TITLE: COMPARATIVE EVALUATION OF CALCIUM LOSS AND ITS EFFECT ON MICROHARDNESS OF RADICULAR DENTIN FOLLOWING TREATMENT WITH VARIOUS IRRIGATING SOLUTIONS- AN IN VITRO STUDY

INTRODUCTION

An amorphous layer known as smear layer is formed on root canal walls after shaping and cleaning (1), which is 1-2 µm in thickness upto 40 µm into dentinal tubules and composed of inorganic particles of calcified tissue, and organic elements like pulp debris, odontoblastic processes, microorganisms, & blood cells (2).

Despite controversy over maintaining smear layer, it is shown that smear layer itself may contain bacteria, and protect those within dentinal tubules (3). It can also hinder penetration of intracanal disinfectants & sealers into dentinal tubules and compromise seal of the root canal filling (4,5) A recent meta-analysis of leakage studies concluded that smear layer removal improves the fluid tight seal of the root canal system (6). Thus it is currently considered important to promote techniques and products that prevent the formation or eliminate the smear layer.

Complete removal of smear layer demands use of chelating agents or organic acids, followed by tissue solvents to remove both organic & inorganic materials (7). Alternative use of EDTA and NaOCl has gained wide acceptance to remove the organic & inorganic remnants (8,9). Niu *et al* studied the ultrastructure on canal walls after EDTA and EDTA in combination with NaOCl irrigation by SEM: more debris was removed by irrigation with EDTA followed by NaOCl than EDTA alone (10). However, 10 minute application of EDTA caused excessive peritubular & intertubular dentinal erosion and reduced dentin microhardness (11,12). It causes enlargement of dentinal tubules, softening of the dentin, denaturation of the collagen fibres and its pulp dissolving property is questionable.

Various acids have been tried to remove the smear layer effectively. Maleic acid is used as acid conditioner in some adhesive systems, and has been reported to remove the smear layer in adhesive dentistry. Ballal *et al* found that maleic acid was similar to EDTA in endodontic smear layer removal in coronal and middle third of root canal, but a better ability at the apical third (13). Tidmarsh in 1978 reported that 50% citric acid irrigation was effective in removal of superficial smear layer (14). Wayman *et al* said that alternate use of 10% citric acid and 2.5% NaOCl was an effective method for smear layer removal (15). This acid removes the smear layer better than many acids such as polyacrylic acid, lactic acid and phosphoric acid (16). MTAD, a mixture of tetracycline isomer (doxycycline hyclate), an acid (4.25% citric acid) and a detergent (0.5% polysorbate,Tween 80), is a new generation combination product for root canal irrigation. Torabinejad *et al* investigated the ability of MTAD to remove the smear layer and disinfect contaminated root canals (17). Their study showed that a 5 minute application of MTAD is effective as a final rinse in removing the smear layer and bacteria from infected canal.

Calcium and phosphorous present in hydroxyapatite crystals are the main inorganic elements of dentin (18,19). Chelating agents react with Calcium ions in hydroxyapatite crystals and cause alterations in dentin chemical structure (20). Calcium/Phosphorus ratio of the dentin surface is changed, which alters the original proportion of organic & inorganic components. This is turn changes the permeability, microhardness, solubility characteristics of dentin, and adhesion of sealers to dentin (21,22,23). Microhardness determination can provide indirect evidence of mineral loss or gain in dental hard tissues (24). Significant alteration in dentin hardness after irrigation with different chemicals indicates the direct effect of these chemical solutions on the components of dentin structure (25). Chelating agents like EDTA, citric acid, maleic acid and MTAD have been shown to cause erosion of dentin (11,26,27,28).

Thus the purpose of this study was to compare calcium loss and microhardness reduction of radicular dentin following treatment with 17% EDTA, 10% citric acid, 5% maleic acid, and MTAD, by estimating calcium loss, and radicular dentin microhardness.

MATERIALS AND METHODS

Seventy two human mandibular premolars with type 1 canal anatomy extracted for orthodontic treatment were included. Crowns were removed at CEJ using high speed diamond bur under water cooling. A size 10 K file (Dentsply Maillefer, Baillaigues, Switzerland) was placed in the canal until visible at the apical foramen. Working length was established 1 mm short of this length. Root canals were enlarged by using hand Protaper files (Dentsply Maillefer, Baillaigues, Switzerland), upto file size F3. Irrigation during cleaning and shaping was accomplished by 2ml of 3% NaOCl solution after every file. Each root was sectioned longitudinally by starting from cervical with a low speed diamond disc and separated buccolingually to expose entire extent of root canal, one half for calcium loss estimation, and the other for microhardness estimation Each half was weighed on a precision balance and standardized to 0.22g before use.

For calcium loss estimation, cemental surface of specimens were coated with nail polish to prevent entry of irrigating solutions. Specimens were immersed in containers for 5 minutes each containing 5ml of irrigating solutions as follows: Group IA- 17% EDTA, Group IIA- 10% citric acid, Group IIIA- Biopure MTAD, Group IVA- 5% maleic acid, Group VA- Saline (control). Solutions were maintained under constant agitation using magnetic multistirrer to homogenize extracted calcium in solution. The level of calcium in the solutions was determined using Atomic absorption spectrophotometry (GBC 932 Plus) using air-acetylene flame at a wavelength of 422.7nm. The instrument was calibrated with known standards of 0.5, 1 and 1.5 µg/ml.

specimens for microhardness testing were horizontally embedded in autopolymerizing acrylic resin exposing the dentin surfaces, then ground polished with carborundum paper (300, 600, 1200, 2500 grade) and polished using aluminium oxide paste. To determine baseline surface hardness of root dentin, indentations were made with Vicker's diamond indentor a minimum of 3 widely similarly positioned locations (0.5mm level to root canal wall in apical, middle, and cervical root region) using 300g load and a dwell time of 20 seconds. The values were averaged to produce one hardness value for each specimen, and converted to Vicker's numbers.

Acrylic blocks with embedded teeth were kept in containers containing 3% NaOCl for 5 minutes followed by 20ml of various irrigating solutions for 5 minutes- Group IB- 17% EDTA, Group IIB-10% citric acid, Group IIIB- Biopure MTAD, Group IVB- 5% maleic acid, Group VB- Saline (control). Dentin sample microhardness was measured in same manner after immersion and reduction in microhardness calculated.

The results were statistically evaluated using one way ANOVA test and performed at 95% level of confidence. Spearman's correlation test was applied to determine correlation between calcium liberation and radicular dentin microhardness.

RESULTS

Chart 1 depicts Group IVA (5% maleic acid) showing the highest mean calcium loss from radicular dentin followed by group IIA (10% citric acid), group IIIA (MTAD), group IA (17% EDTA) and group VA (saline). Intergroup comparison was done using one way Analysis of variance (ANOVA). There was a statistically significant difference among all the groups ($p<0.05$).

Chart 2 depicts Group IVB (5% maleic acid) showing the highest mean reduction in microhardness, followed by Group IIB (10% citric acid), group IIIB (MTAD), group IB (17% EDTA) and group VB (saline). Intergroup comparison using one way Analysis of variance (ANOVA) revealed Group IVB (5% maleic acid) showing a significantly higher reduction in microhardness as compared to group IB (17% EDTA), Group IIIB (MTAD), and Group VB (saline-control). However, there was no significant difference in microhardness reduction between group IVB (5% maleic acid) and Group IIB (10% citric acid) ($p=0.371$). Group IB (17% EDTA) showed a significantly lower reduction in microhardness as compared to group IIB (10% citric acid) and group IVB (5% maleic acid). However, there was no significant difference between group IB (17% EDTA) and group IIIB (MTAD) ($p=0.141$). Groups IB, IIB, IIIB and IVB showed a significantly greater reduction in microhardness as compared to the control saline (Group VB).

Spearman's correlation test was done to evaluate whether any correlation exists between calcium loss from radicular dentin and microhardness reduction. A positive and significant correlation was observed. ($r=0.794$; $p<0.05$)

DISCUSSION

The occurrence of erosion resulting from chemomechanical preparation can be harmful when they are located on the apical third, especially in teeth with apical cemental and dentinal erosions. The erosion promoted by the irrigant can reach external resorption areas. Moreover, the erosion can reduce the dentin microhardness, consequently causing root fragility (10,29). EDTA is believed to erode dentin if the exposure time exceeds one minute (11). Contrary to this, Scelza *et al* (2004) found that the action of EDTA was not time dependant, although a small nonsignificant increase in decalcifying activity was reported with time (30). Time periods of less than 5 minutes are not recommended for EDTA (31), although chelating activity is observed at between 1 and 4 minutes (11).

Specimens were immersed for a period of 5 minutes. This duration is more realistic in terms of clinical practice (31). Also; it has been shown that the main effect of chelator substances occurred after 5 minutes of application (32). The solutions were not renewed during the 5 minute immersion as that would increase its effectiveness compared with a single continuous application over the same time because it maintains the pH at neutral levels, thereby increasing its moisturizing and decalcifying capacity (33).

EDTA is used to enlarge root canals, remove the smear layer and prepare the dentinal walls for better adhesion of filling materials. Under neutral pH, the sodium salt of EDTA is supposed to exchange hydrogen ion by calcium during chelation with subsequent decrease in pH. Therefore not all the EDTA reacts after few hours. The autolimitation might be due to this acidification of the EDTA solution (34).

Maleic acid is highly acidic, with a very low pH (1.47) and a higher etching potential (pKa-1.8) as compared to citric acid (pka-3.1), which may be responsible for its better demineralizing effect within a short periods of time (35). Ballal *et al* in a similar study found that maleic acid reduced the calcium level significantly more than EDTA upto 5 minutes. However, at 10 and 15 minutes, EDTA caused significantly greater demineralization (27).

10% citric acid showed significantly more calcium loss than 17% EDTA (Chart 1). In a similar study, Machado-Silveiro *et al* found that 10% citric acid solution was a more effective decalcifying agent as compared to 17% EDTA (26). They also said that the decalcifying action of citric acid, due to its low pH, is greater than its chelating action. Scelza *et al* also found a higher decalcifying effect of 10% citric acid over 17% EDTA, and found that the demineralizing action of 10% citric acid significantly increased from 3 to 10 minutes, but was no longer time dependent at 15minutes (36).

MTAD is an acidic solution with a pH of 2.15 that is capable of removing inorganic substances. Manufacturers recommend to use MTAD as a final irrigant before obturation, which could be of concern, owing to the fact that if any remaining traces of MTAD are present in the dentinal tubules even after drying with paper points, they can continue to cause erosion of dentin which could be aggressive (37). In the present study, MTAD showed a significantly higher calcium loss as compared to EDTA (Chart 1). Tay *et al* (2006) concluded that Biopure MTAD is comparatively more aggressive in demineralizing intact radicular dentin, exposing collagen matrices that were 1.5 to 2 times as thick as those produced with 17% EDTA (28). Soumithran N *et al* found that MTAD is a better demineralizing agent than 17% EDTA and recommended that both solutions be flushed out after ten minutes, especially MTAD, which causes severe erosion of dentin after ten minutes (37).

The higher demineralizing effect of MTAD may be attributed to the following factors:

- Doxycycline acts as a chelating agent and citric acid is also used to remove the smear layer. Therefore it exerts exaggerated action on demineralization (38).
- The pH of citric acid is 2.5 rendering a more acidic action as compared to EDTA 17% which has a slightly alkaline pH of 8.5.
- The role of detergent Tween 80 enhances the contact of MTAD to the dentinal walls by lowering the surface tension.

In order to obtain a more reliable reading of dentin microhardness, 3 indentations were taken and the mean calculated. As the microhardness of dentin may vary considerably within the same tooth (39), comparisons of dentin hardness values before and after treatment with irrigating solutions was made within the same root dentin sample. This was done to minimize the effect of structural variations of different teeth, and to establish a reasonable evaluation with respect to baseline data. This was also suggested by Saleh & Ettman (29).

According to Pashley, dentin microhardness, which depends on the amount of calcified matrix per mm^2, is inversely correlated to tubule density (40). The microhardness found next to the lumen in which the tubuli are denser is higher than in the periphery in which the tubuli are less crowded. Thus, readings were recorded at a fixed distance of 500µm from the root canal lumen.

The chelating actions of these solutions induce an adverse softening potential on the calcified components of dentin, and a reduction in microhardness is expected. This could be of clinical benefit because it permits rapid preparation and facilitates the negotiation of tight small root canals (41). However, the degree of softening and demineralization may have an influence on the physical and chemical properties of this heterogenous structure (12,22).

In the present study, a positive correlation was found between microhardness loss and calcium loss from radicular dentin. Similar observations have been reported by Panighi and G'Sell (22). Arends and ten Bosch said that microhardness determination can provide an indirect evidence of mineral loss or gain in the dental hard tissues (24).

Variations in microhardness can be attributed to the concentrations of the solutions used, pH of solutions, and application time (42). Further studies are required to confirm the results of the present study and assess the calcium loss and microhardness over an extended period of time. Scanning electron microscopic analysis would also give a clearer picture of the amount of demineralization taking place. Changes according to pH variation could also be included in further studies. Since this is an *invitro* study, further *invivo* studies are required to assess its clinical significance and its affect on long term prognosis of teeth.

CONCLUSION

- All the experimental chelating agents bring about calcium loss and reduction in microhardness from radicular dentin.
- At 5 minutes, 5% maleic acid as a chelating agent causes the maximum calcium loss and microhardness reduction from radicular dentin, followed by 10% citric acid, MTAD, and 17% EDTA. Thus 5% maleic acid was the most effective chelating agent out of those used in the study.
- According to the results of the present study, there is a positive and significant correlation exists between calcium loss from radicular dentin and microhardness reduction.

REFERENCES

1. Mader CL, Baumgartner C, Peters DD. SEM investigation of the smeared layer on root canal walls J Endod 1984; 10(10): 477-83.
2. Sen BH, Wesselink PR, Turkun M. The smear layer; a phenomenon in root canal therapy. Int Endod J 1995; 28(3): 141-8.
3. Torabinejad M, Handysides R, Khademi AA, Bakland LK. Clinical implication of the smear layer in endodontics: a review. Oral Surg Oral Med Orsl Pathol Oral Radiol Endod 2002; 94(6): 658-66.
4. White RR, Goldman M, Lin PS. The influence of the smeared layer upon dentinal tubule penetration by plastic filling materials. J Endod 1984; 10(12): 558-62.
5. Economides N, Liolios E, Kolokuris I, Beltes P. Long term evaluation of the influence of smear layer removal on the sealing ability of different sealers. J Endod 1999; 25(2): 123-5.
6. Shahravan A, Haghdoost A, Adl A, Rahimi H, Shadifar F. Effect of smear layer on sealing ability of canal obturation: a systematic review and meta-analysis. J Endod 2007; 33(2): 96-105.
7. Zehnder M, Schmidlin P, Sener B, Waltimo T. Chelation in root canal therapy reconsidered. J Endod 2005; 31(11): 817-20.
8. Yamada RS, Armas A, Goldman M, Lin PS. A scanning electron microscopic comparison of a high volume final flush with several irrigating solutions: part 3. J Endod 1983; 9(4): 137-42.
9. Perez-Heredia M, Ferrer-Luque CM, Gonzalez-Rodriguez MP. The effectiveness of different acid irrigating solutions in root canal and two new irrigants containing antibiotic. J Endod 2006; 32(10): 1091-3.
10. Niu W, Yoshioka T, Kobayashi C, Sauda H. A scanning electron microscopic study of dentinal erosion by final irrigation with EDTA and NaOCl solutions. Int Endod J 2002; 35(11): 934-9.
11. Calt S, Serper A. Time dependent effects of EDTA on dentin structures. J Endod 2002; 28(1): 17-19.

12. Ari H, Erdemir A, Belli S. Evaluation of the effect of endodontic irrigation solutions on the microhardness and the roughness of root canal dentin. J Endod 2004; 30(11): 792-5.
13. Ballal NV, Kandian S, Kundabala M, Bhat KS, Acharya S. Comparison of efficacy of Maleic acid and EDTA in smear layer removal from instrumented human root canal: a scanning electron microscopic study. J Endod 2009; 35(11): 1573-76.
14. Tidmarsh B. Acid-Cleansed and resin sealed root canals. J Endod 1978; 4(4): 117-121.
15. Wayman BE, Kopp WM, Pinero GP, Lazzari EP. Citric and Lactic acids as root canal irrigants in vitro. J Endod 1979; 5(9): 258-65.
16. Meryon SD, Tobias RS, Jakeman KJ. Smear removal agents: a quantitative study in vivo and in vitro. J Prosthet Dent 1987; 57(2): 174-9.
17. Torabinejad M, Khademi AA, Babagoli J, Cho Y, Johnson WB, Bozhilov K, Kim J, Shabahang S. A new solution for the removal of the smear layer. J Endod 2003; 29(3): 170-5.
18. Marshall GW Jr. Dentin microstructure and characterization. Quintessence.Int 1993; 24(9): 606-17.
19. Hennequin M. Douillard Y. Effects of citric acid treatment on the Ca , P and Mg contents of human dental roots. J Clin.Periodontol 1995; 22(7): 550-7.
20. Dogan H, Calt S. Effects of chelating agents and sodium hypochlorite on mineral content of root dentin. J Endod 2001; 27(9): 578-80.
21. Rotstein I, Dankner E, Goldman A, Heling I, Stabholz A, Zalkind M. Histochemical analysis of dental hard tissues following bleaching. J Endod 1996; 22(1): 23-6.
22. Panighi M, G'Sell C. influence of calcium concentration on the dentin wettability by an adhesive. J Biomed Mater Res 1992; 26(8): 1081-9.
23. Meerbeek BV, Lambrechts P, Inokoshi S, Braem M, Vanherle G. Factors affecting adhesion to mineralized tissues. Oper Dent 1992; Suppl 5: 111-24.
24. Arend J, Ten Bosch JJ. Demineralization and remineralization evaluation techniques. J Dent Res 1992; 71: 924-8.
25. Cruz-Filho A, Sousa-Neto M, Saquy P, Pecora J. Evaluation of the effect of EDTAC, EDTA and EGTA on radicular dentin microhardness. J. Endod 2001; 27(3): 183-4.
26. Machado-Silveiro LF, Gonzalez-Lopez S, Gonzalez-Rodriguez MP. Decalcification of root canal dentin by citric acid, EDTA and Sodium citrate. Int Endod J 2004; 37(6): 365-69.
27. Ballal NV, Kundabala M, Bhat KS. Evaluation of decalcifying effect of maleic acid and EDTA on root canal dentin using energy dispersive spectrometer. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011; 112(2): e78-e84.
28. Tay FR, Pashley DH, Loushine RJ, Doyle MD, Gillespie WT, Weller RN, King NM. Ultrastructure of Smear Layer-Covered Intraradicular Dentin after Irrigation with BioPure MTAD. J Endod 2006; 32(3): 218-221.
29. Saleh AA, Ettman WM. Effect of endodontic irrigation solutions on microhardness of root canal dentine. J Dent 1999; 27(1): 43-46.
30. Scelza MF, Pierro V, Scelza P, Pereira M. Effect of three different time periods of irrigation with EDTA-T, EDTA and citric acid on smear layer removal. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2004; 98(4): 499-503.

31. Taner CS, Serper A, Cehreli ZC, Otlu HG. The effect of EDTA, EGTA, EDTAC, and tetracycline-HCl with and without subsequent NaOCl treatment on microhardness of root canal dentin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007; 104(3): 418-24
32. Patterson S. In vivo and in vitro studies of the effect of the disodium salt of ethylenediamine tetraacetate on human dentin and its endodontic implication. Oral Surg Oral Med Oral Pathol 1963; 16: 176-9.
33. Weinreb MM, Meier E. The relative efficiency of EDTA, sulfuric acid, and mechanical instrumentation in the enlargement of root canals. Oral Surg Oral Med Oral Pathol 1965; 19: 247-52.
34. Perez VC, Medina Cardenas ME, Sanchez Planells U. The possible role of pH changes during EDTA demineralization of teeth. Oral Surg Oral Med Oral Pathol 1989; 68(2): 220-2.
35. Breschi l, Gobbi p, Mazzoti G, Falconi M, Ellis TH, Stangel I. high resolution SEM evaluation of dentin etched with maleic and citric acid. Dent Mater 2002; 18(1): 26-35
36. Scelza MFZ, Texeirs AM, Scelza P. Decalcifying of EDTA-T, 10% Citric acid and 17% EDTA on root canal dentin. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2003; 95(2): 234-6
37. Soumithran N, Kundabala M, D' Souza V, Prabhu R, Prabhu A. Comparative evaluation of demineralization of radicular dentin with 17% EDTA and MTAD at different time intervals - An in-vitro Study. Endodontology 2011; 23(1): 47-55
38. Krause TA, Liewehr FR, Hahn CL. The antimicrobial effect of MTAD, NaOCl, doxycycline and citric acid on Enterococcus faecalis. J Endod 2007; 33(1): 28-30.
39. Seaman F, Shannon IL. Fluoride treatment and microhardness of dentin. J. Prosth Dent 1979; 41(5): 528-30
40. Pashley D, Okabe A, Parham P. The relationship between dentin microhardness and tubule density. Endod Dent Traumatol 1985; 1(5): 176-9.
41. Cruz-Filho AM, Paula EA, Pecora JD, Sousa-Neto MD. Effect of different EGTA concentrations on dentin microhardness. Braz Dent J 2002; 13(3): 188-90.
42. Pérez-Heredia M, Ferrer-Luque CM, González-Rodríguez MP, Martín-Peinado FJ, González-López S. Decalcifying effect of 15% EDTA, 15% citric acid, 5% phosphoric acid and 2.5% sodium hypochlorite on root canal dentine. Int Endod J 2008; 41(5): 418-23.