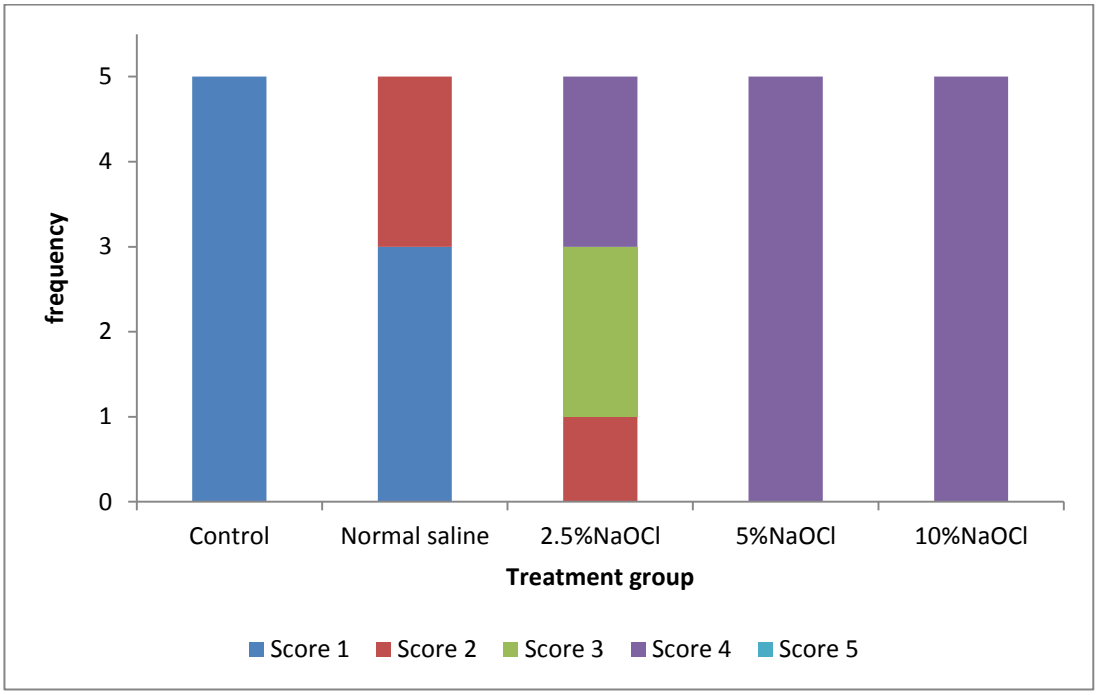

Figure 5.37. Pulp debris and predentine scores for the middle thirds of human root canals after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25°C for 5 minutes. The difference was significant between the experimental groups 3, 4 and 5 and groups 1 and 2. ($p<0.05$, chi square test).

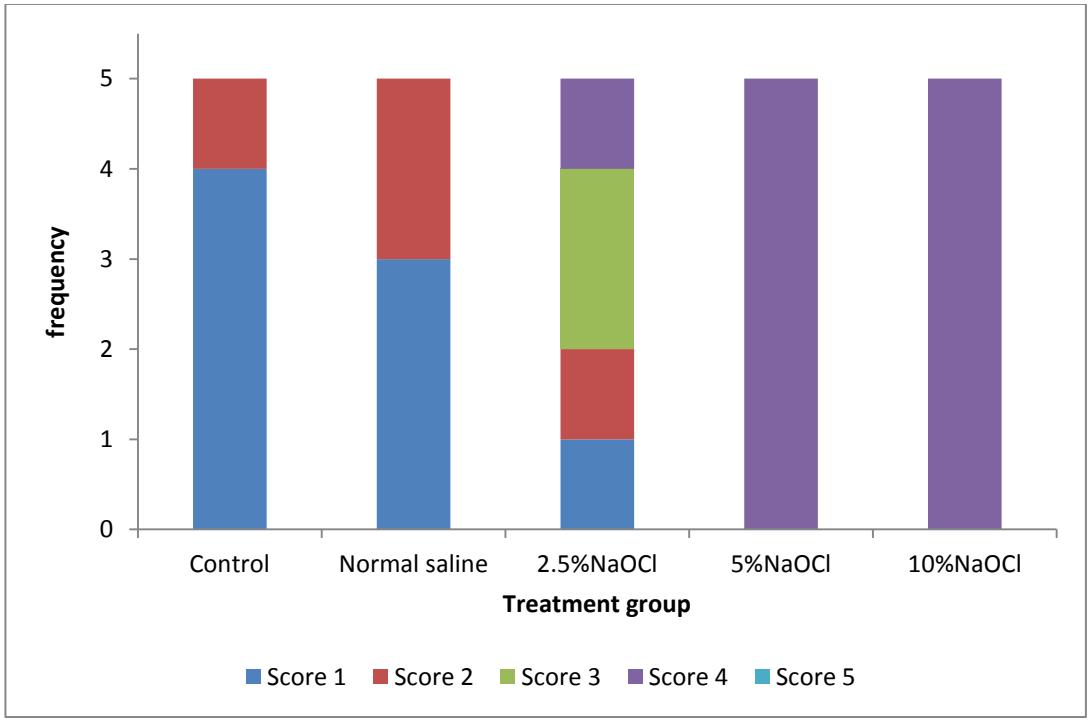

Figure 5.38. Pulp debris and predentine scores for the apical thirds of human root canals after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25°C for 5 minutes. The difference was significant between the experimental groups 3, 4 and 5 and groups 1 and 2 ($p<0.05$, chi square test).

Chi-Square Tests

Place on root		Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)	Point Probability
Cervical	Pearson Chi-Square	26.190[a]	8	.001	.000		
	Likelihood Ratio	34.134	8	.000	.000		
	Fisher's Exact Test	23.007			.000		
	Linear-by-Linear Association	17.602[b]	1	.000	.000	.000	.000
	N of Valid Cases	25					
Middle	Pearson Chi-Square	37.083[c]	12	.000	.000		
	Likelihood Ratio	41.391	12	.000	.000		
	Fisher's Exact Test	27.984			.000		
	Linear-by-Linear Association	19.705[d]	1	.000	.000	.000	.000
	N of Valid Cases	25					
Apical	Pearson Chi-Square	35.960[c]	12	.000	.000		
	Likelihood Ratio	39.224	12	.000	.000		
	Fisher's Exact Test	27.050			.000		
	Linear-by-Linear Association	19.294[e]	1	.000	.000	.000	.000
	N of Valid Cases	25					

a. 15 cells (100.0%) have expected count less than 5. The minimum expected count is .60.

b. The standardized statistic is 4.195.

c. 20 cells (100.0%) have expected count less than 5. The minimum expected count is .40.

d. The standardized statistic is 4.439.

e. The standardized statistic is 4.392.

Figure 5.39. Chi square test to identify significant differences in debris and predentine scores for human root canals in their cervical, middle and apical thirds after no-treatment, irrigation with normal saline, 2.5% NaOCl, 5% NaOCl and 10% NaOCl at 25˚C for 5 minutes. The difference was significant between all groups at the cervical, middle and apical thirds. While, the difference between groups treated with 2.5% and 5% NaOCl , between groups treated with 2.5% and 10% NaOCl, and between groups treated with 5% and 10% NaOCl was not significant at the cervical, middle and apical thirds ($p<0.05$, chi square test).

5.4.3 Discussion

The qualitative assessment of SEM images in previous experiments suggested that ultrasonically activated root canal irrigation risked wall contact and damage compared with conventional syringe irrigation and manual dynamic exchange with a gutta-percha point.

In this study, the agitation of NaOCl at concentrations of 5% and greater with a gutta-percha point was able to eliminate cellular debris and dissolved predentine from the walls of human premolar root canals at cervical, middle and apical levels.

NaOCl solution at 2.5% was as effective as 5% and 10% solutions in the cervical root third, but became progressively less effective in middle and apical thirds. These observations are contrary to those of Hu *et al.* (2010), who considered a 0.5% NaOCl solution as optimal in terms of cleaning, whilst having the least potential to damage dentine (Hu *et al.*, 2010).

Different studies have used different scoring systems for debris removal from root canals. Details of many experiments are lacking in terms of calibration and blinding of observers, and standardisation of viewing conditions (Turek and Langeland, 1982; Goldberg *et al.*, 1988; Ciucchi *et al.*, 1989; Walker and del Rio, 1991; Bertrand *et al.*, 1999; Torabinejad *et al.*, 2003a; Torabinejad *et al.*, 2003b; Lui *et al.*, 2007; Blank-Gonçalves *et al.*, 2011). For studies involving SEM, bias could influence the areas selected for observation, with a focus on 'good' or 'interesting' rather than representative areas (Lumley *et al.*, 1992; Hülsmann *et al.*, 1997; Hulsmann *et al.*, 1999; Hülsmann *et al.*, 2003a; Hülsmann *et al.*, 2005). Most of the studies showed better cleaning of root canal in coronal canal thirds in comparison with apical regions (Haikel and Allemann, 1988; Ciucchi *et al.*, 1989; Walker and del Rio, 1991; Cheung and Stock, 1993; Prati *et al.*, 1994; Wu and Wesselink, 1995; O'Connell *et al.*, 2000; Hülsmann *et al.*, 2003a; Carvalho *et al.*, 2008; Ballal *et al.*, 2009; Kuah *et al.*, 2009; Mancini *et al.*, 2009; Zmener *et al.*, 2009; Rödig *et al.*, 2010; Uroz-Torres *et al.*, 2010; Blank-Gonçalves *et al.*, 2011).

The debris and predentine scoring system introduced for this work was developed because the widely employed Hülsmann *et al.* (1997) system was found to be

unsuitable. This scoring system does, however, require more rigorous validation to ensure its utility and validity in a range of settings.

The current investigation made no attempt to assess surface and sub-surface changes in Ca/P ratios, since earlier studies confirmed no significant changes, even with 10% NaOCl solutions. The effects of NaOCl and acid/EDTA combined treatments were not assessed.

The same size of gutta-percha cone #25 was used in the previous study of ovine teeth and this study of human teeth and the results was effective in all thirds of the root canals, with 5% and 10% NaOCl treatment. In case of 2.5% NaOCl treatment, the exchange did not seem to have helped and the cleaning was poor in the apical and middle thirds.

5.4.4 Conclusion

Within the limitations of this study, 5% was the lowest concentration of NaOCl that was able to predictably eliminate pulp debris and predentine from all thirds of uninstrumented human root canals. The null hypothesis was rejected, since different NaOCl irrigation regimes were able to remove pulp debris and predentine from the walls of human root canals.

Chapter 6: AFM analysis of the effect of irrigants on human root dentine

6.1.1 Introduction

The mechanical, chemical and biological objectives of endodontic irrigation have been discussed in previous sections of this thesis. For brief recap, the mechanical and chemical purposes are to flush out debris, to lubricate of the root canal, to dissolve organic and inorganic tissue, to prevent the formation of a smear layer during instrumentation or to eliminate it once it forms (Basrani and Haapasalo, 2012). The biological objectives are to eliminate anaerobic and facultative microorganisms in their planktonic and biofilm states, and inactivate their toxins, whilst being non-toxic to vital periodontal tissues and low in allergenicity (Basrani and Haapasalo, 2012).

Again, but way of brief recap, NaOCl is the irrigant of choice in root canal irrigation, because of its ability to meet most of the objectives highlighted (Basrani and Haapasalo, 2012), though its capacity to degrade the organic component of the dentine matrix has long been recognised (Shellis, 1983).

The chelating agents EDTA and citric acid are often used in conjunction with NaOCl and are active on the mineralised component of dentine and its smear layer (Hülsmann *et al.*, 2003b; Basrani and Haapasalo, 2012). EDTA is most active at neutral or alkaline pH (Parmar and Chhatariya, 2004), while acids such as citric acid (Sousa and Silva, 2005), and phosphoric acid (Marshall Jr *et al.*, 1997) are most active at acidic pH. The disodium salts of EDTA forms stable complexes with calcium ions derived from dentine and dissolution of its mineralised component results (Hülsmann *et al.*, 2003b), but after binding of all available ions, an equilibrium results and dissolution is arrested. The dissolution of minerals by the EDTA is therefore understood to be limited under some circumstances (Hülsmann *et al.*, 2003b).

Citric acid is a weak organic acid which reacts quickly with calcium (Papagianni, 2007; Pimenta *et al.*, 2012) to remove it from dentine (Machado-Silveiro *et al.*, 2004), with the formation of calcium citrate (Papagianni, 2007). The decalcifying efficacy of citric acid is influenced positively by its concentration (Machado-Silveiro *et al.*, 2004). The

decalcifying efficiency of chelating agents is influenced by exposure time, concentration and pH (Parmar and Chhatariya, 2004; Pérez-Heredia *et al.*, 2008). Citric acid may be more potent than EDTA at like-for-like concentration, though both are highly effective. Chelating agents can be used either as a liquid or paste (Basrani and Haapasalo, 2012).

Atomic force microscopy (AFM) can enable processes such as demineralisation, denaturation, collapse and renaturation in dentine collagen to be monitored at high resolution and in real time (El Feninat *et al.*, 1998). It can operate both in the air and in fluids under a hydrated environment (Breschi *et al.*, 1999). It has been used in both contact and intermittent-contact modes for the analysis of biological samples such as human dentine (El Feninat *et al.*, 2001a). In other studies, the tapping mode was used (Eliades *et al.*, 1997; Silikas *et al.*, 1999; Pyne *et al.*, 2009), while in the current project a quantitative imaging mode was employed.

The studies described in this chapter were undertaken to monitor the effect of NaOCl, citric acid and EDTA irrigants on the stiffness of dentine in a number of locations within human dental root canals.

6.2 Effect of NaOCl on human root dentine surfaces

6.2.1 Aim

To analyse the effect of NaOCl on the stiffness of human root dentine at different depths from the lumen of the root canal by AFM after irrigating root canals with 2.5%, 5% and 10% NaOCl for 5 minutes. This study tested the null hypotheses that NaOCl application at different concentrations (2.5%, 5% and 10%) would have no effect on the stiffness of dentine at different depths from the root canal lumen.

6.2.2 Materials and methods

6.2.2.1 Tooth collection and storage

Twenty five freshly extracted human premolar teeth were collected for this study. See section 3.2.2 for tooth collection and storage.

6.2.2.2 Specimen preparation

See section 3.2.2 for specimen preparation.

6.2.2.3 Treatment

See section 5.4.1 for treatment of the root canals. The exception in this study was that after treatment, the dental root specimens in the current study were stored in 1% chloramine-T at 4˚C instead of the 2% glutaraldehyde (2% glutaraldehyde in Sorenson's phosphate buffer, pH 7.3).

AFM analysis usually takes 1 day per group, so in this experiment each group was treated on a different day to regulate the duration of storage in 1% chloramine-T and keep it constant for each group, at each step.

Table 6.1. Summary of treatment regimens of human roots.

Group	Treatment	Temperature (˚C)	Time (minutes)
1	**Non-treated**	**N/A**	**N/A**
2	**Normal saline**	**25**	**5**
3	**2.5% NaOCl**	**25**	**5**
4	**5% NaOCl**	**25**	**5**
5	**10% NaOCl**	**25**	**5**

6.2.2.4 Storage of the samples and AFM preparation

See section 3.4.2 and section 3.5.2 (storage of the samples and material preparation).

6.2.2.5 Analysis of the of the samples by the AFM

See section 3.2.2 for details of analysis of the samples by the AFM. Results were analysed using the Kruskal-Wallis test ($p<0.05$).

6.2.3 Results

AFM images of dentine surfaces are presented in Figures 1.1, 1.3, 1.5, 1.7 and 1,9, with corresponding force-distance curves presented in Figures 1.2, 1.4, 1.6, 1.8, and 1.10. The median stiffness values for horizontal sections of human cervical root dentine are presented in table 1.2 and Figure 1.11.

When compared with the non-treated control (Figure 6.1) there was no apparent changes in the structure of dentine resulting from treatment with of NaOCl at 2.5%, 5% and 10% concentration (Figure 6.5, Figure 6.7, Figure 6.9 and respectively). Exposure to normal saline also brought no change relative to the non-treated control (Figure 6.3 and Figure 6.1 respectively).

The force-distances curves of all groups (1-5) showed similar patterns of vertical deflection (extended) per distance and vertical deflection (retract) per distance, although there were differences in their absolute values. In addition, the fluctuations of these curves were slightly different in the l group treated with 10% NaOCl in comparison with the other groups (Figure 6.10, Figure 6.2, Figure 6.4, Figure 6.6 and Figure 6.8 respectively). Analysis of median stiffness data presented in Table 3.8 and Figure 6.11 showed no significant difference between the groups ($p>0.05$), with no difference between experimental groups and non-treated controls. Variation within experimental groups at different depths from the canal lumen was not statistically significant ($p>0.05$). The differences between different experimental groups at comparable depths from the lumen were not statistically significant ($p>0.05$), although the variation between Group 5 (treated with 10% NaOCl) and all other groups was more prominent. It was noted in Group 5 that the stiffness of dentine was lower than in all other groups at all depths from the lumen up to 1000µm. At this depth, all of the non-treated and experimental groups had median values ranging from 126 to 136 nN/µm.

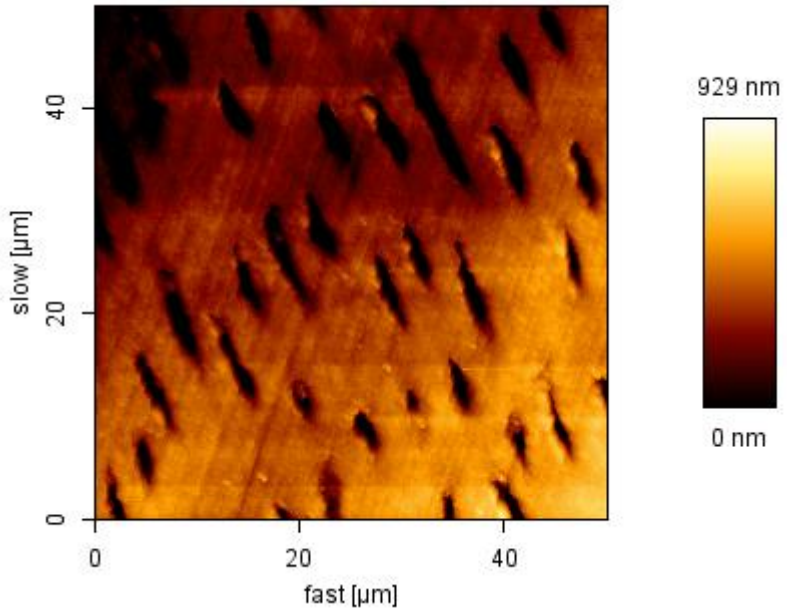

Figure 6.1. AFM image to show the structure of untreated root canal dentine walls (Group 1) in the cervical third of non-treated human teeth, at 100µm depth from the root canal lumen.

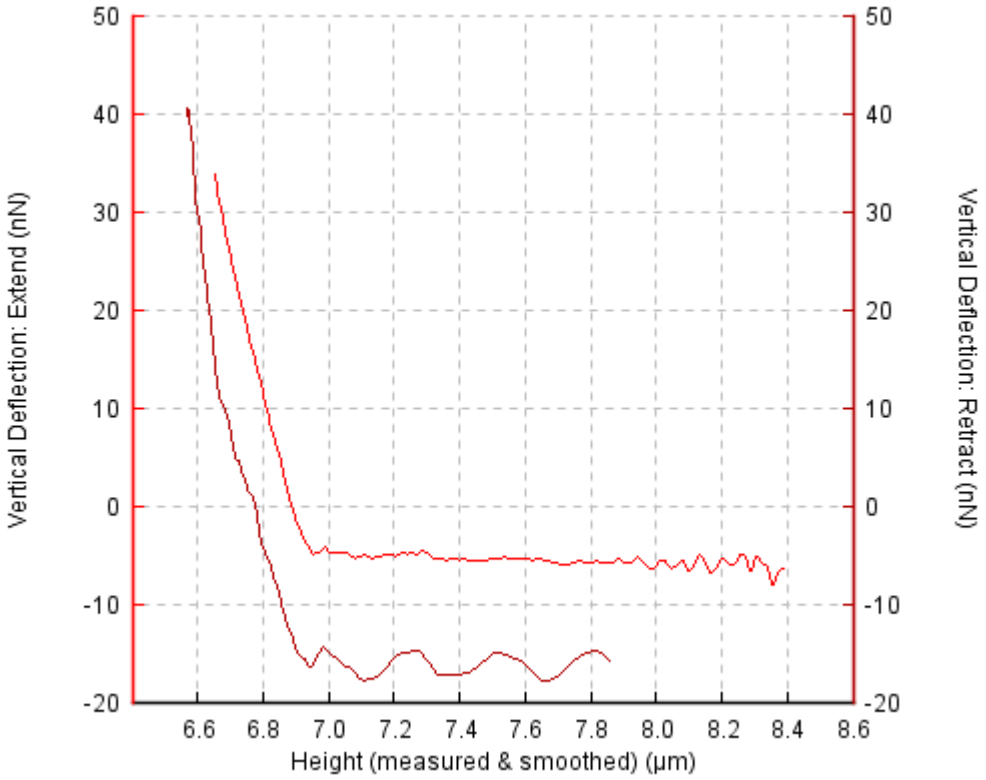

Figure 6.2.Typical force curve from horizontal section of root dentine in the cervical third of non-treated human teeth (Group 1).

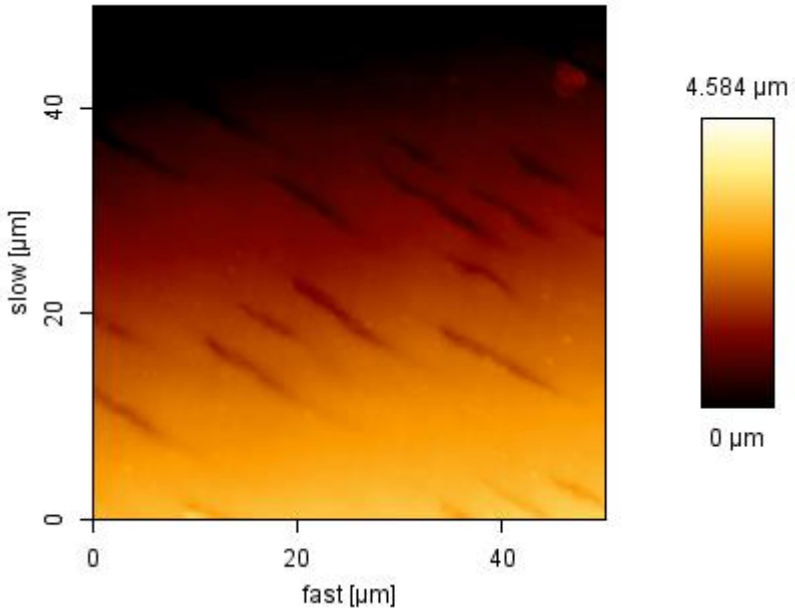

Figure 6.3. AFM image from horizontal section of root dentine to show the structure of root dentine in the cervical third of human teeth, at 200µm depth from the root canal lumen, after 5 minutes treatment with normal saline (Group 2).

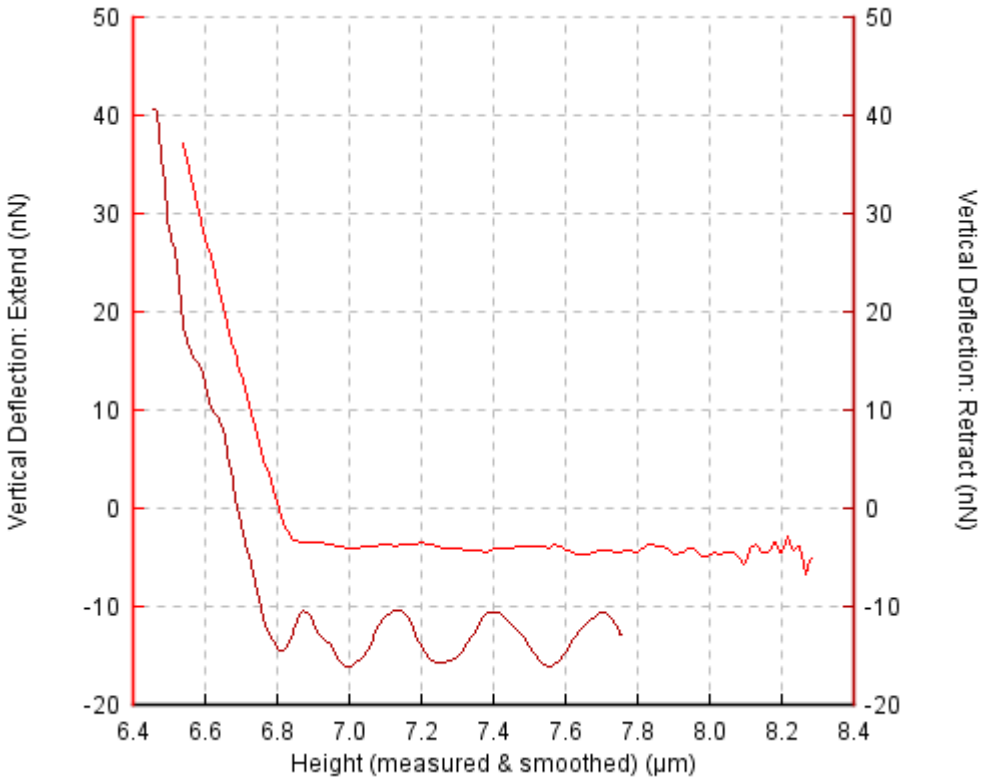

Figure 6.4.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, at 100µm depth from the root canal lumen, after 5 minutes treatment with normal saline (Group 2).

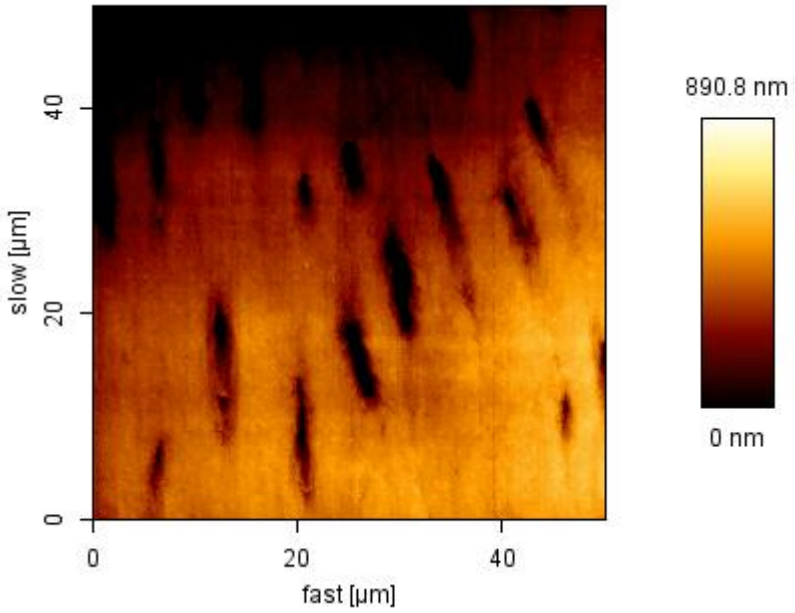

Figure 6.5. AFM image from horizontal section of root dentine to show the structure of root dentine in the cervical third of human teeth, at 100µm depth from the root canal lumen, after 5 minutes treatment with 2.5% NaOCl (Group 3).

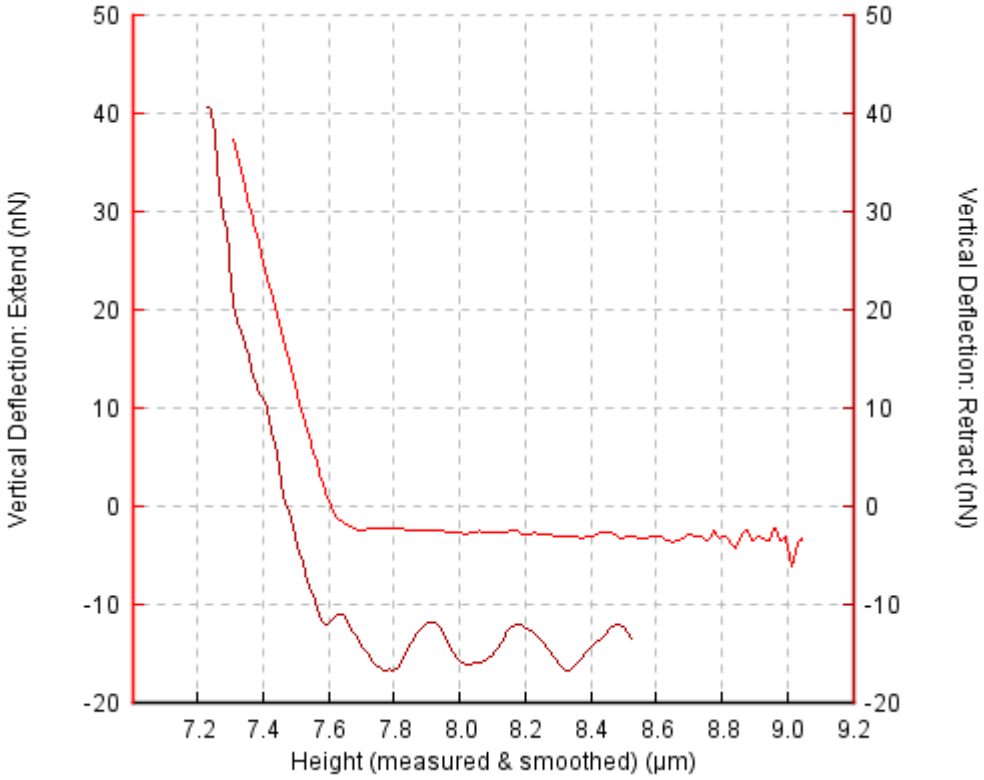

Figure 6.6.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, at 200µm depth from the root canal lumen, after 5 minutes treatment with 2.5% NaOCl (Group 3).

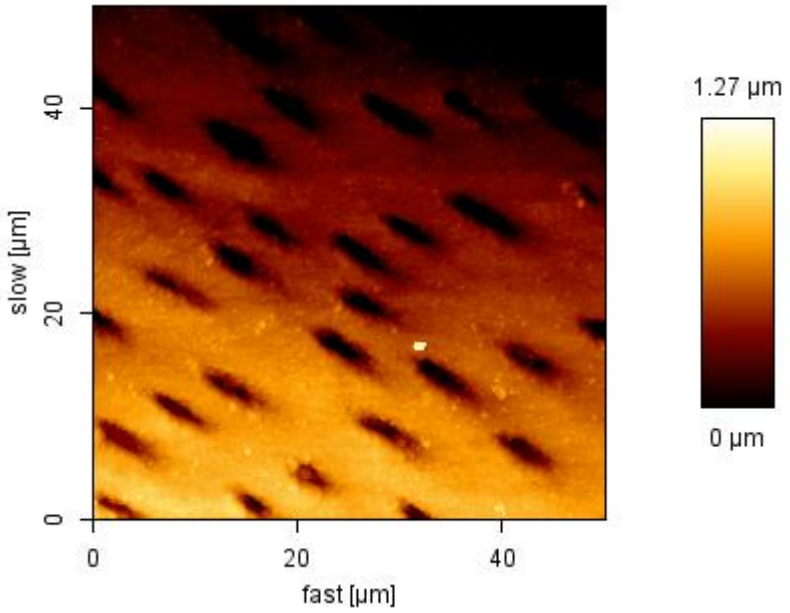

Figure 6.7. AFM image from horizontal section of root dentine to show the structure of root dentine in the cervical third of human teeth, at 100µm depth from the root canal lumen, after 5 minutes treatment with 5% NaOCl (Group 4).

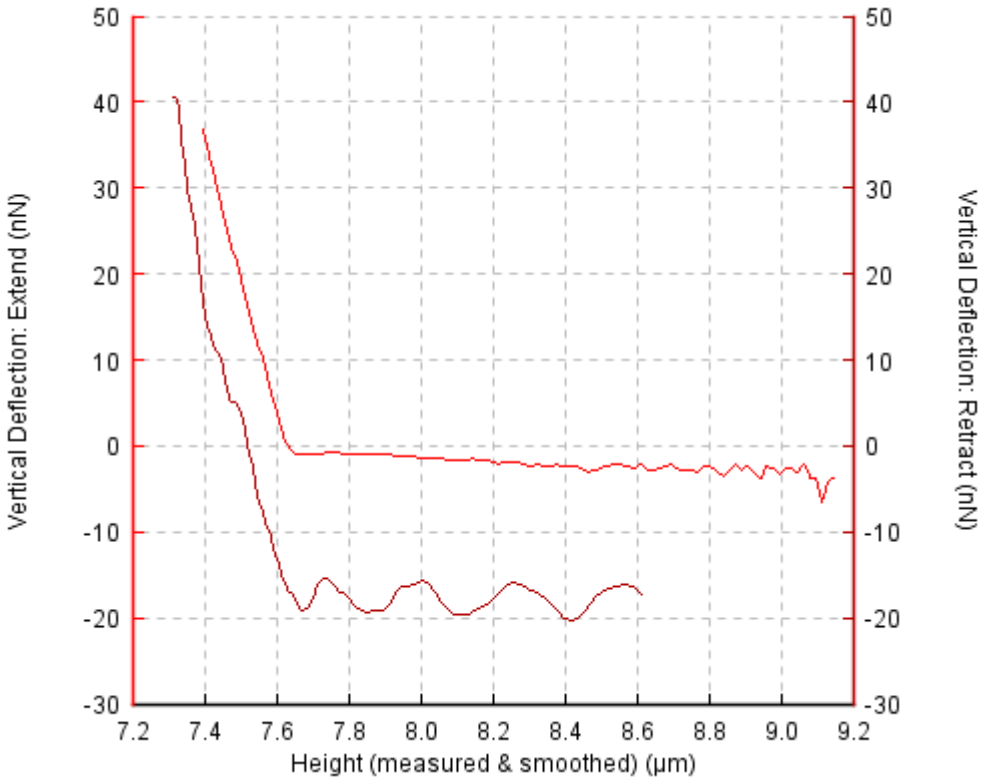

Figure 6.8.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, after 5 minutes treatment with 5% NaOCl (Group 4).

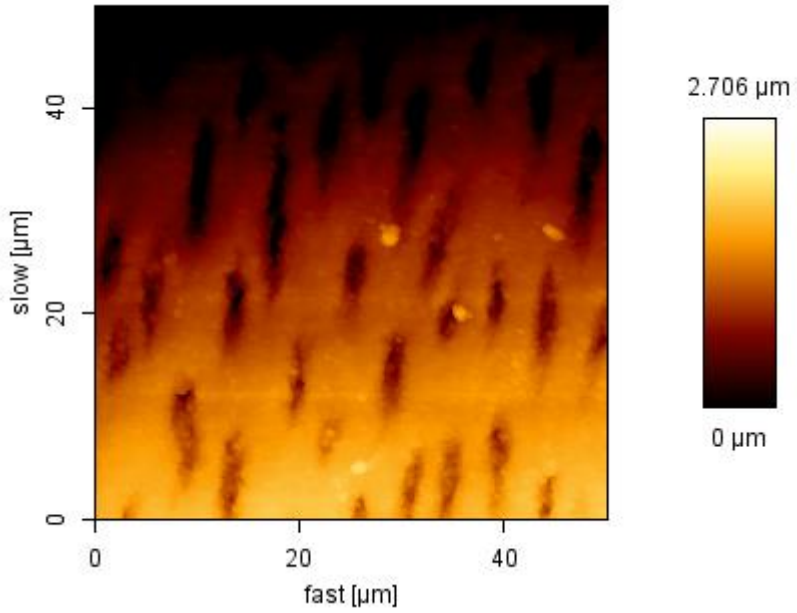

Figure 6.9. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, at 200µm depth from the root canal lumen, after 5 minutes treatment with 10% NaOCl (Group 5).

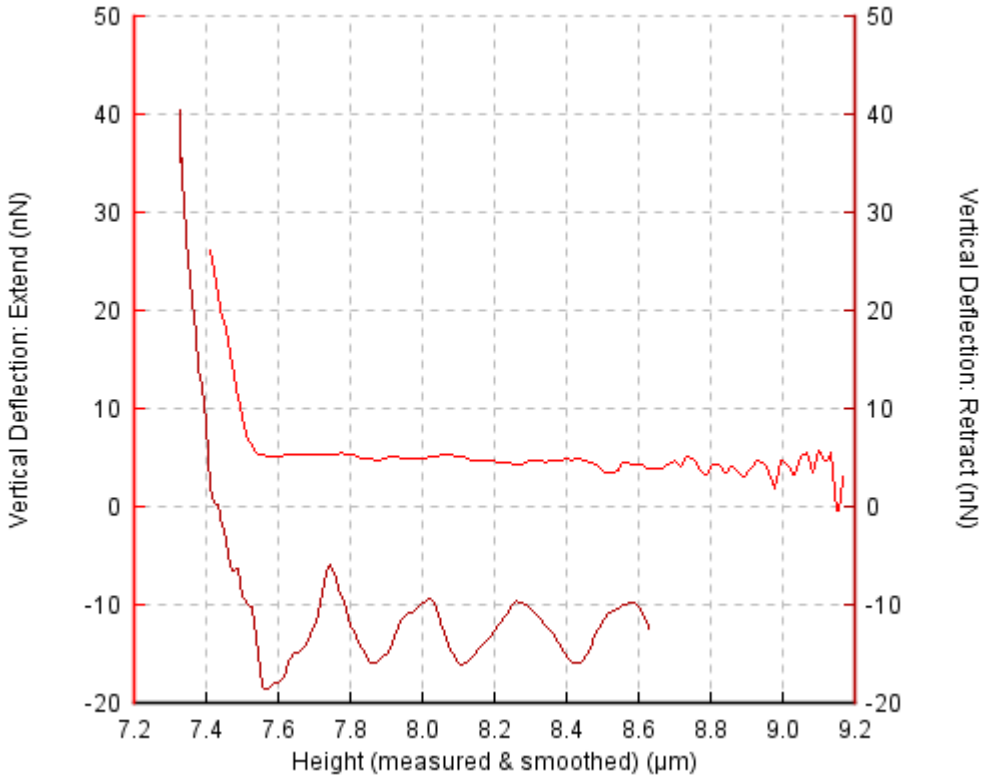

Figure 6.10.Typical force curve from horizontal section of root dentine in the cervical third of human teeth, after 5 minutes treatment with 10% NaOCl (Group 5).

Table 6.2. Median (upper and lower quartiles) dentine stiffness of horizontal cervical dentine sections of human teeth at, 0µm, 100µm, 200µm, 300µm and 1000µm from root canal lumen, after root canal irrigation with normal saline and different concentrations of sodium hypochlorite, 2.5%, 5% and 10% (Groups 1-5 respectively).

Group	Treatment	At 0µm	At 100µm	At 200µm	At 300µm	At 1000µm
1	Control	132 (146 & 126)	130 (139 & 126)	128 (136 & 126)	127 (127 & 124)	127 (131 & 122)
2	Normal saline	142 (147 & 136)	138 (142 & 132)	137 (139 & 130)	136 (141 & 129)	136 (140 & 126)
3	2.5% NaOCl	132 (146 & 129)	135 (143 & 127)	132 (136 & 128)	132 (137 & 127)	129 (135 & 126)
4	5% NaOCl	142 (151 & 130)	137 (147 & 129)	131 (141 & 130)	132 (140 & 127)	130 (140 & 129)
5	10% NaOCl	117 (153 & 108)	116 (151 & 105)	117 (153& 102)	113 (154 & 97)	126 (165 & 104)

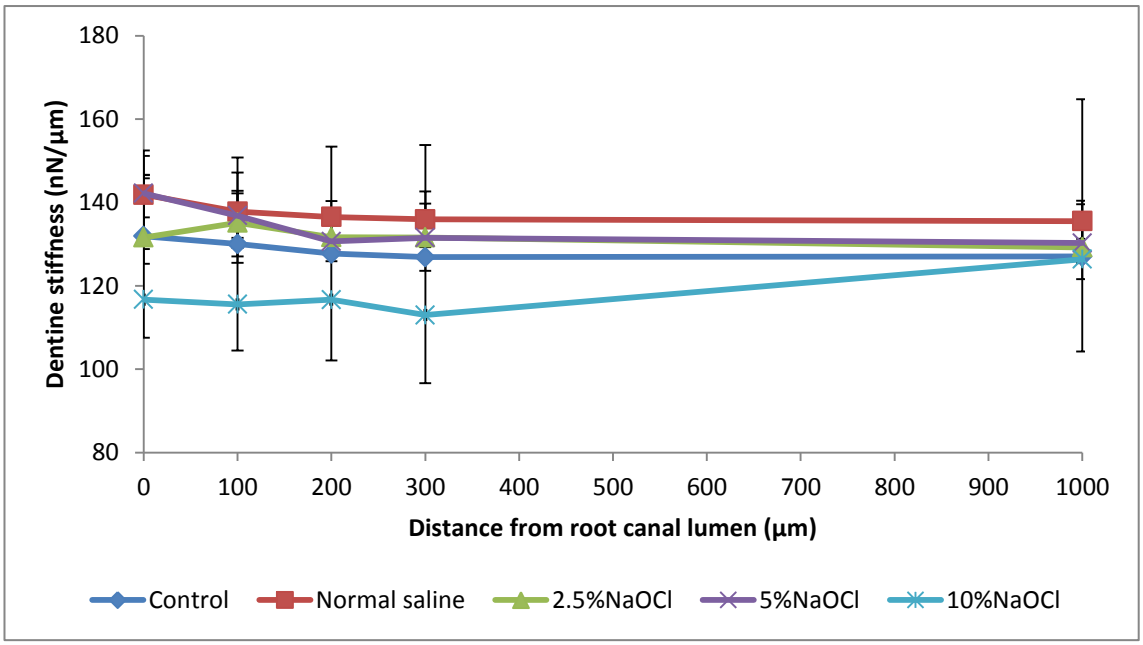

Figure 6.11. Median dentine stiffness at different depths from the lumen of horizontal cervical human root dentine 0µm, 100µm, 200µm, 300µm and 1000µm, after no treatment or root canal irrigation with normal saline and different concentrations of sodium hypochlorite, 2.5%, 5% and 10% (Groups 1-5 respectively). The differences were not significant ($p>0.05$, Kruskal-Wallis test). The error bars are the upper and lower quartiles for each group.

6.2.4 Discussion

As in a previous experiment (section 3.4) which analysed Ca/P ratios of cervical root dentine at 5 different depths from the lumen, this study examined dentine in similar locations, but this time measuring stiffness by AFM after root canal irrigation with NaCl or NaOCl.

AFM has the ability to measure the viscoelastic properties of dentine surfaces, such as stiffness, which is equal to the initial linear portion of the unloaded part of the force-distance curve. It has the ability to image samples as making measurements, so the exact location under analysis can be determined. All analyses can be made in a wet environment to prevent the dehydration of dentine and the collapse of collagen fibres (Kinney *et al.*, 1999).

Teeth had been stored for approximately 1 month between extraction and preparation for this study. Investigations on bone have shown a decreased elastic modulus, when storage extended from 24 to 72 h in water (Gustafson *et al.*, 1996); a changed attributed to possible demineralisation by water. Prepared specimens employed in this study were therefore stored for more than 24 hours in 1% chloramine-T before AFM analysis in this study. Other studies on dentine have also restricted pre-analysis storage to 24 h (Kinney *et al.*, 1999).

Preliminary investigations for the current work confirmed the pH of chloramine-T as neutral (approximately 7.2), whilst distilled water had a pH of approximately 6.5 and may have presented a demineralisation risk. Pilot studies for the current work also evaluated the effects of storing dentine specimens in 1% chloramine-T, 2% glutaraldehyde (pH 7.3) and DPBS (pH 7.2). This work confirmed that chloramine-T did not affect the stiffness of dentine, while the other solutions reduced it significantly. The precise mechanisms responsible for these observed changes are unclear.

Another risk in the current study was the influence of NaOCl on the AFM tips. NaOCl is believed to damage the silicon nitride coating of AFM tips, and precautions with water flushing were undertaken to avoid contact of AFM tips with NaOCl.

The AFM method was convenient for the analysis of horizontal slices of root dentine directly after irrigating the canals, and without the need to cut dentine into bars. In

addition, and unlike sample preparation for SEM, there was no need for chemical preparation or coating of specimens for AFM. The AFM can be considered as a dynamic tool, since it can analyse the effect of NaOCl on dentine surfaces directly after treatment. Analysis of tissues at different depths from the root canal lumen was also readily achieved.

The current experiment found no changes in the stiffness of dentine after NaOCl treatment, and found no significant effects compared to control on dentine close to the canal lumen and at distances up to 1000µm from the lumen. Nanomechanical properties did not appear to have been significantly affected.

Marshall *et al* (2001), measuring hardness and elastic modulus, made conflicting observations. Working on human coronal dentine after etching dentine of surfaces with 10% citric acid for 15 sec followed by 6.5% NaOCl for periods between 5 sec and 30 minutes, they showed with a significant reduction in both parameters after exposure to NaOCl. This was attributed to initial swelling in depth measurements. With further exposure to 6.5% NaOCl, values increased due to the removal of collagen fibrils; values arriving at a constant level of approximately 70- 80% the values of un-etched dentine (0.7-0.9 GPa and 14-16 GPa, compared with pre-treatment values of 1.1 GPa and 20 GPa respectively (Marshall *et al.*, 2001c).

This behaviour of an initial decrease, followed by an increase in the nanomechanical properties of dentine was not observed in the current experiment, and this may be related to the fact that specimens in the current study were not treated initially with 10% citric before exposure to NaOCl (Marshall *et al.*, 2001c). The lower exposure times could also be relevant.

In terms of tissue microstructure, the combined etching and deproteinating effects of citric acid and NaOCl employed by Marshall *et al* (2001) created porous dentine surfaces, with exposure of anastomosing secondary dentinal tubules and canaliculi, which may have made the tissue more susceptible to chemical treatments. Similar observations were made by Perdigão *et al.*, 1999, who noticed that canaliculi were more prominent in superficial areas of intertubular dentine than in deep areas of intertubular dentine, after exposure of dentine to phosphoric acid (H_3PO_4) and NaOCl (Perdigão *et al.*, 1999). Impingement of the AFM tip into these canaliculi during the scanning

resulted in force displacement-curve changes and large reductions in nanomechanical properties. This phenomenon was not observed after treating dentine only with NaOCl (without citric acid) which showed the typical pattern of the force displacement-curve (Marshall *et al.*, 2001c), corroborating the observations of the current investigation. Another AFM study was also in agreement with the current work, finding that the application of 5.25% NaOCl for 1 minute had no significant effect on dentine stiffness (Baron *et al.*, 2013). Details of the NaOCl exposure are however different, making direct comparison difficult.

AFM studies are few, but researchers have often applied other tools such as microindenters and microhardness testers to investigate the mechanical properties of dentine. Slutzky-Goldberg *et al* (2004) measured the microhardness of bovine cervical root dentine at distances of 500µm, 1000µm, and 1500µm from the lumen of the root canal after irrigation with 6% and 2.5% NaOCl for 5, 10 and 20 minutes (Slutzky-Goldberg *et al.*, 2004).

Slutzky-Goldberg *et al* (2004) observed that the microhardness of dentine decreased at 500µm from the lumen after irrigation with both NaOCl solutions and after all periods of exposure (Slutzky-Goldberg *et al.*, 2004). At 500µm from the lumen, the reduction in microhardness in comparison with the control group was not statistically significant after 5 minutes treatment with either 2.5% or 6% NaOCl solutions, whilst it was significant after 10 and 20 minutes irrigation. The response to 6% NaOCl was more marked than after exposure to 2.5% NaOCl. At 1000µm and 1500µm from the lumen, there was no statistically significant difference among groups at any period and this was attributed to limited penetration of NaOCl into the tissue (Slutzky-Goldberg *et al.*, 2004). In addition, there were no significant differences found for the microhardness of dentine at 500µm, 1000µm and 1500µm from the root canal lumen after 5 minutes treatment.

In the current experiment, it was also found that 5 minutes treatment with NaOCl did not affect the mechanical properties of dentine at any depth from the lumen.

Direct comparison between the current study and that of Slutzky-Goldberg *et al* (2002) are complicated by differences in experimental design (instrumented versus non-instrumented canals, bovine vs human dentine, different irrigation regimes) and testing

methods (indentation vs AFM) Consensus has not, however, been reached on the best experimental models and most appropriate testing methods, and until this is the case, the comparison of studies will be subject to much speculative interpretation.

The decision to explore the effects of irrigant on dentine at different depths from the lumen, recognised the porous, tubular structure of dentine and the recognition that NaOCl may exert antimicrobial properties 400µm or 500µm inside dentinal tubules (Haapasalo and Ørstavik, 1987). The influence of tubular size and number in different areas of roots has been recognised (Berutti *et al.*, 1997), and for this reason, cervical dentine with its relatively wide and numerous dentinal tubules as employed in the current study. One explanation for the insignificant effect of NaOCl on dentine at different depths from the lumen in the current study may have been a loss of potency after its first contact with and residual organic debris and with predentine at the root canal lumen. A study showed that 5.25%, 2.6% and 1.3% NaOCl dissolved approximately 90.1%, 90.3% and 83.2% (weight percent) of pulp tissue from bovine teeth and dissolved 21.8%, 17.7% and 11.8% (weight percent) of the organic part of dentine structure (Beltz *et al.*, 2003). More work is necessary to understand the detailed dynamics of interaction between NaOCl and surface/subsurface organic materials in clinically realistic settings (Hu *et al.*, 2010).

6.2.5 Conclusions

Within the limits of this study, the null hypothesis was supported, since exposure of dentine to different concentrations of NaOCl (2.5%, 5% and 10%) resulted in no significant change of root dentine stiffness at different depths from root canal lumen.

6.3 **The effect of citric acid on human root dentine surface**

6.3.1 Aim

To analyse the effect of citric acid on the stiffness of dentine surface of human root wall after exposure of the root canal for 1 and 2 minutes using AFM. This study tested the null hypothesis that 6% citric acid had no effects on the dentine stiffness of dentine surface of human root canal wall.

6.3.2 Materials and methods

6.3.2.1 Tooth collection and storage

Five freshly extracted human premolar teeth were used for this study. See section 3.2.2 for tooth collection and storage.

6.3.2.2 Specimen preparation

See section 3.2.2 (in the first chapter of the results). The roots were then split into two halves by grooving them longitudinally with a flex diamond disc (Skilldenta, Skillbond®, Skillbond Direct Ltd Dudley House, UK) until just before the lumen and then cracking apart with a chisel.

6.3.2.3 Storage of the samples and AFM preparation

Subsequently, each half was embedded in polystyrene based clear resin and the pulpal side of the canal was directed upwards to be analysed by the AFM, see the details of embedding the samples in clear resin and their polishing and AFM preparation in section 3.4.2 and section 3.5.2 (storage of the samples and preparation).

6.3.2.4 Treatment

Samples were analysed by AFM before treatment and after treatment with 6% (wt/v) citric acid (SIGMA-ALDRICH, UK), pH 2.4 for 1 minute, and 2 minutes. The 6% citric acid solution was prepared by dissolving 6g citric acid powder in 100 mL distilled water, and the pH was measured with a pH meter (Orion 4 Star).

Treatment of dentine was undertaken by withdrawing DPBS by syringe from the AFM liquid cell, and replacing with 6% citric acid. After the allotted time of treatment (1 or 2 minutes), the citric acid was aspirated by syringe and replaced with DPBS liquid.

6.3.2.5 Analysis by AFM

See section 3.5.2 for details of sample analysis by AFM. In the current experiment 9 areas of each specimen were analysed, using the x-direction and y-direction moving scales of the AFM analysing tip after elevation using the z-direction moving stage. The distance between each point was 100μm vertically and horizontally in the same third (the basis of their selection being explained in the discussion section) (Figure 6.12). The 9 points of each specimen were analysed before treatment, after 1 minute and after 2 minutes treatment with citric acid. Results were analysed using Kruskal-Wallis and Mann-Whitney tests ($p<0.05$).

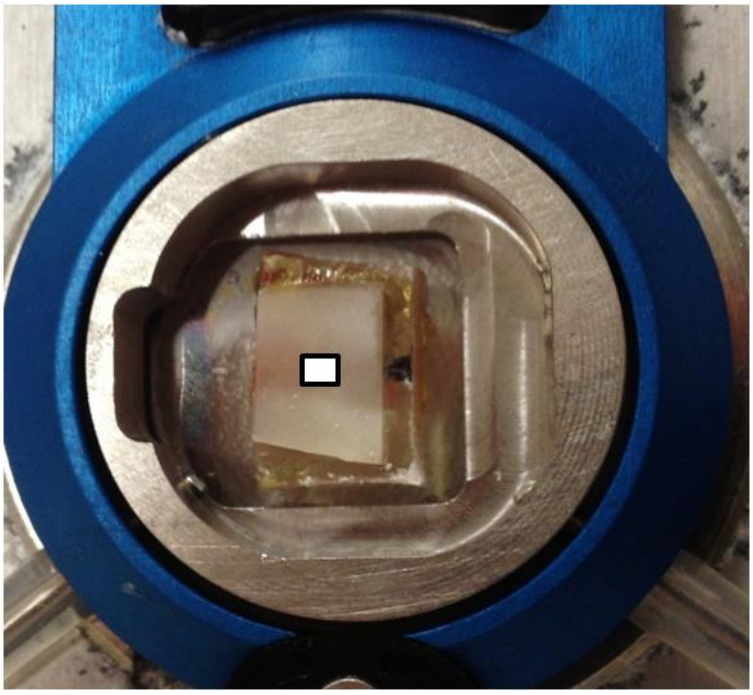

Figure 6.12. Shows a specimen within the AFM liquid cell, with a white square on the surface which represents the area in which the 9 points of surface analysis were positioned.

6.3.3 Results

Figure 6.13, Figure 6.15 and Figure 6.17 show AFM images of the dentine surface before treatment, after 1 minute treatment, and after 2 minutes treatment with 6% citric acid respectively. Figure 6.13 shows dentinal tubules plugged with smear layer. After 1 minute of citric acid treatment, the dentinal tubules were opened (Figure 6.15). This figure does not show whether this represents opening of dentinal tubules by smear plug removal alone or if there was additional opening of dentinal tubules with increase in their diameter due to dissolution of the highly mineralised peritubular dentine. After 2 minutes treatment with citric acid, there was no apparent further increase in the diameter of tubular openings compared with 1 minute treatment.

Figure 6.14, Figure 6.16 and Figure 6.18 show the force distance curves of the dentine surfaces before treatment with 6% citric and after treatment for 1 minute and 2 minutes respectively. They show similar patterns of vertical deflection (extended) per distance and vertical deflection (retract) per distance.

The median human root dentine stiffness (upper and lower quartiles) of all groups can be seen in Table 6.3. The table shows the median stiffness of the surface of the root canal wall at 9 points on the cervical third of human root dentine. Dentine stiffness was reduced after treatment with 6% citric acid for 1 minute compared with control ($p<0.05$), but did not reduce significantly further after 2 minutes of treatment ($p>0.05$) (Figure 6.19).

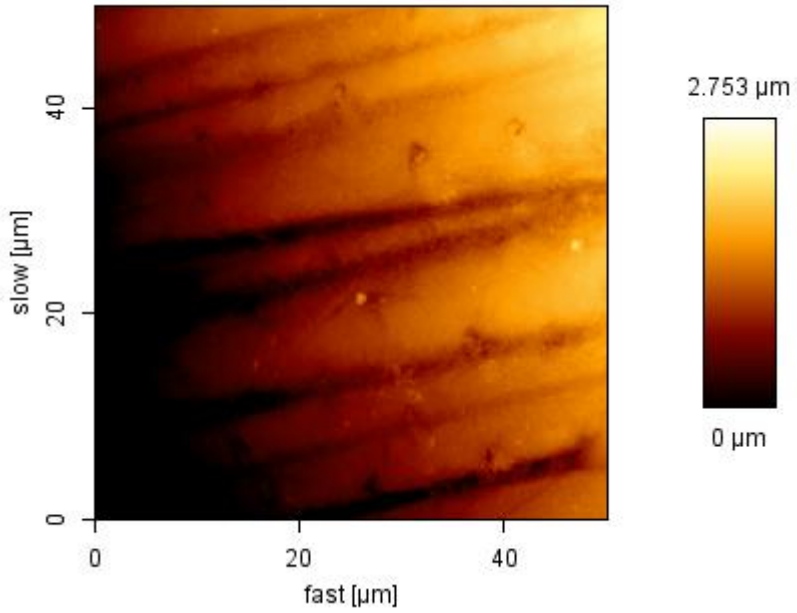

Figure 6.13. AFM image to show the structure of root canal dentine walls in the cervical third of non-treated human teeth (Group 1).

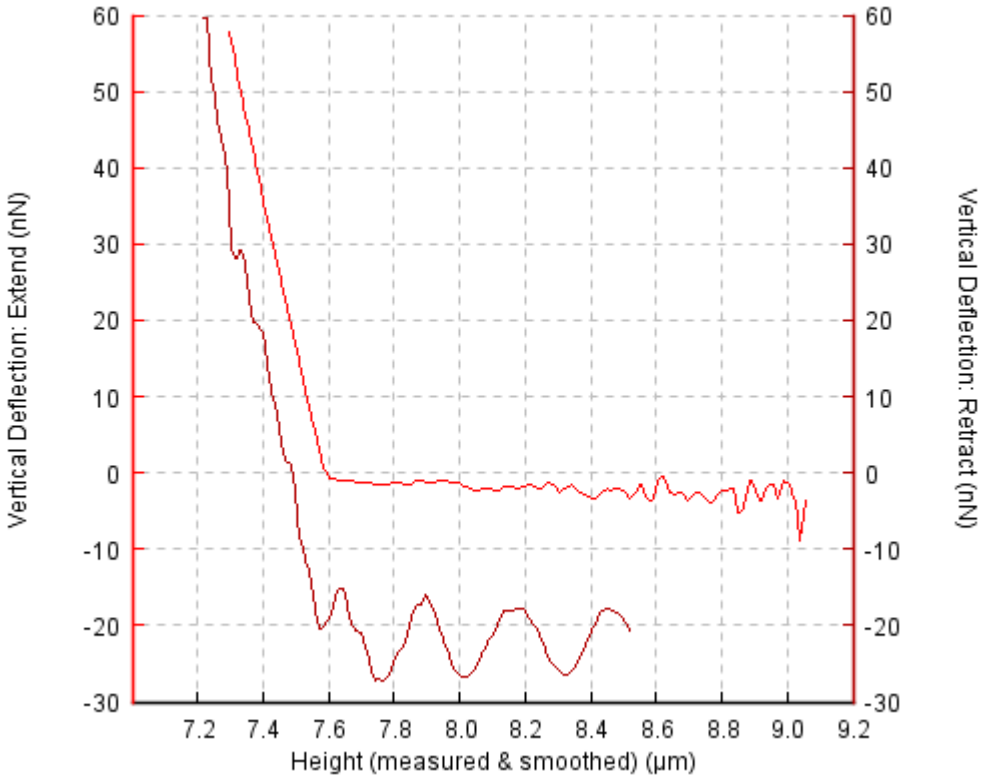

Figure 6.14. Typical force curve of root canal dentine walls in the cervical third of non-treated human teeth (Group 1).

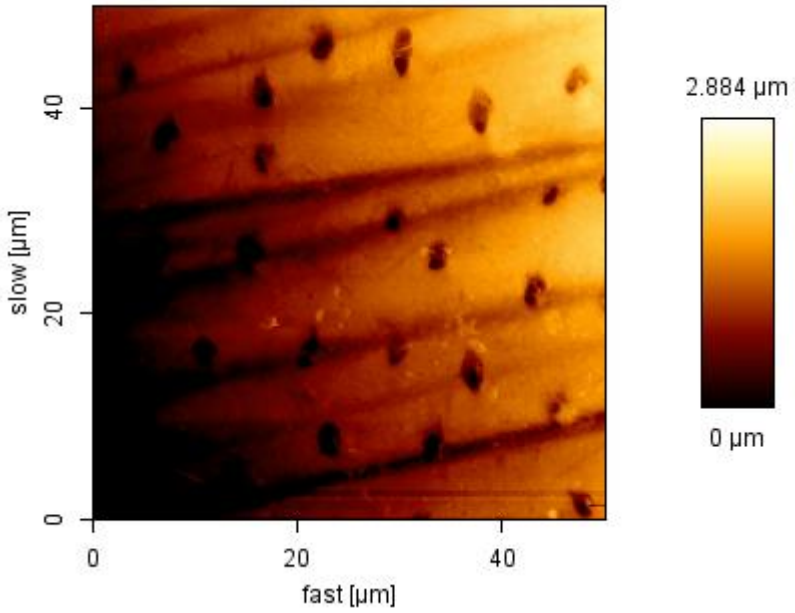

Figure 6.15. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 6% citric acid (Group 2).

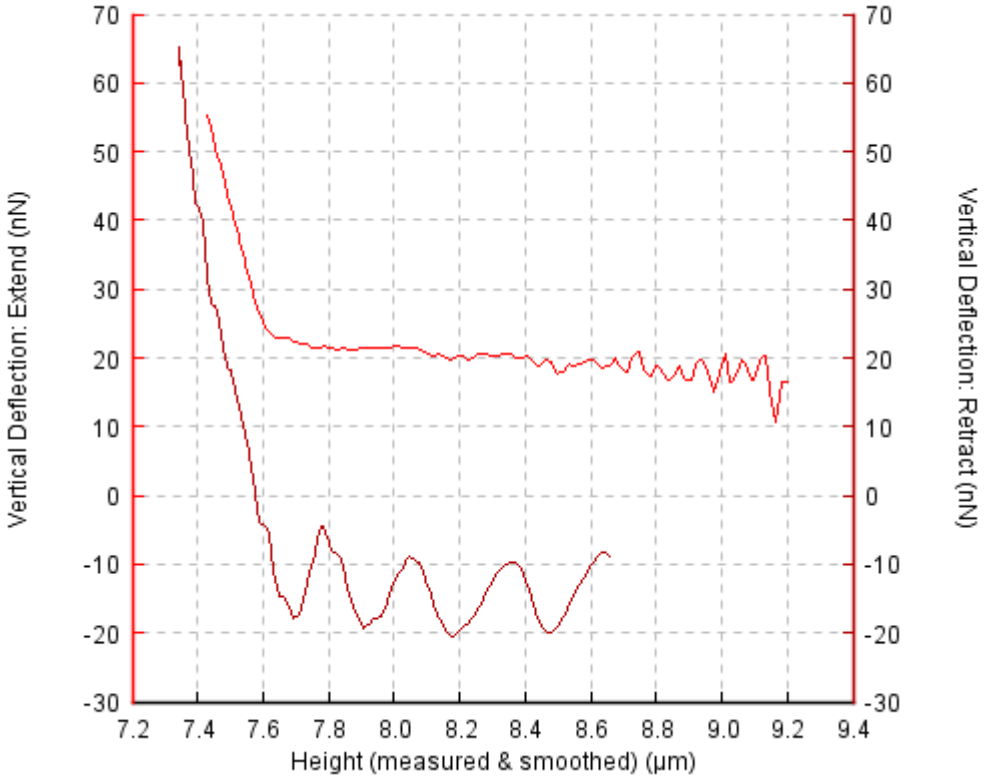

Figure 6.16.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 6% citric acid (Group 2).

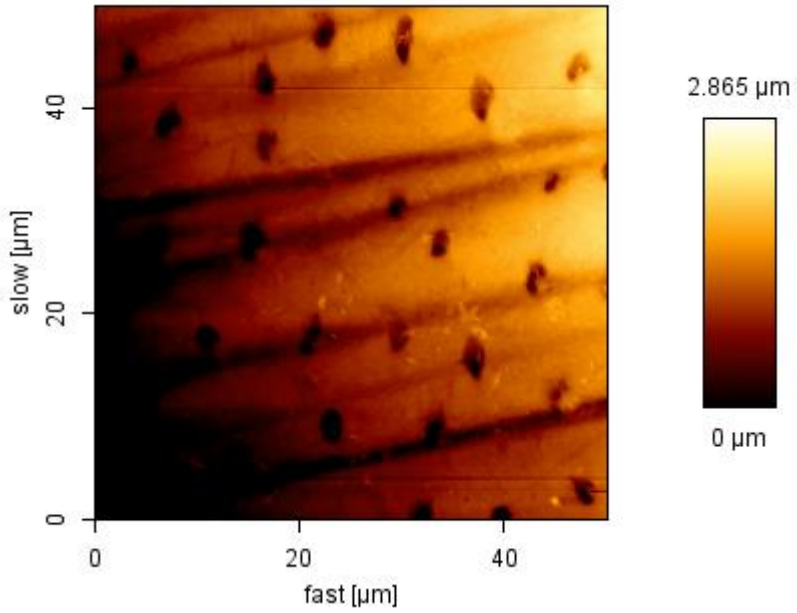

Figure 6.17. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 6% citric acid (Group 3).

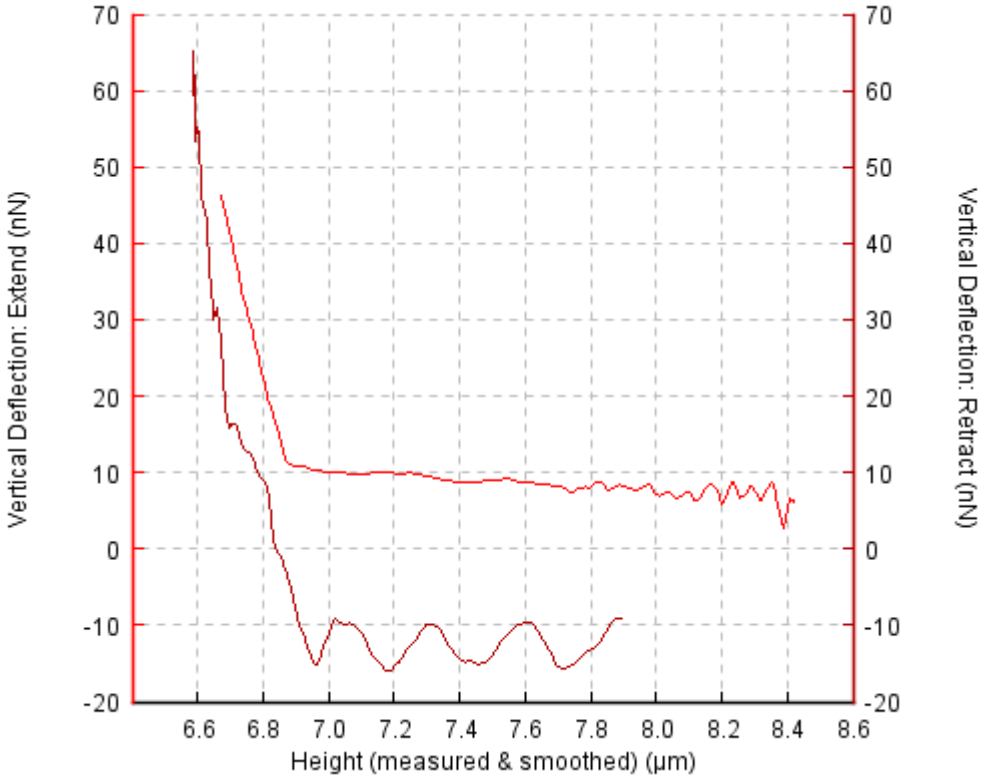

Figure 6.18.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 6% citric acid (Group 3).

Table 6.3. Median (upper and lower quartiles) dentine stiffness of human cervical root canal walls, with no treatment, and after 1 and 2 minute treatments with 6% citric acid.

Group	Treatment	Median dentine stiffness (upper and lower quartiles)
1	Control	176 (191 & 161)
2	6% citric acid, 1 minute	153 (156 & 148)
3	6% citric acid, 2 minutes	153 (156 & 150)

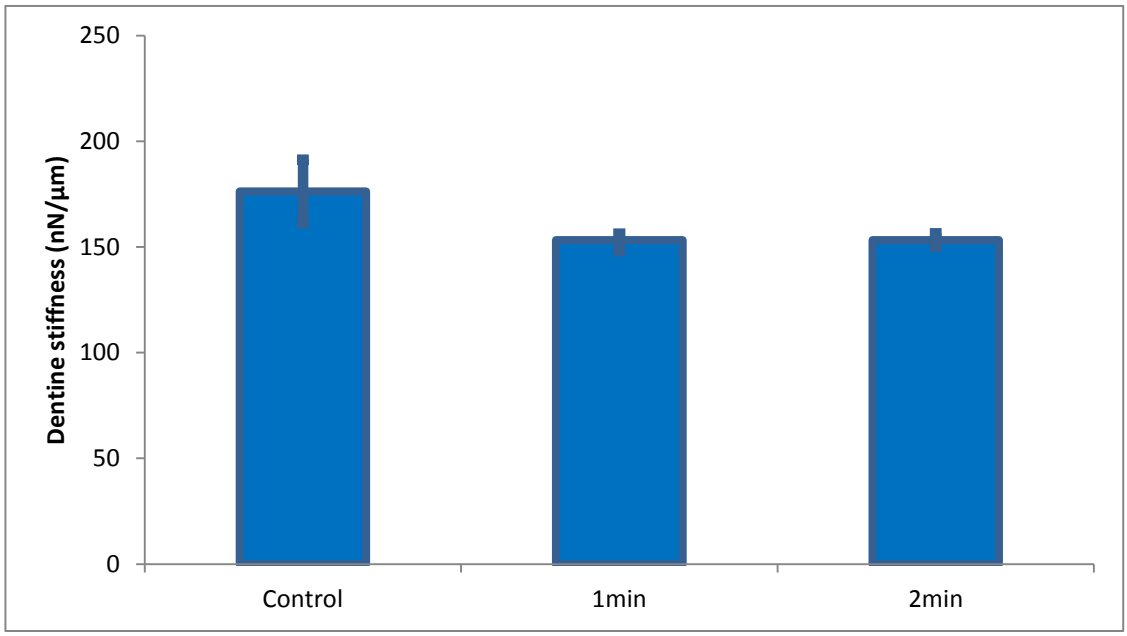

Figure 6.19. Median dentine stiffness (nN/µm) of human cervical root canal wall, with no treatment, 1 and 2 minute treatments with 6% citric acid. The difference was significant between the control group and the 1 minute treatment, and between the control group and the 2 minute treatment ($p<0.05$), but between the 1 minute and 2 minute treatments, the difference was not significant ($p>0.05$, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the upper and lower quartiles.

6.3.4 Discussion

Dentine can be demineralised by an acid etching agent to form a porous collagenous surface layer (Habelitz *et al.*, 2002a). AFM has been used successfully to study microstructural changes and etching rates of peritubular dentine and intertubular dentine during acid demineralisation (Marshall Jr *et al.*, 1993; Marshall *et al.*, 1995; Marshall Jr *et al.*, 1997; Marshall *et al.*, 1998). It has also been used in endodontic research to evaluate dentine changes following the use of acids such as citric acid, EDTA and EDTAC (De-Deus *et al.*, 2006). It may be considered as a dynamic tool to study the effects of irrigants on dentine surface, since it has been used to monitor demineralisation, drying, collapse, denaturation and bonding (Kinney *et al.*, 1993; Marshall *et al.*, 1998; El Feninat *et al.*, 2001a).

In the current experiment, analysis was undertaken in real-time as demineralising events took place; a technique has been described previously (Cassinelli and Morra, 1994; De-Deus *et al.*, 2006). In the current experiment, the analysis of dentine surface stiffness was undertaken at the same time as imaging, and samples were fully hydrated. Such real-time, hydrated analysis is once again not possible in conventional SEM (Silikas *et al.*, 1999; Crumpton *et al.*, 2005; Teixeira *et al.*, 2005), although this may be possible with environmental SEM. The current AFM method also allowed repeated measurements of the same point. Shortcomings within this experiment included the limited number of human root dentine samples available for analysis, variation in the likely age and degree of mineralisation of the teeth and likely variation in storage times, and conditions.

Results of this study suggested that one minute treatment with 6% citric acid reduced the surface stiffness of dentine significantly in comparison with untreated control specimens ($p<0.05$). A 2 minute exposure did not reduce surface dentine stiffness to a significantly greater degree than the 1 minute treatment ($p>0.05$). The observed change in the mechanical properties of dentine following 6% citric acid application broadly supported the findings of another study which employed microhardness measurements and found a significant reduction from 69.73 VHN (control group) to 46.53 VHN following the application of 19% (1 M) citric acid at pH 1.3 for 2.5 minutes, followed by 5.25% NaOCl at pH 11.85 for 2.5 minutes (Eldeniz *et al.*, 2005). But the irrigation protocols were not really comparable.

In a study that analysed the roughness of dentine, it was concluded that it is better to undertake AFM analysis on different points within the same sample, in order to represent the sample properties, because dentine surfaces are non-uniform (heterogeneous) structures with surface variation. Even relatively homogenous structures such as aluminium have been shown to have surface variation (El Feninat *et al.*, 2001b). Therefore, in this study 9 points were selected for analysis. The specimen size permitted the selection of a maximum of 3 points horizontally, with 3 points on a cervical line, 3 points on a second, middle line, and the last 3 point on an apical line. In each line, the central points coincided with the canal wall and the other 2 points were away from the canal wall as a result of polishing. The criteria for selection of these points was by selecting points on the dentine surface with enough distance from each other with at least 100µm to prevent the overlap during the scan of each point, since the dimension of each scan was 50µm * 50µm.

One study identified that undertaking acid etching of the dentine surface with 10% citric acid for 15 sec can produce 3 zones. The superficial layer consisted of a collagen rich zone, the second layer contained a small concentration of minerals and the third layer was a mineralised dentine structure (Habelitz *et al.*, 2002a). Surface and sub-surface changes of this sort could not be assessed in the current study, which only analysed surface changes. However, the limitation of 2 minutes citric acid treatment in bringing about further measurable change may have reflected the exposure of a collagen rich zone which could limit further tissue demineralisation. The exposure of collagen fibrils by demineralisation of dentine with acids has also been confirmed in studies employing 37% phosphoric acid for 15 sec (El Feninat *et al.*, 2001a). Habelitz *et al* (2002) also assumed that the extrafibrillar mineral was dissolved initially by acid, whilst the intrafibrillar mineral demineralised more slowly. This is due to the fact that the intrafibrillar mineral remained protected by collagen molecules (Habelitz *et al.*, 2002a), and this was supported by a study which showed that 5 minutes irrigation of root canals with 10% citric acid resulted in loss of calcium ions (Spanó *et al.*, 2009).

The arrival to plateau after 1 minute treatment agreed with the finding of another study which applied 0.018 M citric acid (pH 2.5) for periods up to 30 minutes to etch normal dentine surfaces, with the observation of peritubular dentine demineralising rapidly, the diameter of dentinal tubules increasing, the less mineralised intertubular dentine

demineralising slightly and the recession of intertubular dentine plateauing after 10 minutes (Marshall *et al.,* 2000). Similar observations have been noted in a number of studies (Marshall *et al.,* 1995; Marshall Jr *et al.,* 1997; Marshall *et al.,* 1998).

There are no published AFM studies which have analysed nanomechanical properties of dentine after treatment with citric acid alone. Previous work focused on the use of microindenters to analyse micromechanical properties such as microhardness after irrigation with 10% citric acid and showed significant reductions in comparison with untreated control specimens (Cruz-Filho *et al.*, 2011).

6.3.5 Conclusions

Within the limitations of this study, the null hypothesis was refuted, since citric acid treatment made significant changes to the surface stiffness of human root dentine specimens. A two minute exposure did not result in further significant change compared to a 1 minute treatment.

6.4 **Effect of EDTA on human root dentine surface**

6.4.1 Aim

To analyse the effect of EDTA on the stiffness of human root canal dentine walls after exposure for 1 and 2 minutes, using AFM. This study tested the null hypothesis that 17% EDTA would have no effect on the stiffness human root canal dentine walls.

6.4.2 Materials and methods

6.4.2.1 Tooth collection and storage

Five freshly extracted human premolar teeth were used for this study. See section 3.2.2 for tooth collection and storage.

6.4.2.2 Specimen preparation

See section 3.2.2 (in the first chapter of the results) and section 6.3.2 (in this chapter) for specimen preparation

6.4.2.3 Storage of the samples and AFM preparation

See section 6.3.2, section 3.4.2 and section 3.5.2 (about the storage of the samples and its preparation).

6.4.2.4 Treatment

See section 6.3.2 for treatment of the samples. In this experiment, 17% (wt/v) EDTA (SIGMA-ALDRICH, UK), pH 7.3 was applied to dentine specimens.

6.4.2.5 Analysis of the of the samples by the AFM

See section 3.5.2 for details of analysis of samples by AFM. In the current experiment, 9 areas of each specimen were analysed, in common with the description in section 6.3.2 for citric acid treatment (Figure 6.12). Results were analysed using Kruskal-Wallis and Mann-Whitney tests ($p<0.05$).

6.4.3 Results

Figure 6.20, Figure 6.22 and Figure 6.24 show AFM images of dentine surfaces before and after treatment with 17% EDTA for 1 minute and 2 minutes respectively. The non-treated dentine surface (Figure 1.20) shows open dentinal tubules. After 1 minute EDTA treatment, the diameter of the dentinal tubules increased in comparison with pre-treatment specimens (Figure 6.22). After 2 minutes treatment, it was noted that the diameter of dentinal tubules increased further in comparison with the 1 minute specimens (Figure 6.24). Figure 6.21, Figure 6.23 and Figure 6.25 show the force distance curves before and after treatment with 17% EDTA respectively. All show similar patterns of vertical deflection (extended) per distance and vertical deflection (retract) per distance.

The median human root dentine stiffness (upper and lower quartiles) of all groups can be seen in Table 6.4. Graphical depiction in Figure 6.26, shows a trend towards reduction in dentine stiffness following treatment with 17% EDTA for 1 minute. There appeared to be a plateauing after 1 minute of treatment.

Statistical analysis revealed that 1 minute exposure to 17% EDTA resulted in a significant reduction in dentine stiffness compared to untreated control ($p<0.05$). Dentine stiffness after 2 minute treatment was not significantly different form that after 1 minute treatment ($p>0.05$), but was significantly different from the un-treated control ($p<0.05$).

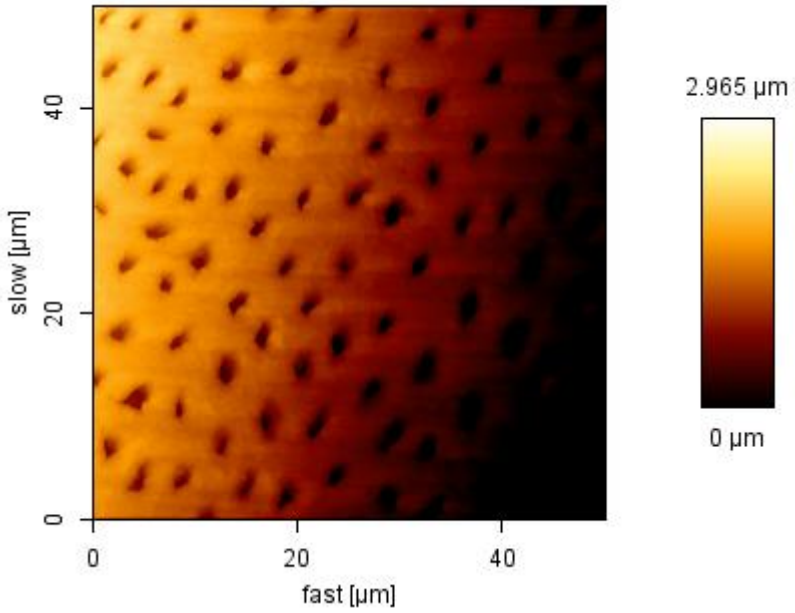

Figure 6.20. AFM image to show the structure of root canal dentine walls in the cervical third of non-treated human teeth (Group 1)

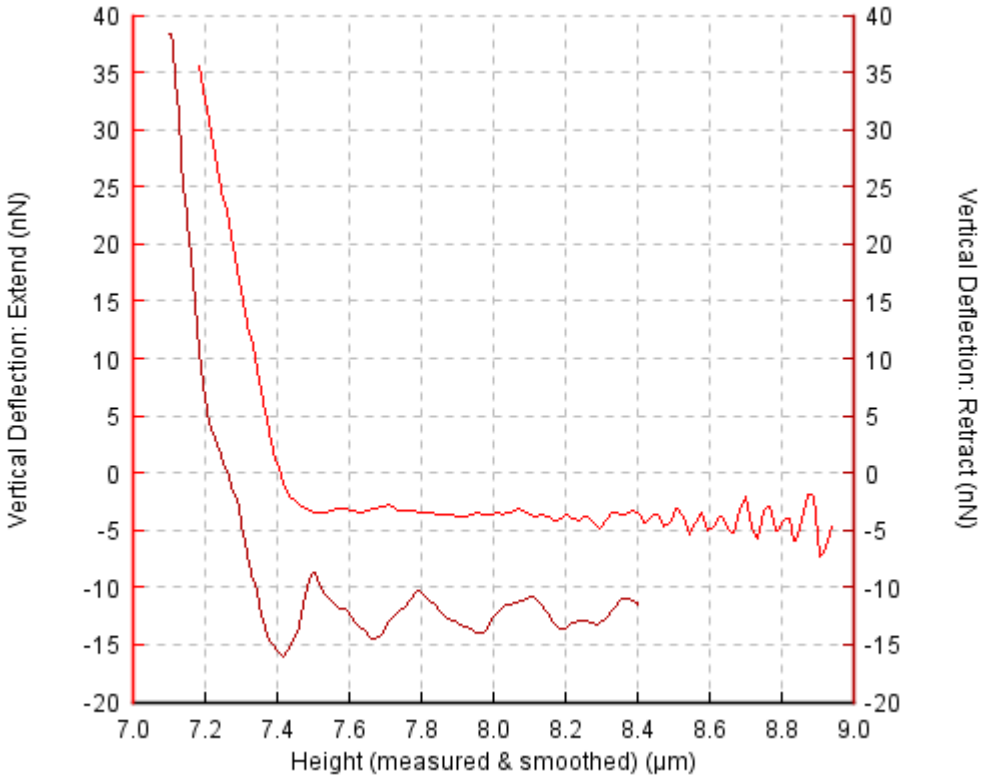

Figure 6.21.Typical force curve of root canal dentine walls in the cervical third of non-treated human teeth (Group 1).

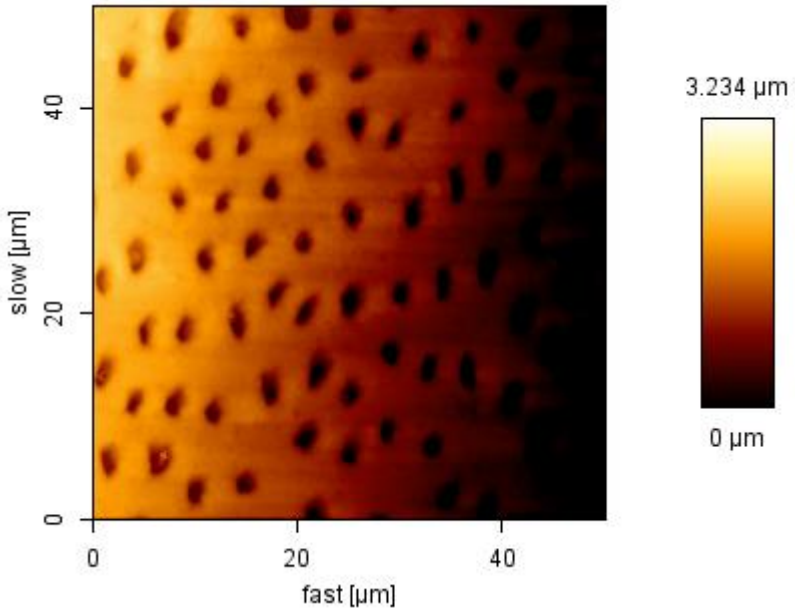

Figure 6.22. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 17% EDTA (Group 2).

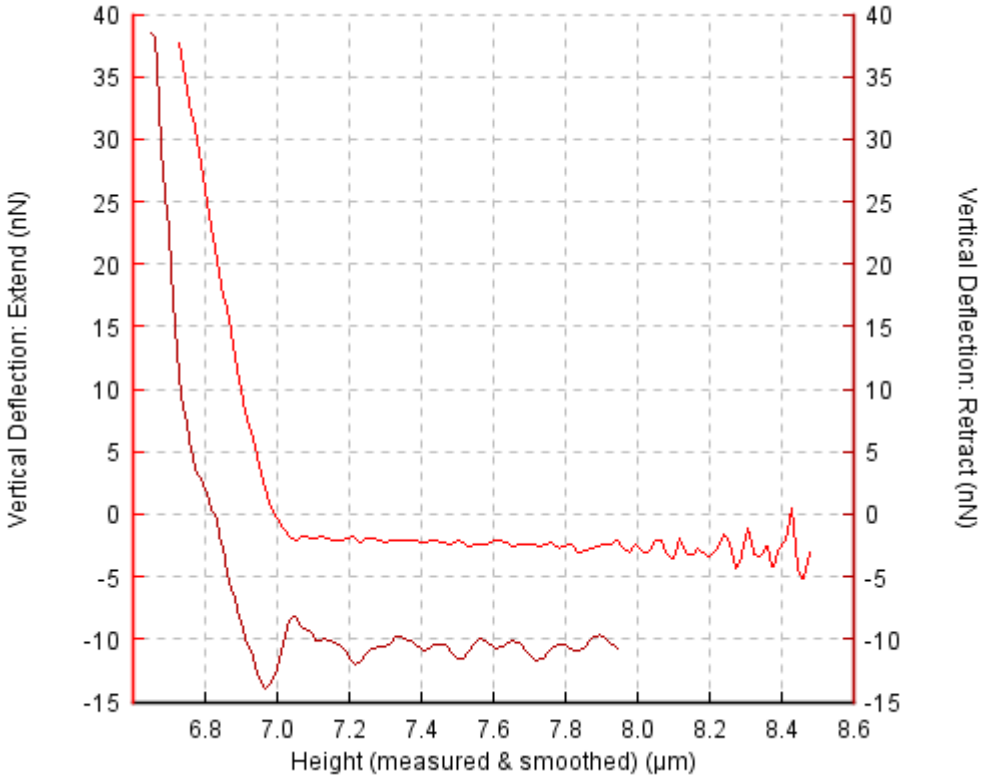

Figure 6.23.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 1 minute treatment with 17% EDTA (Group 2).

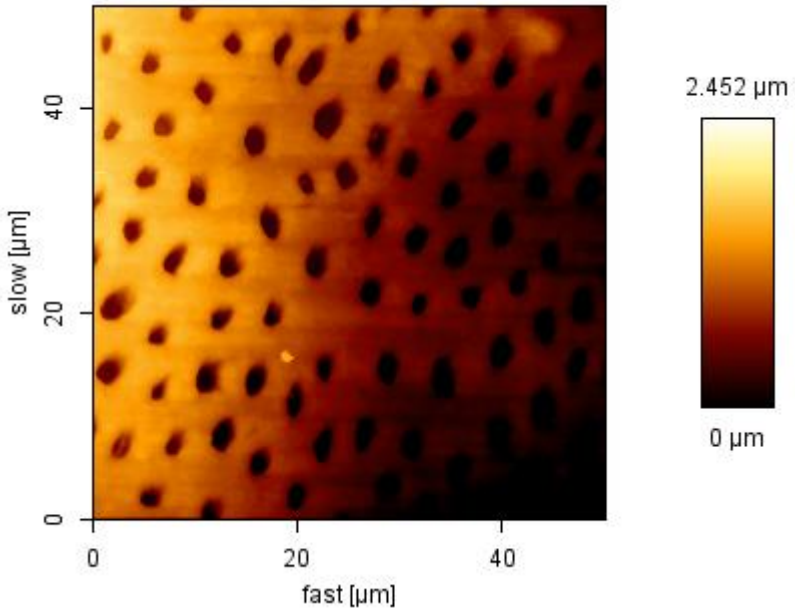

Figure 6.24. AFM image to show the structure of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 17% EDTA (Group 3).

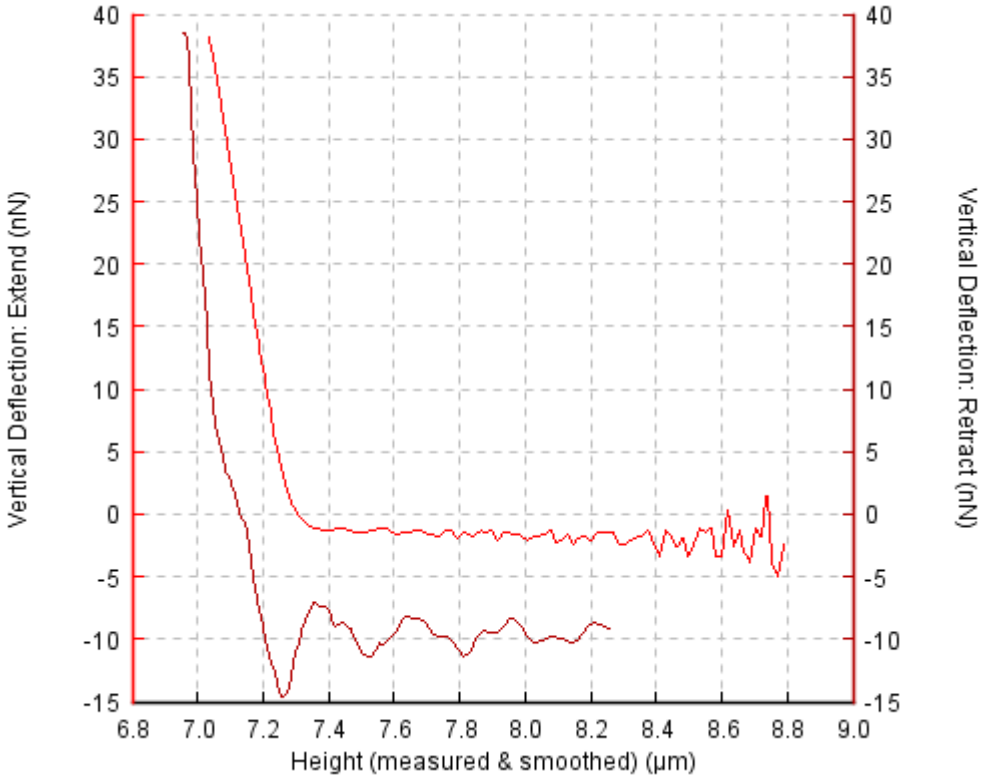

Figure 6.25.Typical force curve of root canal dentine walls in the cervical third of human teeth, after 2 minutes treatment with 17% EDTA (Group 3).

Table 6.4. Median (upper and lower quartiles) dentine stiffness cervical human root canal wall, after no treatment, 1 and 2 minute treatments with 17% EDTA.

Group	Treatment	Median dentine stiffness (upper and lower quartiles)
1	**Control**	**180 (199 & 173)**
2	**17% EDTA, 1 minute**	**159 (188 & 155)**
3	**17% EDTA, 2 minutes**	**159 (202 & 131)**

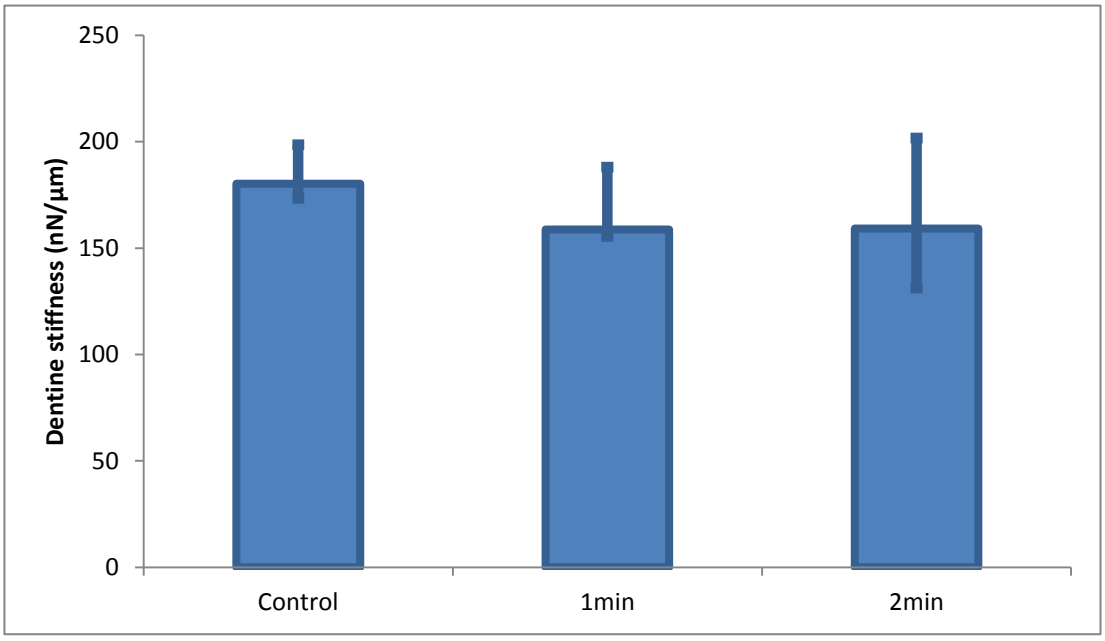

Figure 6.26. Median dentine stiffness (nN/µm) of human cervical root canal wall, after no treatment, 1 and 2 minute treatments with 17% EDTA. The difference was significant between the control group and the 1 minute treatment, and between the control group and the 2 minute treatment ($p<0.05$), but between the 1 minute and 2 minute treatments, the difference was not significant ($p>0.05$, Kruskal-Wallis and Mann-Whitney U tests). The error bars are the upper and lower quartiles.

6.4.4 Discussion

The results of this study suggested that a one minute treatment with 17% EDTA reduced the stiffness of dentine significantly ($p<0.05$) compared to untreated controls. Two minute treatment did not result in significant further reduction ($p>0.05$).

There has been only one published study in which AFM was used to analyse the effects of EDTA on dentine surfaces, only imaging without nanomechanical properties. The study reported that peritubular dentine and intertubular dentine were changed by treatment with 0.05 M and 0.5 M EDTA, dilute solutions of phosphoric (3 mM, 6 mM) and citric (5 mM) acids. In the case of phosphoric and citric acid, the intertubular dentine was smooth, whilst for EDTA it was rough (Marshall *et al.*, 1995). In the current experiment, this difference between 17% EDTA and 6% citric acid was not observed.

The current study revealed plateauing effect, with no further change in dentine stiffness following 2 minute exposure to neutral 17% EDTA compared to 1 minute exposure. This agreed with another study in which neutral 17% EDTA exerted a maximal demineralising effect after 1 minute exposure, with no increase if it was left for up to 25 minutes (Parmar and Chhatariya, 2004). A possible reason for this plateauing was the demineralisation of the dentine surface with the exposure of collagen fibrils which would limit further effects of the EDTA. Other possible explanations may include the demineralisation of extrafibrillar minerals (minerals attached to the type-I collagen fibrils), while the intrafibrillar minerals (minerals in the gaps between collagen molecules) were slowly demineralised because they were protected by collage fibrils. One study involving 5 minute exposure to 17% EDTA supported this view (Spanó *et al.*, 2009).

Deterioration in the mechanical properties of dentine which was observed in section 6.4 of this thesis was supported by another study which investigated similar EDTA treatment using microhardness measurements of horizontal slices of root dentine from the cervical, middle and apical root thirds, and at 500µm and 1000µm from the root canal lumen. It found that there was a significant reduction in the microhardness of dentine after 1 minute, in all of the thirds of the roots and at 500µm and 1000µm from the canal lumen (Saleh and Ettman, 1999). Other studies have also corroborated with

the findings of the current work, based on microhardness testing (Ari *et al.*, 2004) (Sayin *et al.*, 2007; Cruz-Filho *et al.*, 2011). Another study, found that there was an inverse relationship between the microhardness of dentine and the density of dentinal tubules (Pashley *et al.*, 1985), while another study showed that the degree of mineralisation and hydroxyapatite of intertubular substances was a major intrinsic factor influencing the hardness of dentine (Panighi and G'Sell, 1992). The precise origin of dentine employed in studies of this sort (animal or human; site on the tooth) may have a significant bearing on the outcome.

Other studies have again used combinations of EDTA and NaOCl, making direct comparison with the current work difficult (Eldeniz *et al.*, 2005).

It has been proposed that when comparable concentrations of EDTA and citric acid are employed, citric acid may react with more calcium ions and have a greater demineralising and softening effect (Papagianni, 2007). Under normal conditions, 1 mol of citric acid chelates 1.5 mol of calcium ions, while 1 mol of EDTA chelates 1 mol of metallic ions (Voguel, 2004). But it should be kept in mind that the calcium ions do not react completely with citric acid, because in dentine structure actually they are not available as free ions, since they are available as complexes with hydroxyapatite crystals (Cruz-Filho *et al.*, 2011).

Although erosion of dentine was observed from treatment of dentine with 17% EDTA, this did not mean that it could effect on the microhardness or mechanical properties of whole dentine structure, since a previous study concluded that the erosion of dentine was not the determining factor for reduction of dentine microhardness and the main factor may have been the amount of irrigant that penetrated the tissue (Saghiri *et al.*, 2009). A similar process could be expected for stiffness, because if the penetration of irrigant was limited, no effect on the mechanical properties of dentine could be expected. Therefore, future research is recommended to analyse effect of EDTA on dentine at different depths from the root canal lumen.

In a study, it was shown that 15% EDTA and 10% citric acid were the most efficient solutions for the removal of the smear layer when used as a root canal irrigant, when compared with 10% sodium citrate, 5% acetic acid, apple vinegar and 5% maleic acid. In addition, it was shown that 15% EDTA resulted in the greatest concentration of Ca

followed by 10% citric acid (Spanó *et al.*, 2009). In the experiment undertaken in this project, the 6% citric acid could remove the smear plugs or the polishing compound left inside the dentinal tubules, but they had no significant effect on dentine stiffness. Unfortunately, no smear plugs were present in the specimens of the EDTA experiment to observe this phenomenon to compare it with the EDTA.

When monitoring the effect of the 6% citric acid on a dentine surface for 1 minute in this experiment, an abrupt variation in dentine stiffness in point 6 was noted in comparison with the adjacent points. This was attributed to hypermineralized dentine in this area and it was speculated that this finding could make this area susceptible to rapid demineralisation in comparison with the surrounding areas.

From the collection of experiments in this chapter, it appeared that NaOCl had no significant effect on the stiffness of dentine, while citric acid and EDTA had significant effects. The NaOCl, experiment showed this non-significant effect with a range of concentrations (2.5%, 5% and 10%), while the acid experiment showed the significant effect with single concentrations of 6% for citric acid and 17% concentration for EDTA. An analysis of the effects of combined or alternating NaOCl/acid treatments was not included in this series of investigations, but further work should be conducted to explore the effects of such treatments.

6.4.5 Conclusions

Within the constraints of this study, the null hypothesis was refuted, since EDTA treatment had a significant effect on the stiffness of human cervical root canal dentine when compared with untreated control. Exposure of dentine for 2 minutes resulted in no greater reduction in stiffness than 1 minute treatment ($p<0.05$).

Chapter 7: General discussion

This section includes a general discussion of the investigations included in this thesis, and recommendations for future research.

7.1 The suitability of ovine and bovine teeth as a model for human teeth

One of the primary aims of this work was to establish whether root dentine originating from animal sources would be a suitable alternative to human root dentine. This is a consequence of the increasing difficulty in obtaining human dentine for laboratory investigations (Schilke *et al.*, 2000).

The positive advantages of animal teeth may include their relative uniformity if large batches of teeth can be obtained from animals derived from the same flock and therefore, of a similar age, sex, and with similar nutritional and environmental exposure (Rahman *et al.*, 2005). Other positive advantages include the ability to harvest large numbers of similar teeth in a single session and to store and process them under uniform conditions. Another significant consideration relates to the use of animal teeth in the research laboratory, where the need to control risks such as blood-borne viruses may be less stringent than with human tissues (Al-Kilani *et al.*, 2003).

When reviewing the literature, it appears that ovine teeth have been used infrequently, whereas bovine teeth have been used extensively as a model for human teeth. Even though they have not previously been subject to systematic validation as a model for human teeth, the relative availability of ovine teeth has given support for their use in current investigations, even if this was to develop other methods before translating to smaller samples of human teeth (Al-Kilani *et al.*, 2003).

Thus, the early experiments in this work involved characterising ovine and bovine roots and root dentine as models for human tissues. In this project the density and diameter of the dentinal tubules was investigated first, in order to make a comparison between human and animal root dentine. The studies observed in the literature have often focussed strongly on these two parameters, particularly when researchers have been

interested in the interactions between adhesive dental materials and dental hard tissues (Schilke *et al.*, 2000).

For the purpose of the current work, tubular diameter and density was considered significant, as this may influence the penetration depth of irrigant solutions into a body of dentine after application to the root canal lumen. This may in turn have allowed irrigant solutions with deproteinating or chelating effects to alter the mechanical properties of dentine not just at the root canal lumen, but at varying depths within the wall of the root. The change in the diameter and density of dental tubules or the amount of intertubular dentine in different regions of the dentine can have an outcome on the values of the mechanical properties in certain areas (Pashley *et al.*, 1985; Kinney *et al.*, 1996a; Kinney *et al.*, 1996b) and inter-species variation is recognised.

Other parameters which were considered in the comparison between human and animal teeth included the volume of the root canals. This is because different root canal volumes could accommodate different volumes of irrigant, which could in turn influence the effectiveness of the irrigant on root canal cleanliness and/or damage. The latter was investigated by assessing Ca/P ratios and the stiffness of dentine.

The results of the current studies showed that there was no significant difference in the dentinal tubular density and diameter of the ovine, bovine or human teeth. This corroborates the findings of a previous study, which found human and bovine teeth to have similar tubular density and structure (Schilke *et al.*, 2000). This suggests that ovine and bovine teeth may provide a suitable alternative to human for some types of experimental work. Although the current results informed that there was no significant difference between human and animal teeth in terms of the density and diameter of dentinal tubules, investigation of other parameters would need to be considered, in order to assess whether animal teeth were a suitable alternative for human teeth in all types of experimental investigation.

It is acknowledged that tubular diameter and density can be variable within individual teeth and that a precise like-for-like comparison between samples from the same or different species may be challenging (Garberoglio and Brännström, 1976; Carrigan *et al.*, 1984; Pashley, 1989; Caiado *et al.*, 2010). For example, Caiado *et al* (2010) found the human tubular density of cervical third root dentine to be 43948 tubules mm^{-2},

whilst it was 35084 tubules mm^{-2} for cervical third coronal dentine. This difference was not statistically significant (Caiado *et al.*, 2010).

Historical reports have calculated the tubular density and diameter of dentine by traditional, manual methods from projected SEM images and scales (Forssell-Ahlberg *et al.*, 1975). More recent studies, including Caiado *et al* (2010) employed image analysis software. Such methods offer simplicity and accuracy compared to the traditional methods, and they control many of the risks of human bias (Caiado *et al.*, 2010).

In the current work, the dentinal walls of the root canal were not treated with any chemical agent following the removal of the pulp tissue. This allowed analysis of the dentine surface in its native state. However, the root canal walls were often covered with cellular debris, necessitating the search for windows of clean dentine for imaging and analysis. Some samples could not be used due to the absence of suitable windows.

In the literature, it is common for root canal walls to be treated with irrigants such as NaOCl and/or demineralising agents in order to expose the dentinal tubules for analysis. For example, Caiado *et al.* (2010) used a combination of 5.25% NaOCl and 32% phosphoric acid to prepare their specimens. Exposure to phosphoric acid changed the size of dentinal tubules through demineralisation of the peritubular dentine, and this, combined with the de-proteinating effects of NaOCl resulted in surfaces that were greatly altered, when compared to the native state. The results of the current work were different from those of Caiado *et al.* (2010), and this may partly reflect differences in sample preparation.

7.1.1 Estimation of the root canal volume

While many clinically-orientated studies have reported on the shape and configuration of the root canal system, few have reported directly on pulp volume (Fanibunda, 1986).

Traditional 2-dimensional radiographic images are limited by the need to estimate dimensions that cannot be directly measured, and by assumptions, for example, about the cross-sectional shape of a pulp space (Dowker *et al.*, 1997). Destructive methods are often appropriate for laboratory studies, and methods including resin injection and dissolution of tooth tissues, and instillation of elastomeric impression materials have been described (Fanibunda, 1986; Melton *et al.*, 1991).

Perhaps the most suitable way to assess pulp volume is by high-resolution 3-dimensional imaging, which provides a non-destructive means of making such measurements, albeit at a considerable cost (Dowker *et al.*, 1997; Bergmans *et al.*, 2001). Further considerations such as radiation exposure are not relevant for *in vitro* investigation, but image resolution and the time required for image reconstruction may present a number of challenges (Dowker *et al.*, 1997; Bergmans *et al.*, 2001; Versiani *et al.*, 2013).

Advanced imaging techniques of this sort were not available for the current investigations. Two alternative methods were therefore employed. The first method involved the application of distilled water in order to estimate the canal volume. Potential errors included air entrapment within the canal, which could have reduced the fluid volume and thus, the estimation of the canal volume. To some extent, water could also have been absorbed within the porous tubular structure of the dentine, with the potential for over estimation of the pulp volume.

In the second method, 2 dimensional images were analysed with ImageJ software, based on the assumption that the canals were conical in shape. This analysis was likely to have been greatly flawed by the irregular conical-like configuration of the pulp spaces, and by the considerably different cross-sectional profiles of ovine, bovine and human pulp spaces. In both of these methods, it was established that the root canal volume of human teeth was significantly smaller than bovine teeth, but not significantly different to ovine teeth.

7.1.2 Analyses of the Ca/P ratios of root dentine

There were significant differences in the Ca/P ratios of inner root dentine (close to the root canal) in ovine, bovine and human teeth. In deeper areas of dentine, Ca/P ratios of bovine and human teeth were not significantly different, but in ovine teeth Ca/P ratios became similar to human dentine only at 1000µm from the root canal lumen. Differences in the ratio of Ca and P may reflect the difference in mineralisation between predentine and dentine (Arnold and Gaengler, 2007), as it does between dentine and enamel (Dauphin and Williams, 2007).

- The ovine teeth used in the current project were from sheep 2-3 years old,

- The bovine teeth were from cattle 18-20 months old,
- The human teeth were from people 12-14 years old.

These differences in age may be partly responsible for the observed differences in Ca/P ratios. In addition, the ovine and bovine teeth employed were incisors, whilst the human teeth were premolars; a further possible factor in Ca/P ratio variation. Furthermore, the degree of maturity of the teeth and regions sampled may be significant. This was supported by a study completed by Ryou *et al.* (2011) who used Fourier transformation infrared microscopy to analyse the mineral/collagen ratio of human dentine. They showed that this ratio was lower for dentine near the enamel than dentine near the pulp (Ryou *et al.*, 2011).

In the current work, immature ovine and bovine tooth areas near the root canal lumen could represent predentine. In this experiment the Ca/P ratios close to the lumen were lower than in deeper areas; a finding observed by others, who found that the Ca/P ratio of areas near the root canal lumen (in predentine) were higher than in deeper areas from the root canal lumen (2.5 vs 2.1 respectively) (Arnold and Gaengler, 2007). Arnold and Gaengler (2007) have shown by EDAX that the distribution of Ca and P in predentine was homogenous and that the concentration of Ca and P becomes higher towards the mineralising dentine, whilst the carbon decreases (Arnold and Gaengler, 2007).

7.1.3 Analysis of dentine stiffness

There were significant differences in the stiffness of the inner and middle root dentine in ovine, bovine and human teeth. Human root dentine was significantly stiffer than that of ovine teeth at all depths from the root canal lumen, whilst it was significantly stiffer than bovine teeth up to 300µm from the root canal lumen As a result, this may have implications for the transferability of research concerning the physical properties of dentine, which has been conducted on non-human teeth.

Previous studies have reported using solutions of 2% glutaraldehyde to preserve tissues during storage (Torabinejad *et al.*, 2003a). However, in pilot studies for the current work, when the effect of exposure to 2% glutaraldehyde was analysed, it was noted that the stiffness of dentine reduced. It was discovered that 1% chloramine-T was a better

solution for storing teeth, because it had a neutral pH and little capacity to demineralise teeth. Chloramine-T did not reduce the stiffness of dentine.

Consequently, it was recommended that further studies are carried out to analyse other mechanical properties to estimate the effect of this solution on dental tissues and to find whether they can be changed.

7.1.4 Summary

To sum up, no significant differences were found between ovine, bovine and human teeth in terms of the structure of dentine. There were, however, significant differences in Ca/P ratios and stiffness. In addition, there was a significant difference between the volumes of human and bovine teeth, although there was no significant difference between human and ovine teeth. Therefore, it could be concluded that ovine and bovine teeth are not identical to human teeth, meaning they are imperfect models for use in endodontic research. They may be best suited to preliminary studies before conducting further work on human specimens.

7.2 **The preparation of root dentine specimens to analyse the effects of irrigants**

One of the primary aims of this work was to establish whether there is a suitable method of producing a horizontal slice from root dentine to analyse the effect of the irrigant on dentine at different depths from the root canal lumen. As a result, specimens were prepared in subsequent investigations by splitting; however, this method did not create the smooth surfaces necessary for analysis. A polishing protocol was therefore necessary to create a horizontal flat surface of dentine, free of a smear layer, in order to analyse the underlying surface of dentine. Consequently, horizontal sections were cut from root dentine and subsequently polished to remove the smear layer that had been generated during sectioning.

Polishing was found to be an effective way of removing the smear layer from laboratory sections of dentine. However, it was not without some disadvantages, including the potential to heat dentine, with the collapse and denaturation of fibrillar and nano-fibrillar proteins, change the orientation of these proteins, and bring about changes to the structure and biomechanical responses of the tissue during analysis (Ho *et al.*,

2004). Macro polishing can result in smear plugs, which can enter the dentinal tubules and close them, potentially influencing the results.

The results of this investigation suggest that smear layers generated during the sectioning of dental root specimens can be removed by macro and micro polishing of specimens. It was also found that that smear layers generated during the sectioning of dental root specimens can also be removed by micro polishing alone. Both of the approaches successfully removed the smear layer and created a flat surface suitable for AFM analysis. The difference between the macro and micro polishing and the micro polishing alone was that the number of steps for polishing the sample was less in the second method. Therefore, the possibility of heating the dentine could be less, in comparison with the first method, although this was not formally investigated.

Ho *et al* (2004) suggested that the creation of a smear layer can be avoided by cryo-fracturing specimens. However, this resulted in the production of a rough surface which was difficult to analyse by AFM (Ho *et al.*, 2004). Thus, polishing could be the best solution to produce flat and smooth surfaces free of smear layers.

7.3 **The effect of different irrigants on the root dentine**

Another of the primary aims of this work was to establish the most suitable irrigation regime to remove cellular debris and predentine from root canal walls, whilst doing the least damage to root dentine.

This series of investigations studied the potential to remove cellular debris and predentine from the walls of freshly extirpated root canals. A number of variables are considered in the following section.

7.3.1 Selection of the correct type of needle to deliver the irrigant

Irrigation needles with a 27 gauge were discovered by Hsieh *et al* (2007) to exchange the irrigant throughout the canal following insertion at about 3mm from the apex of the root in human teeth. It was noted that 25 and 23 gauge needles were not able to penetrate to within 3mm of the root end and were not able to exchange the irrigant apically, 6 or 9mm from the apex (Hsieh *et al.*, 2007).

It was found that using a side-vented needle to irrigate the root canal with conventional syringe irrigation did not have the ability to clean root canals with closed apices effectively (Torabinejad *et al.*, 2003a). Therefore, the notched-end needle was selected in this experiment. However, no influence was observed on the amount of debris removal from the root canals.

7.3.2 Selection of irrigant

Sodium hypochlorite has been used commonly in endodontics as a root canal irrigant, since it has the ability to dissolve organic tissue, to destruct microorganisms and as it works as a lubricant during root canal instrumentation (Haapasalo *et al.*, 2005; Clegg *et al.*, 2006; Dunavant *et al.*, 2006).The chlorine (Cl_2) is responsible for the antibacterial activity and tissue dissolving capacity, and this capacity is consumed during tissue dissolution (Moorer and Wesselink, 1982). Continuous refreshment is therefore recommended (van der Sluis *et al.*, 2007).

In the literature, concentrations of NaOCl between 0.5% and 5.25% are the most commonly recommended concentrations for root canal irrigation (Spangberg *et al.*, 1973; Harrison, 1984; Baumgartner and Cuenin, 1992), although an ideal root canal irrigant which covers all of the requirements does not exist (Spangberg *et al.*, 1973; Harrison, 1984; Baumgartner and Cuenin, 1992; Al-Kilani *et al.*, 2003).

Therefore, in the experiments in this project, the NaOCl was selected and the relationship between the concentration, temperature and time of application was investigated. Two concentrations of NaOCl (0.54% and 2.86%) were selected and applied either at 25˚C or 60˚C at exposure times of 10, 20 or 30 minutes, in order to find the most suitable regime for tissue removal whilst minimising dentine damage. In addition, the irrigant was agitated with ultrasonication for 30 second periods. However, the results of this study showed that these concentrations were ineffective at cleaning the root canals at both low and high temperatures, even when the time was increased to 30 minutes. This was in agreement with a study which showed that the dilution of 5.25% NaOCl to various lower concentrations resulted in a reduction in its ability to dissolve pulp tissue (Hand *et al.*, 1978).

A similar finding was observed when a pilot study was completed in this project to assess the ability of different concentrations of NaOCl (0.5%, 2.5%, 5% and 10%) to

dissolve pieces of freshly extirpated dental pulp tissue. The pulp tissue was removed and immersed in irrigant directly to standardize the volume of the irrigant. It was found that with a higher concentration of NaOCl, speed of tissue dissolution was increased. A suitable effective time for each concentration to dissolve the pulp was considered in the next experiment.

This showed that the application of a higher concentration of NaOCl with continuous irrigation of the root canals resulted in clean canals, even when used for a short period-of-time. This was in comparison with the pilot study where a low concentration of NaOCl (0.54% and 2.86%) was used. It was established that flushing the root canal with an irrigant is more effective in cleaning the root canal than by applying the irrigant statically in a brim-full root canal (Baker *et al.*, 1975).

7.3.3 Method of application of the irrigant

In this experiment involving continuous irrigation of the root canal, ultrasonic activation was used to agitate the irrigant for 30 second periods. Various other methods can also be employed to exchange and agitate irrigants, including sonic (de Gregorio *et al.*, 2009; Gu *et al.*, 2009), an ultrasonic (Gutarts *et al.*, 2005; van der Sluis *et al.*, 2007) or negative pressure irrigation (Fukumoto *et al.*, 2006; Nielsen and Baumgartner, 2007; Townsend and Maki, 2009).

However, in the current work, damage to the root canal walls was frequently noted, probably resulting from wall contact with the ultrasonically activated file. Therefore, there was a need for the substitution of ultrasonic activation with other methods. Therefore, in the next experiment different methods of application of the irrigant were compared to discover suitable methods which were less damaging to the root canal walls.

The lowest concentration of NaOCl (5%) was selected for this experiment to find an alternative agitation method that had less obvious effects on the dentine walls. It was established that the manual agitation of the irrigant with a gutta-percha point was the most suitable method, with no evidence of damage to canal walls. Manual agitation with a gutta-percha cone seems to have the ability to displace the apical gas entrapment from the closed root apex (Huang *et al.*, 2008; McGill, 2008).

Finally, NaOCl was used to remove the debris and predentine from human root canals. The result of this study showed that the 5% and 10% NaOCl solutions were more effective than a lower concentration (2.5%) in cleaning the root canal walls, even in the apical third. In addition, manual agitation of the NaOCl with a gutta-percha cone was effective in cleaning the root canals in the apical third. Furthermore, there was no obvious damage to the root canals with both the 5 and 10% concentrations of NaOCl.

7.3.4 Efficiency of the irrigant and its effect on root canal walls

It is necessary to balance the efficiency of irrigants in cleaning root canals walls with aspects of patient safety (Al-Kilani *et al.*, 2003). In the case of the current work, the risks concerned chemical damage to the root canal walls. When Ca/P ratios were analysed, it was established that 5 and 10% NaOCl solutions at low and high temperatures had no effect on the dentine minerals after application for 0, 5 and 10 minutes. In addition, when the root canal was irrigated with NaOCl, none of the treatment groups were associated with significant changes in dentine stiffness at any depth from the root canal lumen. The change in dentine stiffness was more apparent in the group treated with 10% NaOCl than in other groups; nevertheless it was still not significant. This discovery agreed with the findings of a study which showed that 3%, 5.1% and 7.3% NaOCl had no significant effect on tooth surface strain (Goldsmith *et al.*, 2002). Therefore, these concentrations of NaOCl did not appear to affect the mechanical properties of the dentine.

These results agreed with the finding of other studies which showed that higher concentration NaOCl solutions could be more effective, although these studies also indicated that the higher concentration could affect the properties of dentine surfaces (Moorer and Wesselink, 1982; Baumgartner and Cuenin, 1992; Sim *et al.*, 2001).

7.3.5 The effect of other irrigants on dentine

The effects of 2 commonly used acids EDTA and citric acid on dentine surfaces were analysed by AFM. Citric acid had no effect on the AFM tip, and specimens could be directly analysed after treatment. The results of this study identified that there were significant differences in the stiffness of root canal dentine treated with 6% citric acid for 1 minute, whilst after a 2 minute treatment, there was no further significant change.

In the experiment with 17% EDTA, a 1 minute exposure of root canal wall dentine had a significant effect on dentine stiffness, whilst a 2 minute exposure produced no further significant decrease.

There is some controversy about the effect of the pH of EDTA on the demineralisation of dentine. Consequently, some authors have reported that it is not pH dependent (Seidberg and Schilder, 1974), whilst others have observed that the maximum demineralising effect of EDTA on dentine was produced at a neutral or alkaline pH (Sreebny and Nikiforuk, 1951; Nikiforuk and Sreebny, 1953; Rubin *et al.*, 1979; Serper and Çalt, 2002; Parmar and Chhatariya, 2004). Nevertheless, others have shown that EDTA with a neutral pH reduced mineral and non-collagenous proteins of dentine and resulted in dentine softening without erosion of the dentine surface (Kawasaki *et al.*, 1999; Verdelis *et al.*, 1999). However, the ETDA used in this study resulted in erosion of dentine, in spite of its neutral pH.

Both of these experiments showed that the demineralisation rate of dentine could be high for the first minute, although after this period the rate of demineralisation reduced rapidly and flattened out. This could be due to the presence of collagen material, which could work as a limiting factor for the efficiency of the demineralising agent.

In addition, the crystals in dentine are found in two areas; between collagen fibrils (extra-fibrillar mineral) and within the fibrils (intra-fibrillar mineral) (Katz and Li, 1973; Katz *et al.*, 1989; Landis, 1996). Therefore, the demineralising agent could harm the extra-fibrillar minerals, but may not have had an effect on the intra-fibrillar minerals, because they are protected by collagen fibrils.

A previous study proposed that the intra-fibrillar mineral can be an important factor, responsible for the normal mechanical properties of dentine. Moreover, it can provide a firm association for the newly formed minerals with the demineralised matrix that could enable the recovery of the mechanical properties of the dentine (Bertassoni *et al.*, 2009).

It could be concluded from the results of previous experimental work that the duration, concentration and volume of the irrigant had a critical effect on the physical properties of the root dentine surface (Marending *et al.*, 2007b), as it showed that dentine in the apical third is more highly mineralised than the middle and cervical thirds and more sclerosed (Vasiliadis *et al.*, 1983). As a result, future research is recommended to compare the effect of the demineralising agent between the 3 thirds of the root dentine.

In this project, it was found that the Hülsmann *et al.* (1997) scoring system (Hülsmann *et al.*, 1997) was unsuitable for scoring the cleanliness and predentine removal from root canal walls, because there was no mechanical instrumentation of the root canals. Thus, cellular debris and predentine were abundant and the smear layer was absent. In addition, in this work, it was important to identify signs of dentine demineralisation caused by the irrigant regimes. Therefore, a new scoring system was developed to score pulp debris, predentine and dentine damage resulting from treatment by the irrigant.

It is recommended that standard parameters are developed in future work to assess whether animal teeth are a suitable model for human teeth. It is necessary to develop a standard method that can be analysed by different machines such as the AFM, SEM and EDAX to polish the dentine surface and to produce a flat surface free of a smear layer. Developing a set of tables is necessary that includes ideal concentrations of NaOCl to remove debris and predentine from the root canals at a certain time and volume.

Future work is also recommended to analyse the effect of NaOCl on the collagen fibres. It is also suggested that other mechanical properties of dentine such as nanohardness and elastic modulus are analysed by the AFM after treatment with the NaOCl. The new scoring system for root canals is recommended in future work and it is proposed that it should be evaluated for its utility in different settings and is subjected to systematic examination of inter and intra-examiner reliability.

Chapter 8: Conclusions and recommendations

8.1 Conclusions

Within the limitations of this work, it can be concluded that:

1. Ovine and bovine teeth are not identical to human teeth, meaning they are imperfect models and may be best suited to preliminary studies in endodontic research.
2. Polishing was found to be the best method to produce flat and smooth dentine surfaces free of a smear layer, which can be analysed by AFM, SEM and EDAX.
3. A 5% NaOCl solution was the minimum concentration with the ability to remove pulp debris and predentine from all thirds of uninstrumented human root canals.
4. Different concentrations of NaOCl (2.5, 5 and 10%) did not result in a significant change in root dentine stiffness at different depths from the root canal lumen. In addition, dentine treatment with 6% citric acid or 17% EDTA resulted in a significant change in surface stiffness after 1 minute of exposure, whilst there were no further significant changes observed after 2 minutes of exposure.

8.2 Recommendations for future work

It is recommended that a reference for important parameter is developed to be used for analysing the suitability of animal teeth as a substitute for human teeth in dental research.

It is proposed that a standard method is developed and universally adopted to produce a dentine surfaces that can be analysed by any technique such as AFM, SEM and EDAX.

Further work on root canal debridement and damage should be undertaken to develop a clinically-relevant guideline on treatment protocols. Investigations of potential damage should incorporate additional parameters, including detailed analysis of the effects of

NaOCl on collagen and non-collagenous proteins, and physical properties of dentine nanohardness and elastic modulus.

Assessments in which deproteinating and demineralising agents are used together or successionally should also be undertaken to supplement the current work on individual agents.

Furthermore, it is recommended that the new scoring system for root canals is used in future studies and is refined as necessary for utility and reliability.

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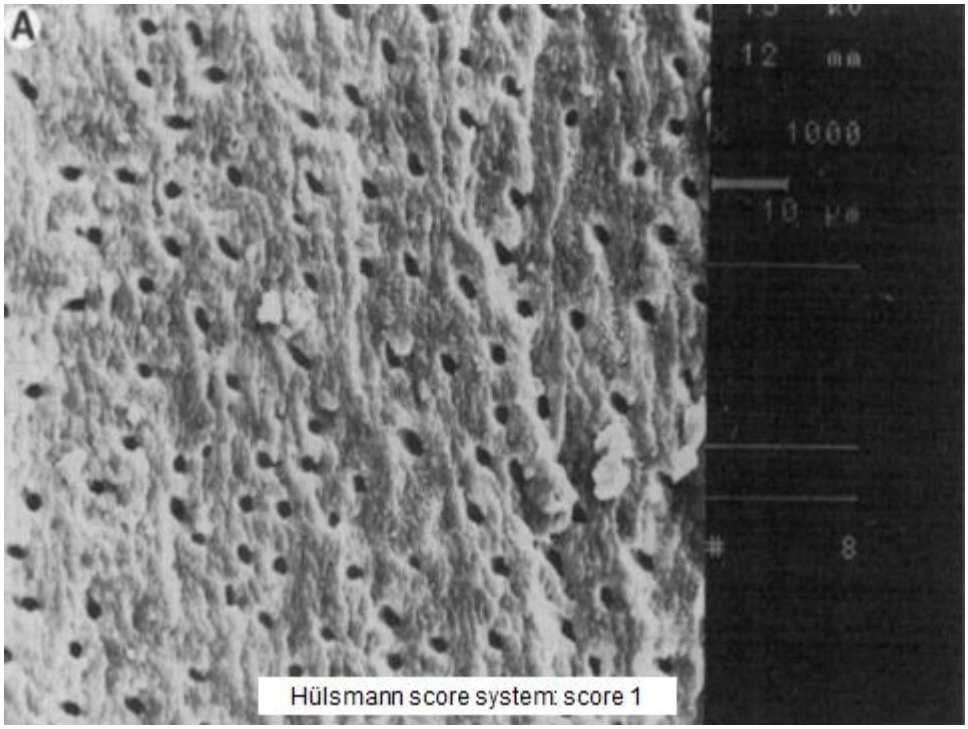
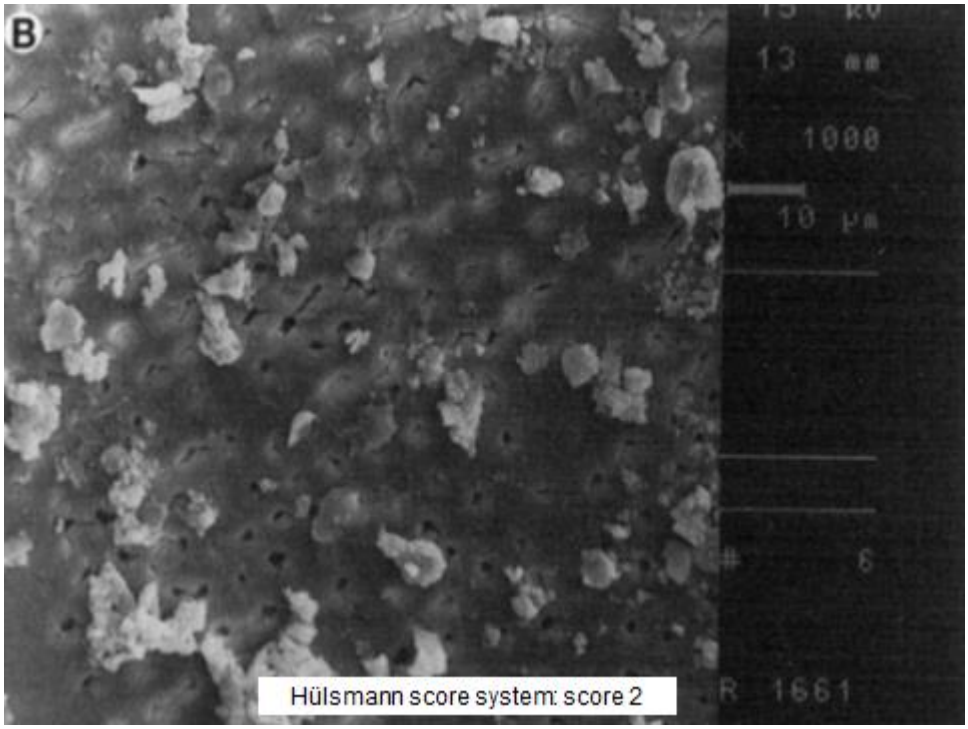
Chapter 10: Appendices

10.1 **Appendix A**

Hülsmann scoring system (Hülsmann *et al.*, 1997)

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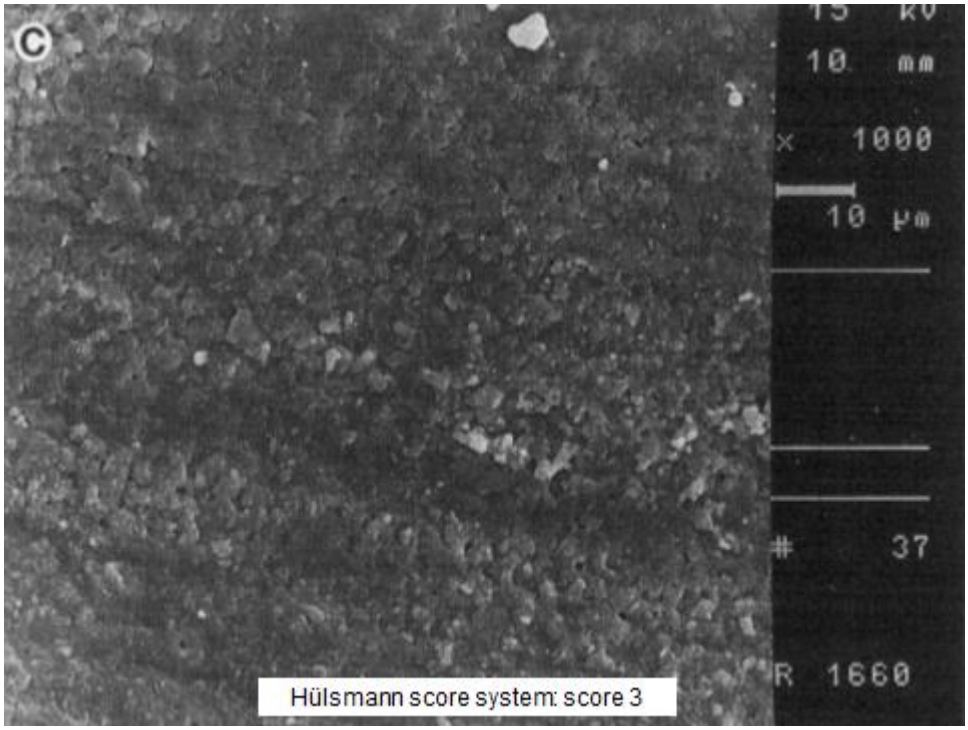
x 1000
10 µm
6
R 1661
Hülsmann score system: score 2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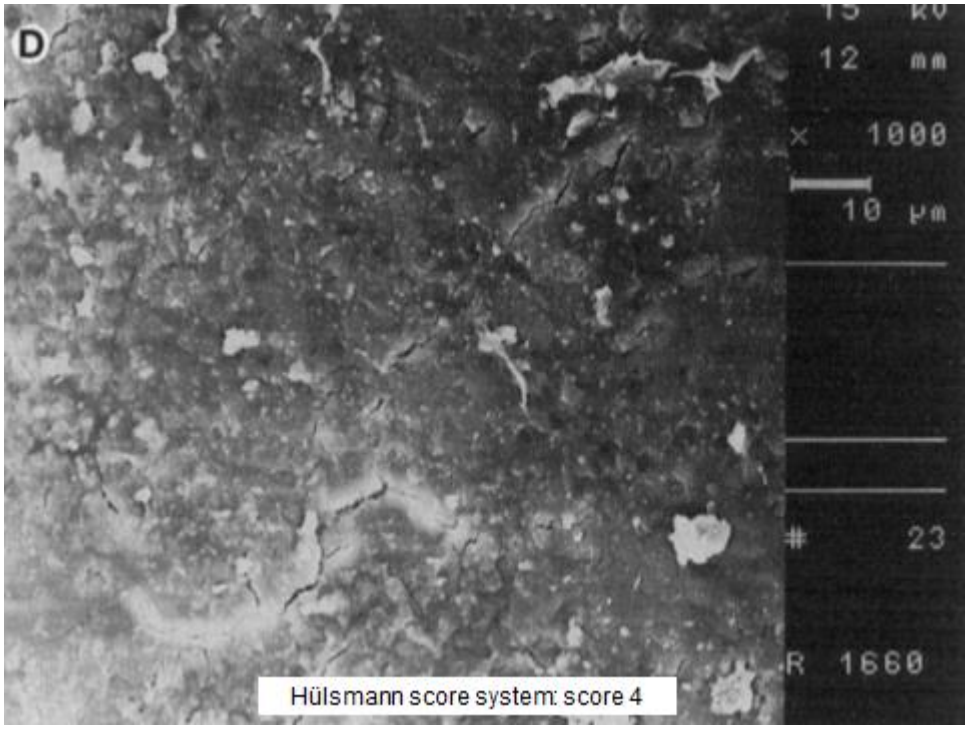
37
R 1660
Hülsmann score system: score 3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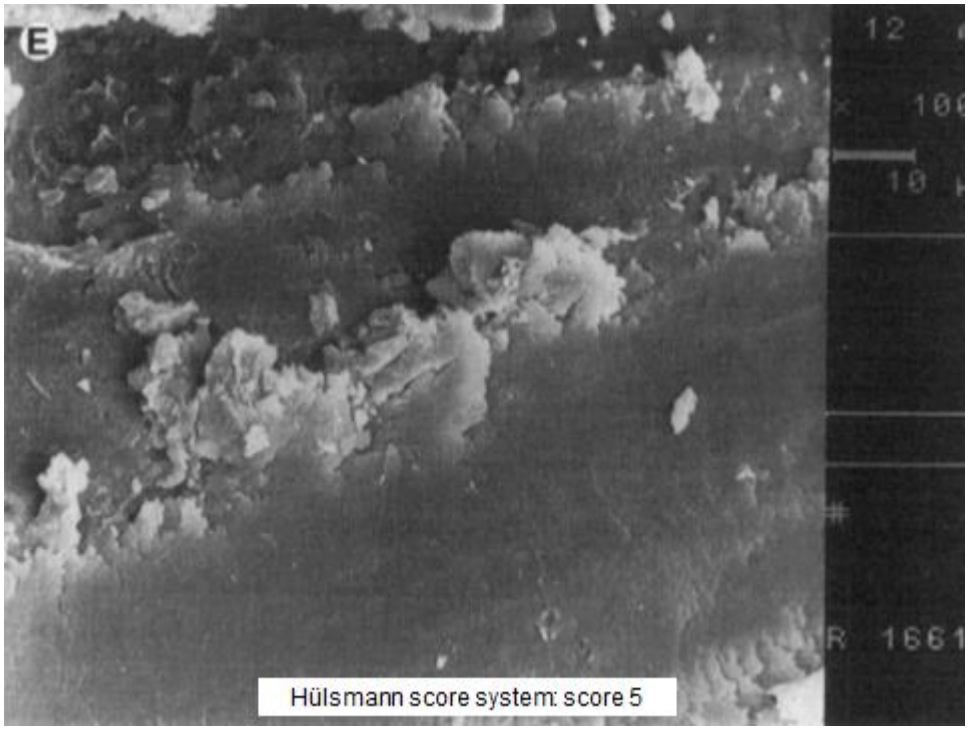
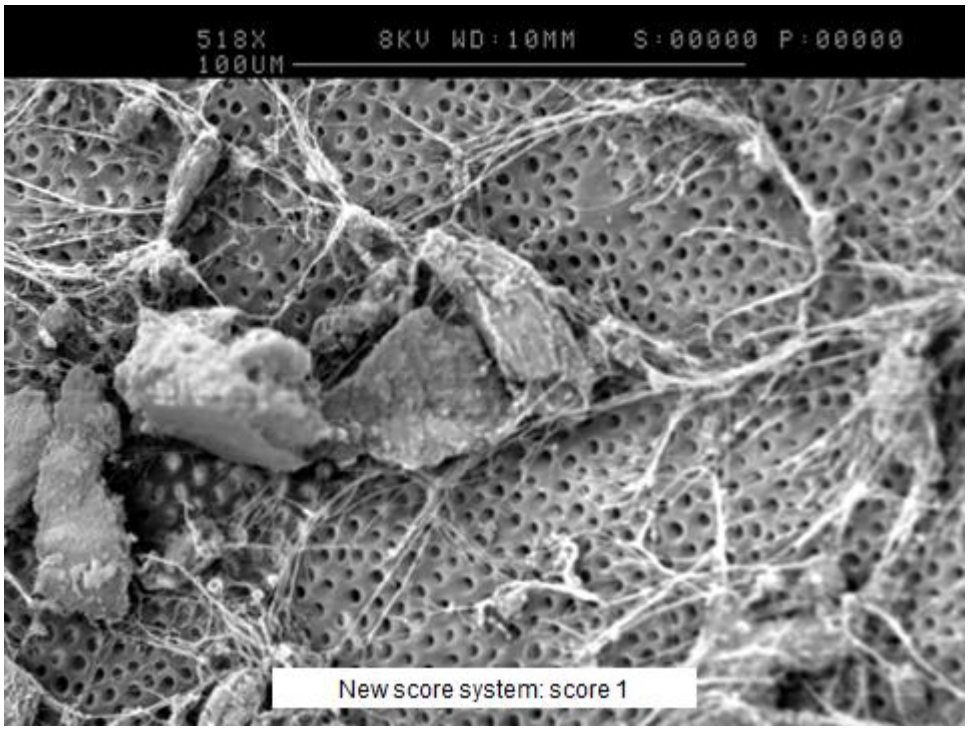
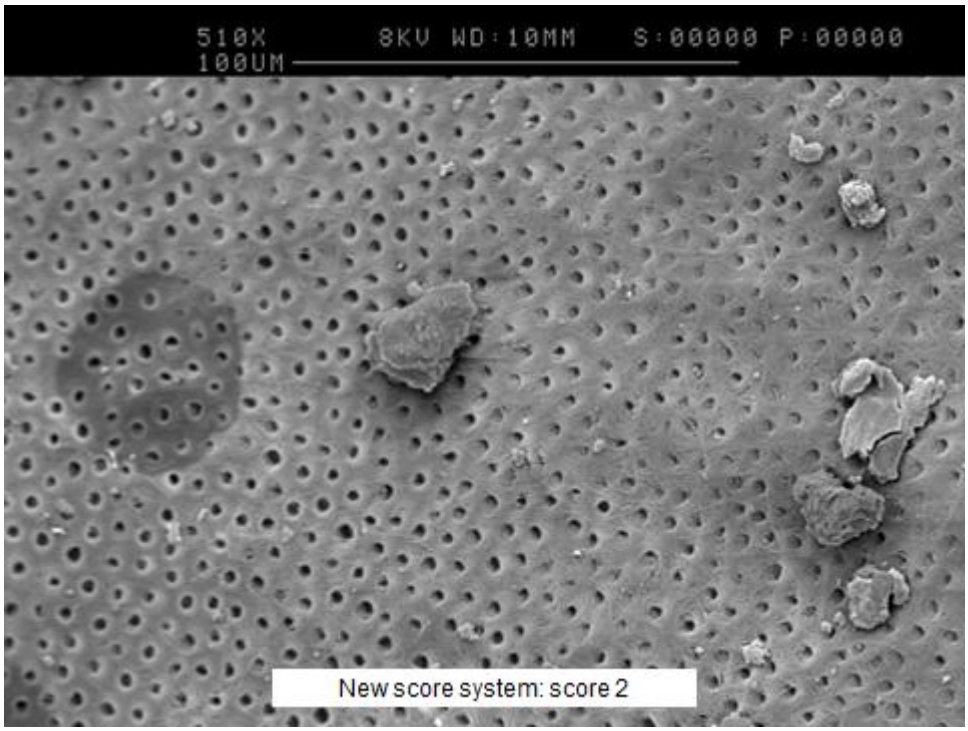
10 µm
23
R 1660
Hülsmann score system: score 4

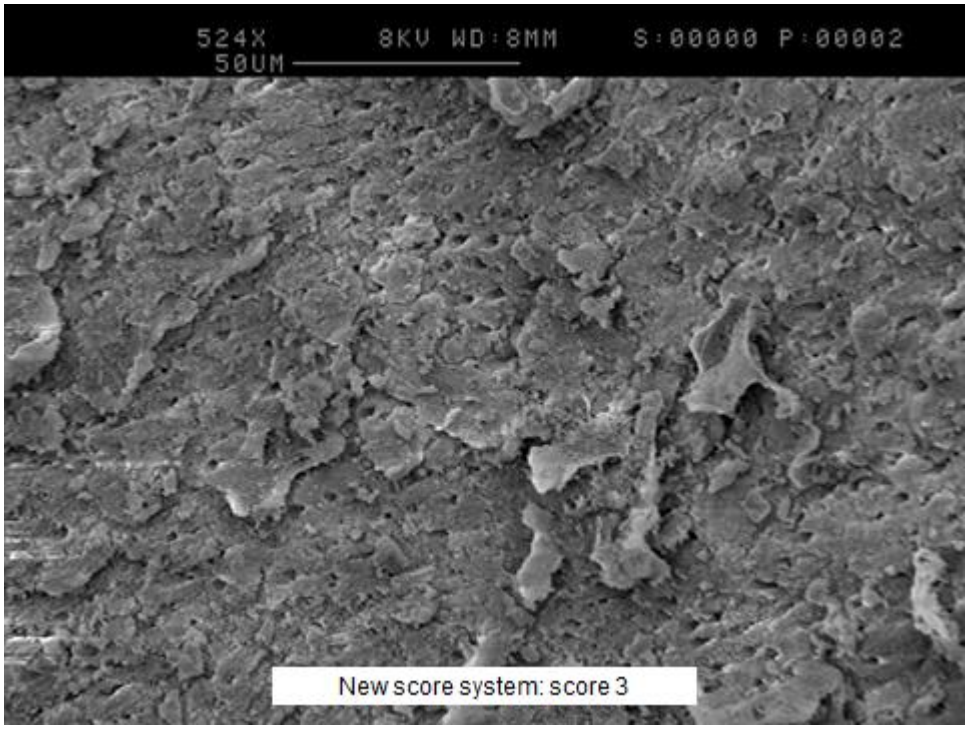

E
12
10
R 1661
Hülsmann score system: score 5

10.2 **Appendix B**

The new scoring system



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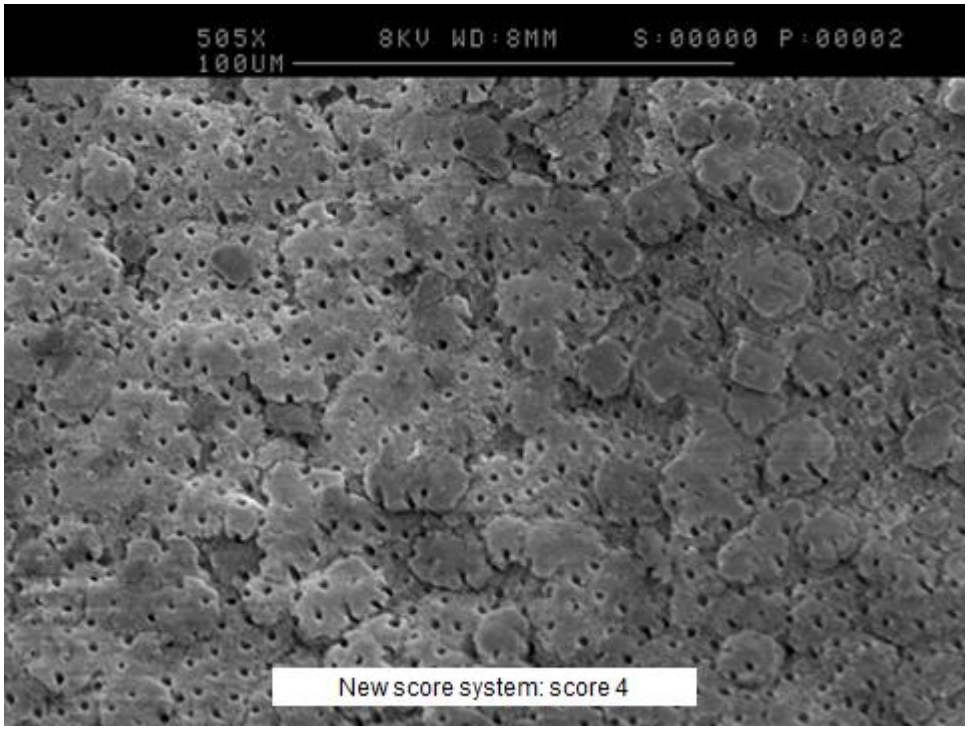
50UM
New score system: score 3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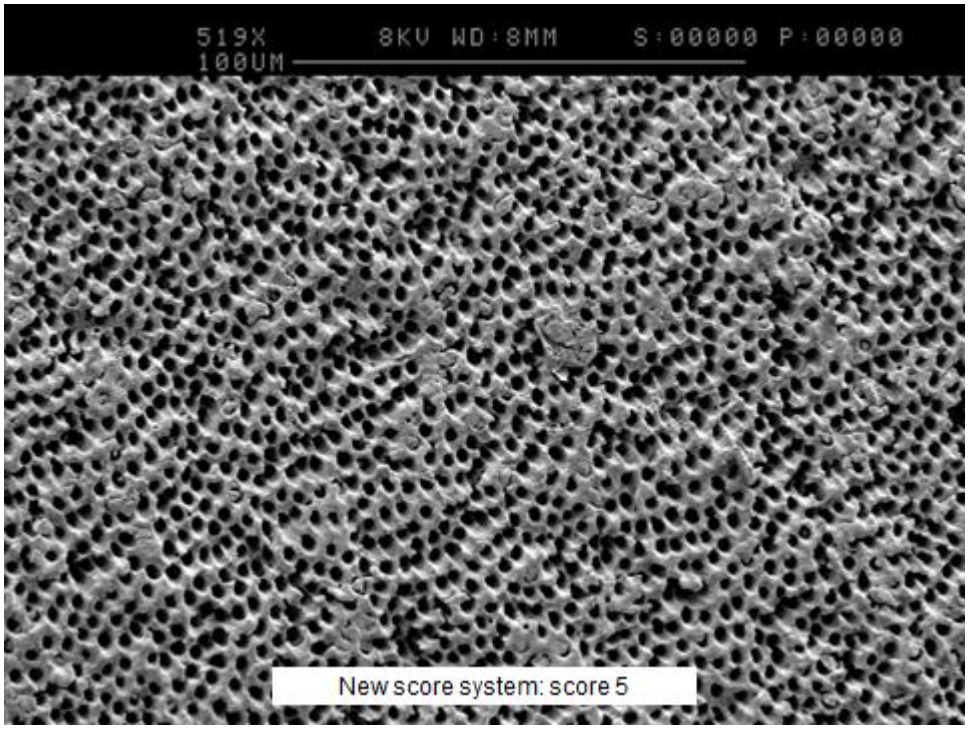
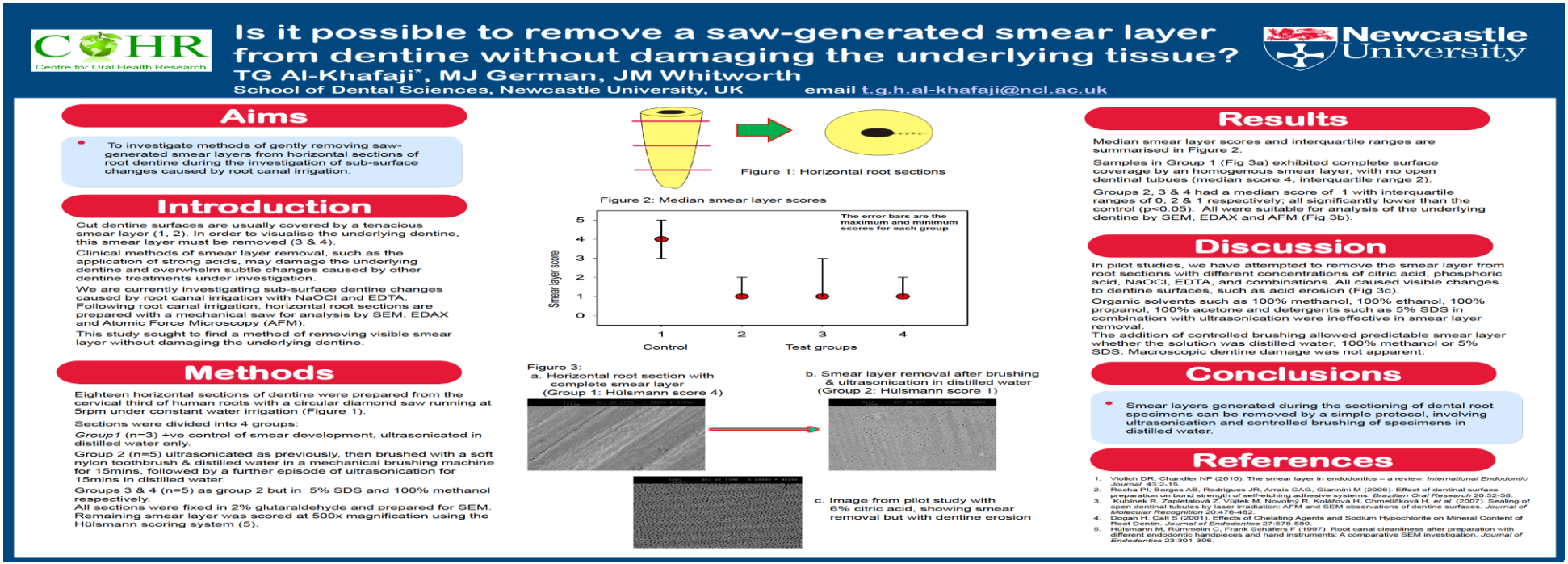
100UM
New score system: score 4


519X
100UM
8KV WD:8MM
S:00000 P:00000
New score system: score 5

10.3 **Appendix C**

This is to certify that: Mr Thulficar Al-Khafaji attended the Spring Scientific Meeting on Saturday 10th March 2012 at The Institution of Civil Engineers, London and was awarded 5.5 hours of verifiable CPD



Is it possible to remove a saw-generated smear layer from dentine without damaging the underlying tissue?

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CHR Centre for Oral Health Research

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Aims

To investigate methods of gently removing saw-generated smear layers from horizontal sections of root dentine during the investigation of sub-surface changes caused by root canal irrigation.

Introduction

Cut dentine surfaces are usually covered by a tenacious smear layer (1, 2). In order to visualise the underlying dentine, this smear layer must be removed (3 & 4).

Clinical methods of smear layer removal, such as the application of strong acids, may damage the underlying dentine and overwhelm subtle changes caused by other dentine treatments under investigation.

We are currently investigating sub-surface dentine changes caused by root canal irrigation with NaOCl and EDTA. Following root canal irrigation, horizontal root sections are prepared with a mechanical saw for analysis by SEM, EDAX and Atomic Force Microscopy (AFM).

This study sought to find a method of removing visible smear layer without damaging the underlying dentine.

Methods

Eighteen horizontal sections of dentine were prepared from the cervical third of human roots with a circular diamond saw running at 5rpm under constant water irrigation (Figure 1).

Sections were divided into 4 groups:

Group1 (n=3) +ve control of smear development, ultrasonicated in distilled water only.

Group 2 (n=5) ultrasonicated as previously, then brushed with a soft nylon toothbrush & distilled water in a mechanical brushing machine for 15mins, followed by a further episode of ultrasonication for 15mins in distilled water.

Groups 3 & 4 (n=5) as group 2 but in 5% SDS and 100% methanol respectively.

All sections were fixed in 2% glutaraldehyde and prepared for SEM. Remaining smear layer was scored at 500x magnification using the Hülsmann scoring system (5).

Figure 1: Horizontal root sections

Figure 2: Median smear layer scores

Figure 3:

a. Horizontal root section with complete smear layer (Group 1: Hülsmann score 4)

b. Smear layer removal after brushing & ultrasonication in distilled water (Group 2: Hülsmann score 1)

c. Image from pilot study with 6% citric acid, showing smear removal but with dentine erosion

Results

Median smear layer scores and interquartile ranges are summarised in Figure 2.

Samples in Group 1 (Fig 3a) exhibited complete surface coverage by an homogenous smear layer, with no open dentinal tubues (median score 4, interquartile range 2).

Groups 2, 3 & 4 had a median score of 1 with interquartile ranges of 0, 2 & 1 respectively; all significantly lower than the control ($p<0.05$). All were suitable for analysis of the underlying dentine by SEM, EDAX and AFM (Fig 3b).

Discussion

In pilot studies, we have attempted to remove the smear layer from root sections with different concentrations of citric acid, phosphoric acid, NaOCl, EDTA, and combinations. All caused visible changes to dentine surfaces, such as acid erosion (Fig 3c).

Organic solvents such as 100% methanol, 100% ethanol, 100% propanol, 100% acetone and detergents such as 5% SDS in combination with ultrasonication were ineffective in smear layer removal.

The addition of controlled brushing allowed predictable smear layer whether the solution was distilled water, 100% methanol or 5% SDS. Macroscopic dentine damage was not apparent.

Conclusions

Smear layers generated during the sectioning of dental root specimens can be removed by a simple protocol, involving ultrasonication and controlled brushing of specimens in distilled water.

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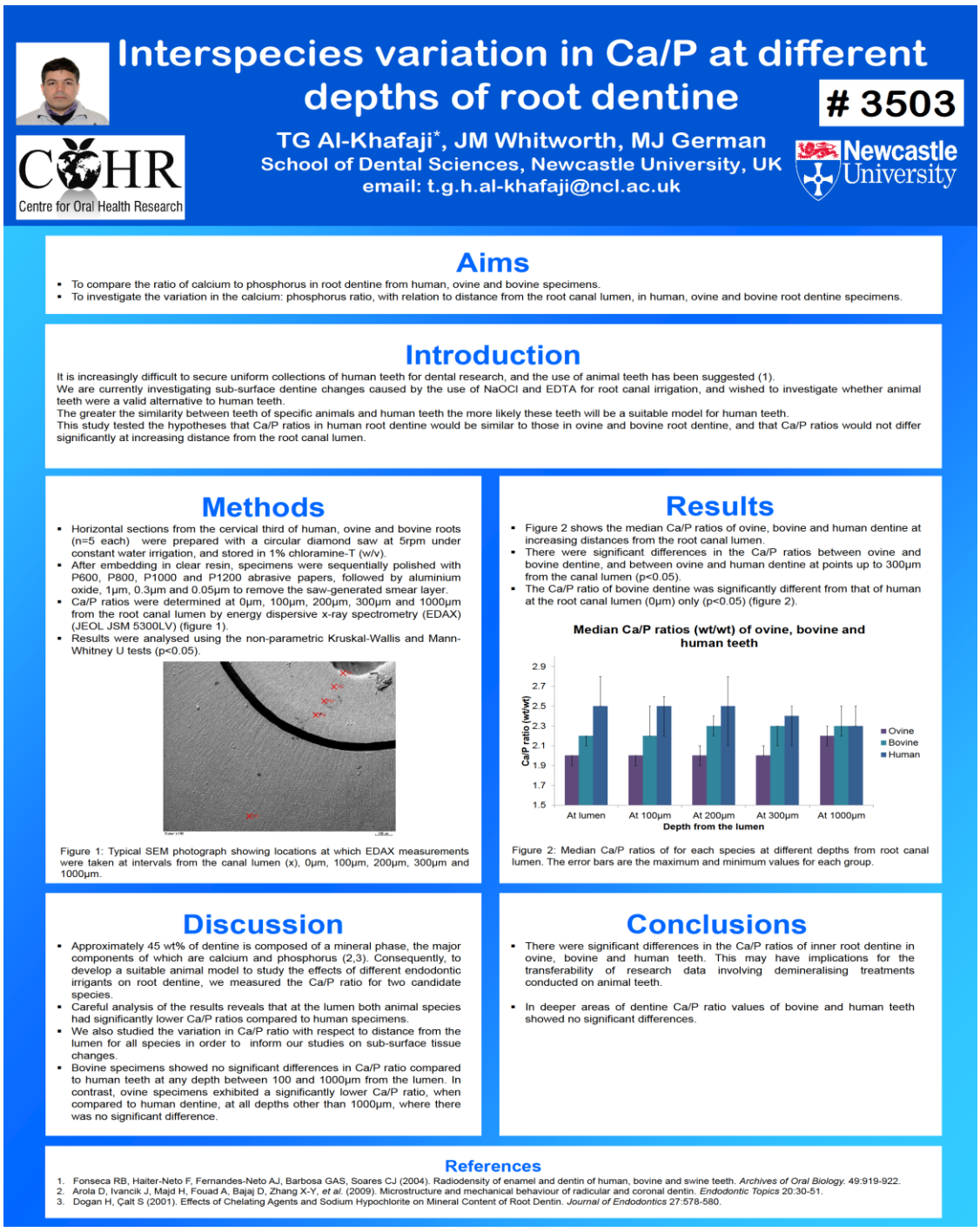
10.4 **Appendix D**

The International Association for Dental Research verifies that: Thulficar Al-Khafaji attended the IADR/AADR/CADR General Session & Exhibition in Seattle, Washington, USA, March 20-23, 2013 and presented the following research:

Interspecies Variation in Ca/P at Different Depths of Root Dentine



Interspecies variation in Ca/P at different depths of root dentine

 # 3503

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Centre for Oral Health Research

Newcastle University

Aims

- To compare the ratio of calcium to phosphorus in root dentine from human, ovine and bovine specimens.
- To investigate the variation in the calcium: phosphorus ratio, with relation to distance from the root canal lumen, in human, ovine and bovine root dentine specimens.

Introduction

It is increasingly difficult to secure uniform collections of human teeth for dental research, and the use of animal teeth has been suggested (1).
We are currently investigating sub-surface dentine changes caused by the use of NaOCl and EDTA for root canal irrigation, and wished to investigate whether animal teeth were a valid alternative to human teeth.
The greater the similarity between teeth of specific animals and human teeth the more likely these teeth will be a suitable model for human teeth.
This study tested the hypotheses that Ca/P ratios in human root dentine would be similar to those in ovine and bovine root dentine, and that Ca/P ratios would not differ significantly at increasing distance from the root canal lumen.

Methods

- Horizontal sections from the cervical third of human, ovine and bovine roots (n=5 each) were prepared with a circular diamond saw at 5rpm under constant water irrigation, and stored in 1% chloramine-T (w/v).
- After embedding in clear resin, specimens were sequentially polished with P600, P800, P1000 and P1200 abrasive papers, followed by aluminium oxide, 1µm, 0.3µm and 0.05µm to remove the saw-generated smear layer.
- Ca/P ratios were determined at 0µm, 100µm, 200µm, 300µm and 1000µm from the root canal lumen by energy dispersive x-ray spectrometry (EDAX) (JEOL JSM 5300LV) (figure 1).
- Results were analysed using the non-parametric Kruskal-Wallis and Mann-Whitney U tests (p<0.05).

Figure 1: Typical SEM photograph showing locations at which EDAX measurements were taken at intervals from the canal lumen (x), 0µm, 100µm, 200µm, 300µm and 1000µm.

Results

- Figure 2 shows the median Ca/P ratios of ovine, bovine and human dentine at increasing distances from the root canal lumen.
- There were significant differences in the Ca/P ratios between ovine and bovine dentine, and between ovine and human dentine at points up to 300µm from the canal lumen (p<0.05).
- The Ca/P ratio of bovine dentine was significantly different from that of human at the root canal lumen (0µm) only (p<0.05) (figure 2).

Median Ca/P ratios (wt/wt) of ovine, bovine and human teeth

Figure 2: Median Ca/P ratios of for each species at different depths from root canal lumen. The error bars are the maximum and minimum values for each group.

Discussion

- Approximately 45 wt% of dentine is composed of a mineral phase, the major components of which are calcium and phosphorus (2,3). Consequently, to develop a suitable animal model to study the effects of different endodontic irrigants on root dentine, we measured the Ca/P ratio for two candidate species.
- Careful analysis of the results reveals that at the lumen both animal species had significantly lower Ca/P ratios compared to human specimens.
- We also studied the variation in Ca/P ratio with respect to distance from the lumen for all species in order to inform our studies on sub-surface tissue changes.
- Bovine specimens showed no significant differences in Ca/P ratio compared to human teeth at any depth between 100 and 1000µm from the lumen. In contrast, ovine specimens exhibited a significantly lower Ca/P ratio, when compared to human dentine, at all depths other than 1000µm, where there was no significant difference.

Conclusions

- There were significant differences in the Ca/P ratios of inner root dentine in ovine, bovine and human teeth. This may have implications for the transferability of research data involving demineralising treatments conducted on animal teeth.
- In deeper areas of dentine Ca/P ratio values of bovine and human teeth showed no significant differences.

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