RESEARCH ARTICLE

A Comparative Evaluation of Microhardness and Chemical Structure of Radicular Dentin with Two Combinations of TAP and MTAP: An *In Vitro* Study

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Abstract

Background: The success of regenerative endodontics depends on various factors and the most vital being the complete eradication of microorganisms in the dentinal tubules. This could lead to changes that leave the radicular dentin prone to fracture.

Aim: The purpose of the present study is to investigate the effects of triple antibiotic paste (TAP) and modified triple antibiotic paste (MTAP) of different concentrations on the microhardness and chemical structure of radicular dentin.

Materials and methods: Human root cylinders were instrumented and randomized into four treatment groups and an untreated control group. Two treatment groups received 1 g/mL TAP or MTAP, and the other two treatment groups received 1 mg/mL methylcellulose-based TAP or MTAP. Cylinders were stored at 100% relative humidity for 4 weeks. Each root cylinder was subjected to a microhardness test before and after treatment. Different sets of radicular dentin specimens were treated as mentioned previously, and were examined using attenuated total reflection Fourier transform infrared spectroscopy.

Results: Significant reductions in microhardness of treated groups was noticed when compared to untreated control roots at 1,000 and/or 500 µm from the pulp-dentin interface.

Conclusion: The use of 1 mg/mL methylcellulose-based TAP and MTAP may minimize the reduction in microhardness of roots compared with the currently used 1 g/mL concentration of these antibiotics.

Keywords: ATR-FTIR, Microhardness, Modified triple antibiotic paste, Triple antibiotic paste.

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Introduction

Population based studies from around the world indicate that the prevalence of dental trauma injuries which are commonly found among young children, accounting for about 4 to 59%, with majority of the cases occurring in the incisors, often leading to pulpal necrosis.[1]

The treatment of necrotic immature teeth has always been a challenge in endodontics because of the difficulty to procure a good apical seal in the teeth with open apices by using only conventional endodontic treatment methods; the discontinued development of dentinal walls in the root part after pulp necrosis can lead to a weak radicular dentin which makes the tooth susceptible to fractures. Revascularization is a regenerative and a biologically based alternative approach to treat necrotic immature teeth unlike apexification and artificial apical barrier techniques as this allows continuation of root development.[2]

One of the major factors associated with endodontic failure is the persistence of polymicrobial infection disseminated within the root canal system and periradicular area. The complex morphology of the root canal system present with accentuated curves and accessory canals make it quite difficult to achieve proper cleansing by mechanical instrumentation and irrigation of canals.[3] There is no definitive evidence in literature that shows mechanical instrumentation alone results in a sterile root canal system.[4] Viable microorganisms may remain in the dentinal tubules particularly in uninstrumented areas and in other irregular spaces after root canal preparation. These micro-organisms may proliferate and re-infect the root canal leading to endodontic failure. It is therefore important that the root canal medicaments should have antimicrobial properties activity against these bacteria seated deeply throughout the root dentin but also be biocompatible to periradicular tissues.[5]

The polymicrobial infection makes it difficult to disinfect the canals effectively with just one type of antibiotic. Therefore, double antibiotic paste was introduced but with a prolonged period of time the DAP also showed bacterial resistance hence a combination of three antibiotics were recommended for the disinfection of the root canals.

This triple antibiotic paste (TAP) is composed of ciprofloxacin, metronidazole, and minocycline in a 1: 1: 1 ratio. The TAP lead to certain drawbacks of the TAP such as discoloration of the crown,

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increase in bacterial resistance, and allergic reactions have been reported and hence a revolution to replace minocycline with clindamycin was advocated and lead to development of modified triple antibiotic paste (MTAP).[6]

However, these chemical agents may negatively affect the chemical, physical, and mechanical properties of radicular dentin. Clinical studies have found that the increase in root wall thickness of immature teeth after endodontic regeneration is limited to the mid- and/or apical root rather than the cervical region, which is the area most prone to fracture in treated immature teeth. Therefore, it is essential to minimize the negative effects of intracanal medicaments on the weak cervical area of necrotic immature teeth.[7] Hence, the purpose of the present study is to investigate the effects of TAP and MTAP of different concentrations on the microhardness and chemical structure of radicular dentin.

Materials and Methods

The present study was conducted in the Department of Pedodontics and Preventive Dentistry, Mamata Dental College, Khammam. Intact human single-rooted mature premolars (n = 85) were selected after obtaining local Institutional Review Board (IRB) approval to use the teeth. Teeth with caries, restorations, hypoplasia, or cracks were excluded. Teeth were stored at 4°C in 0.1% thymol solution (Cipla pharmaceuticals, Sikkim) and were used within 6 months after extraction. A cervical 5-mm root cylinder was obtained from each tooth. The canal of each root cylinder was mechanically prepared using Dentsply 0.04 taper rotary instruments (Dentsply India Pvt Limited) with a master apical size 80 file. One milliliter of 5.25% sodium hypochlorite (Vishal Dentocare Pvt Ltd, Hyderbad) was used for irrigation between subsequent filings and each canal was finally rinsed with 5 mL of sterile water. Instrumented root cylinders were embedded in acrylic blocks. The coronal side of each cylinder was leveled with the surface of the acrylic block in order to maintain easy access to the root canal. The resulting blocks were ground flat and polished with water-cooled abrasive discs (500-, 1,200-, 2,400-, and 4,000-grit Al_2O_3 papers) (Generic Imported No: E_ 140,05439, China) and a polishing cloth with a diamond suspension (1-mm) (Connoisseurs Polishing Cloth, Mumbai).

Blocks were then color coded for easy identification. Blocks were then ultrasonically cleansed for 3 minutes (Sun Sterifab Pvt Ltd Ahmedabad), and pretreatment microhardness measurements were performed using a Vickers microhardness tester (Digital Micro Vickers tester, True Metco New Delhi) on the coronal side of each root cylinder at 500 µm from the pulp-dentin interface (Fig. 1). Three indentations (50 gm, for 10 seconds) were made at each depth and representative hardness values were reported as the mean of the three indentations.

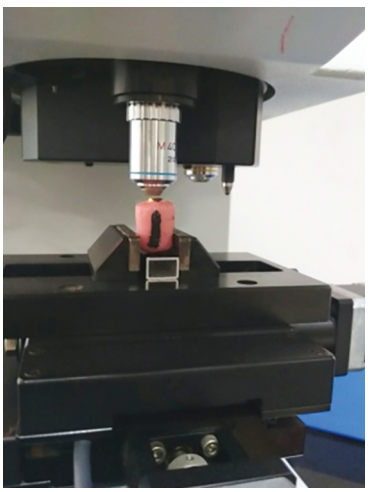
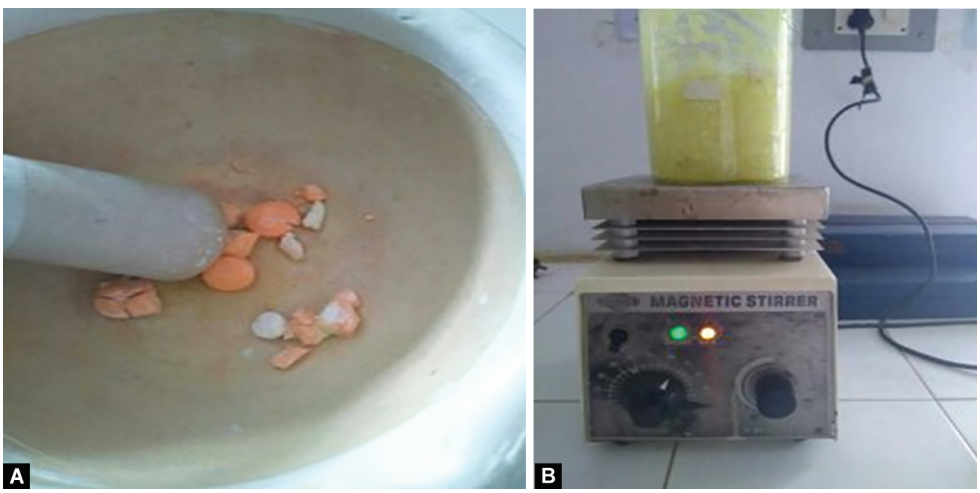


Fig. 1: Pretreatment hardness using Vickers microhardness tester

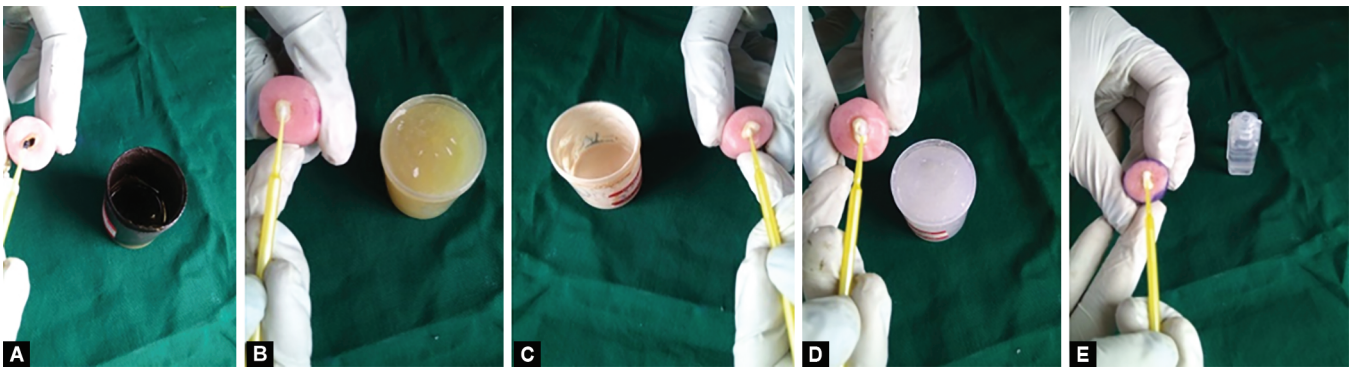
Medicament Application and Final Measurement

In order to prepare 1 gm/mL TAP, 1 gm of antibiotic powders comprising equal portions of metronidazole, ciprofloxacin, and minocycline (USP) were mixed with 1 mL of sterile water. To prepare 1 gm/mL MTAP, 1 g of USP-grade antibiotic powders comprising ciprofloxacin 14%, metronidazole 43%, and clindamycin 43% were mixed with 1 mL of sterile water. To prepare a 1 mg/mL solution of TAP or MTAP, 100 mg of each compounded powder mentioned above was dissolved in 100 mL of sterile water. Then, 8 gm of methylcellulose powder (BFC HPMC K4M, Calcutta) was added to 100 mL of each 1 mg/mL solution under magnetic stirring for 2 hours to obtain a homogenous gel with a 1mg/mL concentration of TAP or MTAP (Fig. 2).

Root cylinders were randomly assigned to four treatments groups (1 gm/mL TAP, 1 gm/mL MTAP, 1 mg/mL TAP, and 1 mg/mL MTAP) and a no treatment control group (n = 17 per group). For



Figs 2A and B: Preparation of medicaments



Figs 3A to E: Medicament application

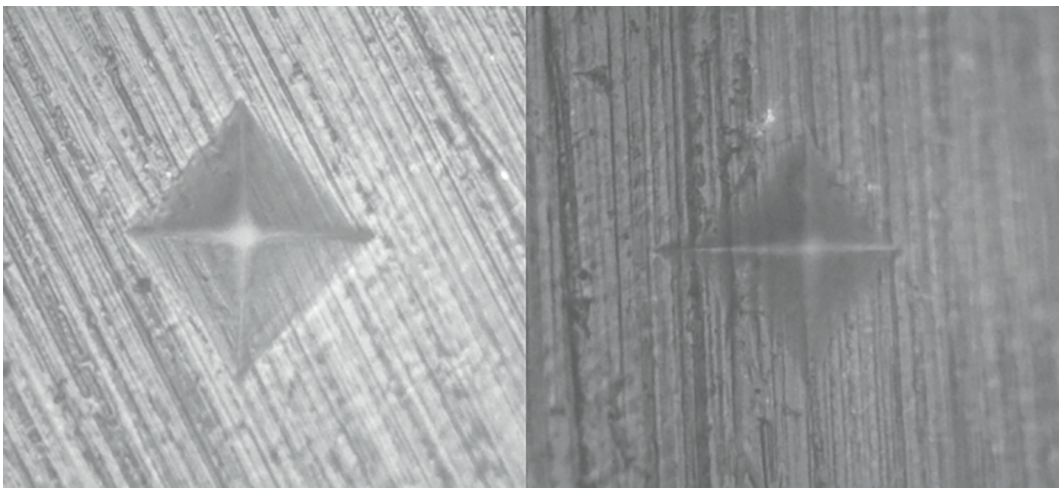


Fig. 4: Pretreatment and posttreatment difference in indentations

the control group, sterile water was applied to the canal. For the treatment groups, the exposed coronal surface of each root cylinder was covered with adhesive unplasticized polyvinyl chloride tape, leaving only the root canal orifice. Each root canal was dried with sterile paper points and the antibiotic paste was tamped into the canal space (Fig. 3). Acrylic blocks were covered with custom made vinyl material caps and stored for 4 weeks at 37°C at an approximately 100% relative humidity. Each root canal was then copiously irrigated with sterile water for 30 seconds to wash out the treatment paste. Adhesive tape was removed and posttreatment microhardness measurements were taken as described previously.

The percentage change in microhardness for each sample was calculated as follows: (Pretreatment microhardness – posttreatment microhardness) × 100 / pretreatment microhardness (Fig. 4).

Fourier Transform Infrared Spectroscopy (FTIR) Experiment

Sample Preparation

An additional 18 intact mature single-rooted premolars were selected according to the same criteria described in the microhardness experiment. A cervical 4-mm root cylinder from each tooth was obtained and sectioned longitudinally across the maximum diameter of the root canal resulting in two specimens. Both sides of each specimen were ground flat to a uniform thickness with 1,200 grit silicon carbide grinding paper (Buehler Ltd., Lake Bluff, IL, USA) under continuous water-cooling. Specimens were ultrasonicated for 5 minutes under sterile water to remove the smear layer.

Medicament Application and FTIR Measurement

Dentin specimens were randomly assigned to the same four treatment groups and a control group (n = 7 per group) as described in the microhardness experiment. Each dentin specimen was placed in a 2 mL conical sample cup containing 0.15 mL of one of the treatment pastes or sterile water (control). The amount of paste selected was sufficient to cover the pulpal surface of each specimen with a 0.2-mm layer of treatment paste. The containers were stored for 4 weeks at 37°C with approximately 100% relative humidity. Specimens were then rinsed thoroughly with sterile water until no visible paste remained, followed by ultrasonication for 15 minutes and air-drying. The chemical structure of treated dentin specimens was analyzed using a 4100 FTIR spectrophotometer with a diamond ATR setup (Perkin Elmer, USA). The pulpal surface of each dentin specimen was placed on a standard FTIR sample holder with a 2.5-mm diameter opening and spectra were collected in triplicate from each treated dentin specimen between 800 and 2,000 cm^{-1} at 4 cm^{-1} resolution using 100 scans. Each obtained spectrum was then processed by smoothing, baseline correction, and normalization against the amide I peak using dedicated Spectra Manager CFR software. The effects of various antibiotic medicaments on collagen and apatite composition of surface dentin were evaluated using the mineral matrix ratio (ratio of integrated areas of phosphate v1 and v3 peaks to the amide I peak). The final ratio assigned for each dentin specimen represented the average of the ratios obtained from the three spectra (Fig. 5).

Scanning Electron Microscopy (SEM)

Two root cylinders from the microhardness experiment were randomly selected from each group for SEM analysis in order to observe any morphological changes in root canal dentin. Each selected root cylinder was sectioned longitudinally without touching the root canal surface. Then, each half of the root cylinder was irrigated with 5 mL of de-ionized water, sonicated in de-ionized water for 5 minutes, and desiccated for 48 hours. Specimens were sputter coated for 70 seconds with gold/palladium using a sputter coater and images were taken from the treated root canal surface area of the specimens with a scanning electron microscope (Ziess, Germany) in secondary electron imaging mode.

Results

All the analysis was done using SPSS version 18. A *p*-value of <0.05 was considered statistically significant. Comparison of values was done using paired t test and ANOVA with post-hoc Tukey's test. Table 1, Figures 6 and 7 shows that the posttreatment

Table 1: Microhardness measurements at 500 µm from pulp-dentin interface

	Pre		*Post*		*Percentage*	
Group	*Mean*	*SD*	*Mean*	SD	*Mean*	*SD*
1 mg/mL MTAP	57.25	4.58	51.88	7.92	12.95	20.52
1 gm/mL MTAP	47.57	4.87	45.38	4.16	5.69	15.06
1 mg/mL TAP	56.28	5.84	49.46	1.32	13.80	11.67
1 gm/mL TAP	59.41	6.13	41.33	9.45	51.76	42.89
Distilled water	64.25	11.74	56.30	1.42	14.30	21.68

SD: standard deviation

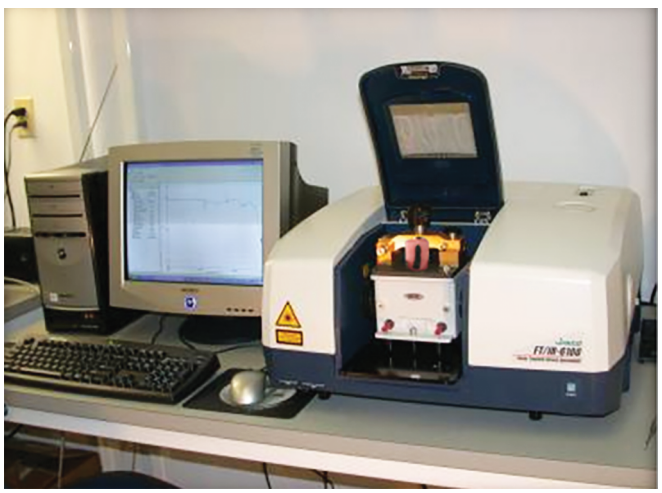


Fig. 5: ATR FTIR measurement

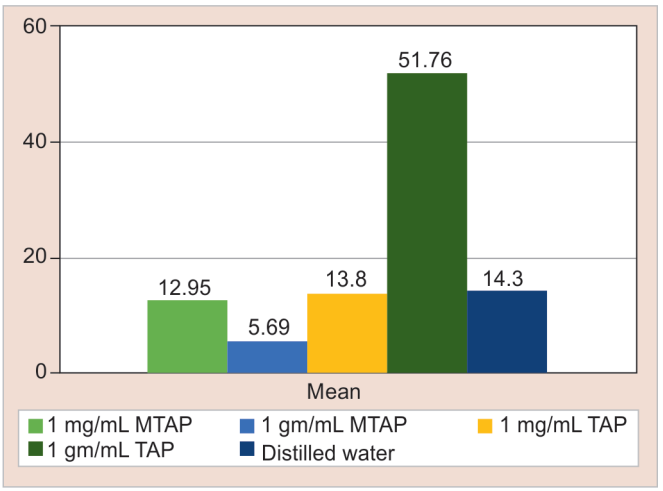


Fig. 7: Bar chart depicting percentage difference in microhardness measurements at 500 µm from pulp-dentin interface before and after treatment

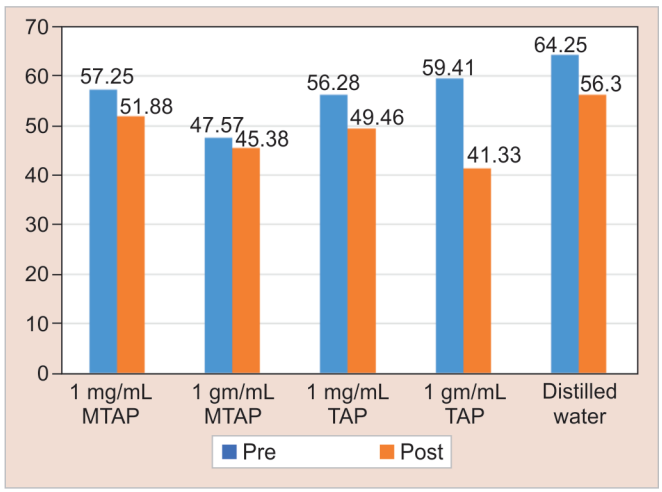


Fig. 6: Bar chart illustrating microhardness measurements at 500 µm from pulp-dentin interface before and after treatment

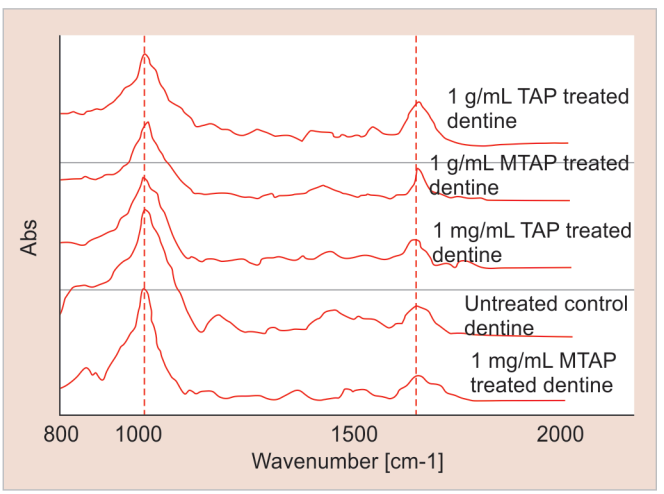
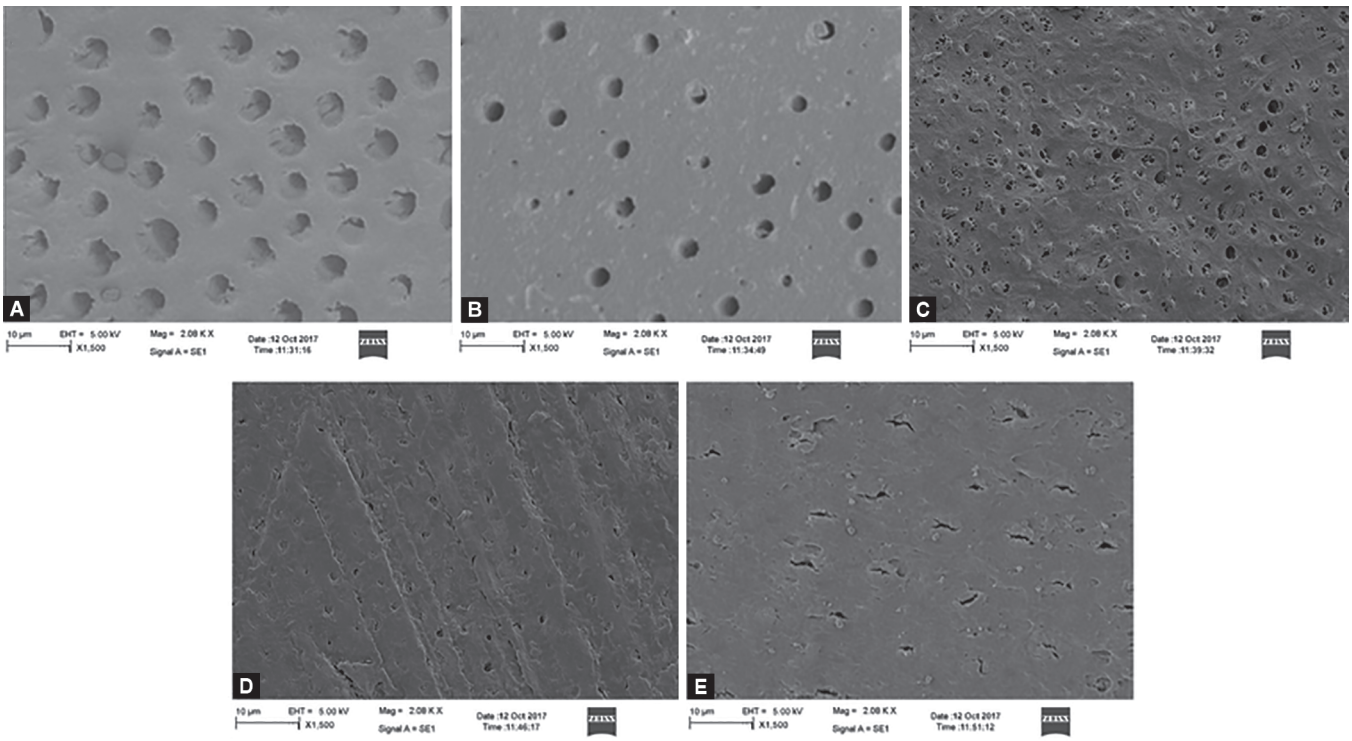


Fig. 8: Representative ATR spectrum of intact radicular dentin for 4 weeks



Figs 9: SEM Analysis

microhardness was significantly lower than the pretreatment microhardness for all treatment groups. The percentage reduction in microhardness was significantly higher for 1 g/mL TAP-treated dentin when compared with 1 g/mL MTAP-treated ($p = 0.0021$) and 1 mg/mL TAP- and MTAP-treated ($p < 0.0002$) dentin. In addition, the percentage reduction in microhardness was significantly higher for 1 g/mL MTAP when compared with 1 mg/mL TAP- ($p = 0.0078$) and MTAP-treated ($p < 0.0001$) roots.

Figure 8: ATR-FTIR Spectroscopy measurements

Representative spectra obtained from untreated control dentin and various dentin treatments shows that the phosphate/amide I ratio in the 1 g/mL TAP-treated group was significantly lower than in all other treatment groups and in untreated control dentin ($p < 0.0001$). Furthermore phosphate/amide I was comparatively high in ratios of 1 mg/mL.

SEM Analysis (Fig. 9)

SEM images showed the presence of a smear layer in instrumented root canals of untreated control dentin, 1 mg/mL MTAP-treated dentin (Figs 9 B and E). No visible smear layer was observed in other treatment In addition, the native structure of collagen fibrils was identified under higher magnification in root canals treated with 1 mg/mL TAP, 1 g/mL MTAP, and 1 g/mL TAP (Figs 9 A and B).

Discussion

Tissue engineering is the restoration of lost tissue function through the delivery of synthetic or natural tissue constructs in the laboratory. It is also developing new frontiers in dentistry known as regenerative dentistry.[8] Although diagnostic ability to differentiate the vital from a necrotic pulp is good, differentiating between reversibly and irreversibly inflamed pulps remains an educated guess at best. But we do know however the younger the pulp, the better is its repair potential.[9]

But the immature root with a necrotic pulp and apical periodontitis presents multiple challenges to a successful treatment, the standard root canal protocol is not sufficient to disinfect the canal simply with the aggressive use of endodontic files, once the microbial phase of the treatment is complete, filling the root canal is difficult because the open apex provides no barrier for stopping the root filling material before impinging on the periodontal tissues and even when the challenges described earlier are overcome, the roots of these teeth are thin with a higher susceptibility to fracture.[10] On the contrary there are studies shows significant amount of root canal walls remained untouched stating therefore that mechanical instrumentation alone cannot result in a disinfected canal.[11] Hence irrigation of root canals with antimicrobial solution might not be sufficient to eliminate all micro-organisms from the root canal. *Enterococcus faecalis* has been detected in asymptomatics and persistent root canal infections.[5]

Biofilm bacteria are a community of microorganisms embedded in an extra cellular polysaccharide matrix and attached to the solid surface. Quorum-sensing is most active when the cell density is high, a situation that is encountered in a mature biofilm.[12]

Sedjley found bidirectional transfer of an erythromycin resistance determinant on the conjugative plasmid pAM81 between *Streptococcus gordonii* and *E. faecalis*. This indicates that horizontal exchange of antibiotic resistance can occur between different bacterial species in the root canals.[13] Elimination of microbial infection from root canal space with aid of antimicrobial agents is considered as a most important phase to achieve thorough disinfection of canals.[14]

Use of antibiotics in endodontics was first reported by Grossman in 1951 which was known as polyantibiotic pasteis

a mixture of penicillin, bacitracin, streptomycin, and caprylate sodium. Antibiotics are used in dentistry both systemically and topically. During systemic administration of antibiotics, negligible concentrations reach the root canal, whereas during the local administration of antibiotics higher concentrations can be used as intracanal medicaments to decrease systemic consequences and complications. Combination of irrigants or medicaments decreases the development of resistant bacterial strains which also produce a synergistic effect whose antimicrobial action last longer and also sustains a release of medicament.[15]

Penicillin was used for targeting against gram-positive organisms, bacitracin penicillin resistant strains, streptomycin for gram negative organisms, and caprylate sodium to target yeasts. Recently TAPs has been used for lesion sterilization.[16]

Hoshino et al. studied the antibacterial effect of ciprofloxacin, metronidazole, and minocycline mixture and has to be found to be effective when nonsurgical management of teeth with nonvital pulp.[17]

Metronidazole was chosen initially for its wide bacterial spectrum against anaerobes commonly found in the oral sites. However, even high concentrates of metronidazole could not fully eliminate bacteria in the lesions therefore two additional antibacterial drugs, ciprofloxacin and minocycline where added in an effort to fully eradicate the bacteria. According to Takushige minocycline has been shown to cause black staining and discoloration of the tooth and gums so he suggested an alternative antibiotic that is, clindamycin due to its effectiveness against streptococci and anaerobes.[18]

The antibacterial effect of various concentrations of antibiotics on biofilm of *E. faecalis* and its action on survival of human dental pulp stem cells concluded that the different concentrations of intracanal medicaments has a critical role in the disinfection of the canal and to reduce its adverse effects on stem cells during regeneration procedure.[19]

SCAPs are the source of primary odontoblasts that are responsible for continuation of root development and as a result of proximity to the periodontal blood supply, can survive pulp necrosis even in the presence of periradicular infections. Cell homing is the use of chemotactic factors like stromal cell derived factor SDF-1 that can induce migration of stem cells from apex into the root canal.[20,21]

Current regenerative endodontic procedures aim is to utilize growth factors found in platelets and dentin which contains a number of bioactive molecules that, when released play an important role in regenerative procedures. The delivery of growth factors is one of the main challenges we are facing now. The direct application of these growth factors often results in temporary release which results in a limited half-life and unstable release of growth factors providing unfavorable conditions for new tissue formation.[22]

The use of medicaments in endodontic regeneration on root fracture and microhardness of radicular dentin found the significance of duration of application and concluded that 3 week application of TAP, DAP, and $Ca(OH)_2$ significantly reduced the resistance to fracture of extracted teeth as compared to 1 week application.[23]

There is paucity of literature regarding the duration of application of these medicaments and its effects on microhardness of dentin. Hence, purpose of the present study was to determine the variation in microhardness and chemical structure of the above mentioned medicaments on radicular dentin after 4 weeks of medicament application.

Both $Ca(OH)_2$ and 0.1 to 1 mg/mL of TAP was recommended to avoid the toxic effects of currently used 1000 mg/mL of TAP on stem cells of the apical papilla.[24]The low concentrations of TAP were also found to be effective against endodontic pathogens according to previous studies. However, the recommended low concentrations of TAP are usually in a liquid form, which makes its use challenging. Therefore, TAP used in the current study was mixed with a methyl cellulose vehicle to create a clinically applicable intracanal medicament.

In the present study, the concentrations of TAP and MTAP currently used in endodontic regeneration at 1 gm/mL concentration caused significant reductions in microhardness at 500 µm depth. This could be explained by the demineralizing effects of these acidic antibiotic mixtures when used at higher concentrations. The present study also showed that TAP caused significantly higher reduction in microhardness at 500 µm from the pulp-dentin interface when compared to MTAP treatment at the same concentration. The result of the present study was in accordance to Prather and Nerness concluded that 1 gm/mL concentration of TAP caused significant higher surface loss and surface roughness when compared to 1 mg/mL of TAP. This is due to the minocycline present in TAP to chelate calcium and demineralize radicular dentin.[24,25]

One of the limitations of studying the chemical structure of dentin with FTIR approach is that the depth of penetration of infrared radiation in the FTIR technique is limited to a few microns. Therefore, the spectral data and the phosphate/amide I ratios reported in our study may only represent the net chemical change of superficial dentin after various treatment protocols rather than the whole chemical change across the total thickness of the dentin specimens. The overall high correlation between phosphate/amide I ratios and microhardness values of dentin implies that the reported reduction in dentin microhardness among all treatment groups could be explained by the superficial demineralization effect following the ER protocols (reduction in phosphate/amide I ratios). These findings generally agree with the studies by Yassen, which also suggested that dentin microhardness depends on mineral concentration. Furthermore, dentin treated with TAP caused significant reduction in phosphate/ amide I ratio compared to all other groups. This could be explained by the strong acidic nature of TAP (pH = 2.9).[26]

TAP treated dentin showed advanced superficial erosion in SEM analyses which might be the consequences of demineralization process accelerated by acidic TAP resulting in the significant reduction in microhardness of TAP treated dentin.

Collectively, the present study showed that the two concentrations of TAP and MTAP cause significant reductions in the microhardness of root when compared to untreated controls. However, 1 mg/mL methylcellulose-based TAP and MTAP caused significantly less reduction in microhardness when compared to 1 gm/mL TAP and MTAP. ATR-FTIR measurements indicated the same when compared to other treatment groups and untreated control dentin.

Summary and Conclusion

The discovery and understanding of pulp stem cells provide us a better insight into the healing potential of the immature teeth. In the same time, clinical and basic research is urgently needed to provide information on the success rate of this treatment modality. TAPs though was proved efficient by various studies had shown detrimental effects on the microhardness and chemical

structural of radicular dentin. It also had shown discoloration of the teeth. Hence a modified TAP substituting minocycline for clindamycin was used.

The result of the present study concludes that MTAP at 1 mg/mL concentration showed the least changes in microhardness and chemical structure followed by TAP at 1 mg/mL. Both TAP and MTAP at 1 gm/mL significantly reduced the microhardness and showed decrease in calcium and phosphate levels of the treated dentin.

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