Endodontic Microbiology

The field of Endodontics is the clinical discipline that deals with management of the pulp and periapical diseases. Bacteria may enter the root canal system directly via caries lesions or via pulp exposure following trauma. The root canal system is in open communication with the periapical tissues through the apical foramen. Bacterial metabolites and toxic products arising from bacteria present within the dentinal tubules may be responsible for persistent root canal infection (12, 13). The bacteria which diffuse into the periapical tissues, results in inflammatory disease, which in turn leads to apical periodontitis and abscess. Endodontic infections are basically caused by a mixed community whose diversity may vary according to the type of infection and clinical situation of the disease (100). Nair PNR (27), Riccuci D et al (101) and Molen et al (102) have studied the endodontic microbiota in untreated (primary infection) and treated (persistent/secondary infection) teeth. They have demonstrated that the dominant pattern of bacterial colonization of the root canal wall resembled that of typical endodontic biofilms by using Light microscope, Electron microscope and Scanning electron microscope.
Biofilm Formation

The formation of a microbial endodontic biofilm can be described as a dynamic process which, involves following steps: i) Bacterial cells get attached to the selected abiotic or biotic surface. Bacteria usually adhere to a conditioning film typically composed of organic molecules (e.g. nutrients, salivary proteins, large macromolecules) that can promote the adherence of bacteria to the surface. The first step of attachment between bacteria and surface is mediated through weak and reversible van der Waals interactions, which can lead to stronger adhesion receptor mediated attachment (24, 103). The bacterial structures like fimbriae, flagella and LPS help in irreversible interactions. ii) Development of micro-colonies is promoted by the growth and division of the first attached cells. The micro-colonies begin to grow faster. iii) When multiple layers of bacterial cells get adhered to the existing surface, the third step of formation is obtained. At this stage, mature biofilm is formed. It is characterized by the presence of macro-colonies surrounded by water channels that help in distribution of nutrients and signaling molecules (24, 104).

*E. faecalis*

*Enterococci* are gram positive cocci that can occur singly, in pairs or as short chains. They are facultative anaerobes; they have the ability to grow in the presence or absence of oxygen (32). It is commonly found in monoinfections, but it is also observed in mixed
infections of the root canal system (36, 105). *E. faecalis* is an opportunistic pathogen and one of the leading causes of nosocomial infections (106). *E. faecalis* is associated with different forms of periradicular disease including primary endodontic infections and persistent infections (33). It has the ability of forming a biofilm structure on root canal walls. *E. faecalis* is a persistent organism that makes a small proportion of the flora in untreated root canals; it plays a major role in the aetiology of persistent periradicular lesions after root canal treatment. It is commonly found in a high percentage of endodontic failures and it has ability to survive in the root canal as a single organism or as a major component of the flora (4). Various studies have shown that *E. faecalis* has been identified in cases of failed endodontic treatment. It has also been proven to be difficult to eradicate during retreatment; hence the failure rate of retreatment is higher when *E. faecalis* has been recovered from the root canal at the time of root filling (4, 6, 106). In the present study, *E. faecalis* was selected as the test organism.

**Role of Instrumentation against Biofilm**

The cleaning and shaping plays a key role in the success of root canal treatment. The objective of root canal instrumentation is to: shape the root canals without altering the original anatomy, clean the root canal system by creating the space for irrigants to flow till the root apex and allow the root filling material to be inside the canal (107). Instrumentation helps disinfectants by
disturbing or detaching the biofilm from the root canal surfaces and it removes a layer of infected dentin, which consists of microbes. Earlier, endodontic instruments were made up of stainless steel; later Ni Ti instruments came into vogue. Dalton et al (108) and Carver et al (109) compared the efficacy of stainless steel hand instruments and rotary instrument to reduce the bacteria inside the canal. They proved that, there was no difference between hand and rotary instruments. The complex anatomy of root canals like isthmuses, C shaped canals and fins creates an environment, that is a challenge to instrument to clean the root canal (116). Peters OA et al (38) compared the effects of four preparation techniques on canal volume and surface area using micro CT. About 35% or more of the canals surface area were unchanged even after use of instrumentation technique. Hence, instrumentation was found to be not enough to completely eradicate the bacteria present inside the canal. Disinfectants were also reported to play a key role in the success of endodontic treatment.

**Challenges faced in root canal disinfection**

**Anatomical complexity of root canals:**

The root canal morphology of teeth is often extremely complex and highly variable. The accessory canals, lateral canals, apical ramifications and fins contribute together to the complexities in root canal anatomy. The root canal lumen will communicate with another root canal lumen via an isthmus (111). These anatomies will
Discussion

be untouched by root canal instruments (38). In flat root canals, rotary instruments often fail to adequately clean and shape the canal, leaving ‘‘fins’’ that may have not been prepared (112, 113). Bacteria present in those areas will tend to grow even after endodontic treatment is completed. The microbes present in apical ramifications, isthumes and accessory canals are usually the common reasons for endodontic failure. These areas were instrument untouched, which might have definitely influenced the disinfection of root canals (114).

Structure of dentin:

The dentin consists of s shaped dentinal tubules. Dentinal tubules contain odontoblast processes, nerve fibers, and unmineralized collagen fibrils. The number of dentinal tubules per mm² varies from 15,000 at the dentino-enamel junction to 45,000 at the pulp. Dentinal tubule is in funnel shaped, diameter will be larger (2.5 µm diameter) towards the pulp, whereas smaller in diameter (0.9 µm diameter) towards dentino enamel or dentino cemental junction. Diameter of the tubule is more compared to the size of $E. \textit{faecalis}$ (0.8–1 µm). So, bacteria can easily penetrate into the dentinal tubules. The inability of antimicrobials to penetrate the infected dentinal tubules results in the survival of the bacteria within the root canals. A persistent intraradicular infection caused by microorganisms located within dentinal tubules has been the most common reason for endodontic disease recurrence (50). George
S et al (36) have highlighted the effect of different growth conditions on the characteristics of *E. faecalis* biofilm formation on root canal and depth of penetration of *E. faecalis* into dentinal tubules. Samples were experimented under nutrient-rich, nutrient-deprived, aerobic and anaerobic conditions for a period of 21 days. Scanning Electron Microscopy with Energy Dispersive X-ray microanalysis, Laser Confocal Scanning Microscopy and Light microscopic examinations have been done. The depth of bacterial penetration was significantly greater in nutrient-rich condition. They have proved that bacteria might penetrate into the dentinal tubule to a maximum of 1483.33 µm (36).

*Lacunae in existing Intra canal medicament:*

Persistent endodontic infection might be attributed to the retention of microorganisms in dentinal tubules. Intracanal medicaments can help in reducing the remaining bacteria remaining even after chemomechanical instrumentation and can provide an environment conducive to periapical tissue repair. The intracanal medicament placed inside the canal, i) has to reach the inaccessible areas like isthmuses, fins, accessory canals and lateral canals ii) has to penetrate deeper into dentinal tubules to eradicate the *E. faecalis* which will be 1400 µm deep. Nanotechnology shows promise as an alternative treatment strategy to overcome the above mentioned lacunae. The word “nano” is used to indicate one billionth of a meter or $10^{-9}$. The use of nanoparticles is gaining
impetus in the present century as they possess defined chemical, optical and mechanical properties. Historically, silver has been used for the treatment of chronic wounds and burns. Various studies have proved the antibacterial effect of AgNps against many organisms (115, 116). Hence, the present study aimed to investigate the ability of the AgNps as an intracanal medicament for root canal disinfection.

**Synthesizing and Characterizing AgNps**

Synthesis of nanomaterials with the desired quality and properties is one of the key issues in current nanotechnology. In the current investigation, the AgNps were prepared by reducing the silver ions in aqueous silver nitrate solution with sodium borohydride and were stabilised with CTAB (47). The golden yellow color confirmed the presence of AgNps. The presence of golden yellow colour was due to the surface of Plasmon resonance of AgNps. This was further confirmed by using UV Vis spectroscopy and HRTEM. UV–Vis spectroscopy is one of the most widely used techniques for optical studies for the characterization of AgNps. The spectra clearly showed the increase in intensity of silver nitrate solution with time, indicating the formation of increased number of AgNps in the solution. In the current investigation, UV visible spectra showed strong absorbance peak located at 405 nm, which confirmed the presence of AgNps (Fig 2). The intensity of the surface Plasmon resonance peaks were varied, which was observed
from the spectra. This was due to the various concentration (4 mM, 6 mM, 8 mM and 10 mM) of AgNps used in each dilution. The spectra was observed after rediluting 10 times to confirm the stability of AgNps. The absorption spectrum after dilution was similar to that before diluting the AgNps. By this we confirmed that the AgNps were not further agglomerated or dimerized.

**HRTEM** is an imaging mode of the TEM that helps in direct imaging of nanoparticles. The samples were systematically analysed by using HRTEM to examine the real particle size and morphology of silver nano particles. HRTEM picture was recorded from silver nanoparticles coated on the carbon coated copper grid. In the present investigation, AgNps were synthesized in the size range between 25 - 40 nm. Its spherical morphology was confirmed by using HRTEM (47) (fig 3).

**Agar diffusion method**

The agar diffusion method is the most commonly used method and provides better flexibility and is cost-effective. In Agar diffusion method, the effectiveness of an antibacterial compound against bacteria is measured in a grown culture. In this method, the diameter of the microbial inhibition zone depends on the solubility and infusibility of the test material placed. In the present study the antimicrobial potential of different concentrations (4 mM, 6 mM, 8 mM, 10 mM and 100 mM) of AgNps was evaluated according to
their zone of inhibition against *E. faecalis* and the results (zone of inhibition in mm) were compared with the anti microbial activity of the CHX and Ampicillin. The results revealed that all the compounds were potent antimicrobial agents against *E. faecalis*. Table 1 showed the zone of inhibition of different concentration of AgNPs, CHX and ampicillin. The zone of inhibition for CHX and Ampicillin against *E. faecalis* was 21.5 mm and 22.5 mm respectively. No zone of inhibition was observed in negative control saline group. The mean diameter of zone of inhibition ranging from 13.66 to 18.66 mm was recorded in various concentrations of AgNps against *E. faecalis* (Table 1). From this preliminary result, we proved that different concentrations (4 mM, 6 mM, 8 mM, 10 mM and 100 mM) of AgNps possessed antibacterial efficacy against *E. faecalis*.

**Serial Dilution method**

Minimum Inhibitory Concentration (MIC) is defined as the highest dilution or least concentration of the medicament that inhibits the growth of organisms. Determination of MIC is important in diagnostic laboratories, as it helps to confirm the resistance of the micro-organism to an antimicrobial agent and it monitors the activity of new antimicrobial agents. The major disadvantages of disk diffusion method include lack of interpretive criteria for some organisms and inability to provide precise data regarding the level of an organism’s resistance or susceptibility.
Thus, methods based on serial dilutions have been developed to overcome these problems. The advantage of this method compared with other methods is that it is cost-effective, feasible and uses basic instruments. In the present study, 96 well plate was used to determine the MIC for AgNps. Figure 4 shows the MIC value for 4 mM, 6 mM, 8 mM, 10 mM of AgNps, CHX and Amphicillin against *E. faecalis*. Loading various different concentrations (4 mM (0.0001 moles of Ag), 6 mM (0.00015 moles of Ag), 8 mM (0.0002 moles of Ag) and 10 mM (0.00025 moles of Ag) of AgNPs in different rows of a 96-well plate allowed the use of single plates for evaluating the sensitivity of different concentrations at the same time. The MIC values for various concentrations of AgNps, CHX and amphicillin ranged between 17 µg to 0.25 µg. Minimum inhibitory concentration of AgNps was 1.05 µg for *E. faecalis*. So, 1.05 µg AgNps (0.0002 moles of Ag) was standardized to check the antibacterial efficacy in tooth models.

**Colony forming unit**

The *in vitro* models developed by Haapasalo & Ørstavik (13) are used to assess the antibacterial abilities of medicaments in the disinfection of dentinal tubules. Lynne et al. (17) modified this model to include quantitative analysis of bacteria in the dentinal tubules to define a percentage of reduction in CFU in infected dentin before and after the application of intracanal medicaments. The model has clear limitations because it does not reflect the
situation in apical dentin, which is mostly sclerotic (118). The CFU method used to count the bacterial cells which have ability to give rise to new colonies. Under microscope, the formed colonies will be counted. Basrani et al. (119) observed that 2% CHX gel was a better antimicrobial when compared to 0.2% CHX gel or calcium hydroxide mixed with 0.2% chlorhexidine. Hence, 2% CHX was selected to compare the antibacterial potential of AgNps. In our previous study, the efficacy of different intracanal medicaments was compared at two different depths. Hence the same protocol was followed for current investigation. The CFU log reduction has been done for saline, CHX and AgNPs at 200 µm and 400 µm in the present study. From the results of the present study, log reduction of colony forming units at 200 µm for saline, CHX and AgNps have been observed as 7.57, 5.4 and 5.3 respectively. The mean log reduction of colony forming units at 400 µm for saline, CHX and AgNps were observed to be 7.5, 5.5 and 5.3 respectively (Table 2). Both CHX and AgNPs showed statistically significant difference compared to saline group. There was no difference in log reduction between 200 µm and 400 µm. On comparing the antibacterial efficacy of CHX and AgNps against *E. faecalis*, no difference was observed. This part of the investigation proved that AgNps synthesized and characterized by the authors had disinfection ability in the tooth model against *E. faecalis*. 
From the previous part of the study, we showed the efficacy of AgNps in tooth model. Further research question would be: i) For how many days, should AgNps be placed inside the root canals? ii) What will be the percentage difference between live and dead bacteria when treated with CHX and AgNps?

Microbiological sampling techniques can estimate the number of log reduction - colony-forming units of bacteria and the quantitative analysis of the dentin infection can be done. Further, polymerase chain reaction (PCR) technology could be used to detect culture-difficult species and uncultivable microorganisms. (120) However, PCR-based methods cannot distinguish between live or dead cells (121, 122). The histological sectioning of the infected tooth shows the bacteria in dentin, but it cannot give status of viable bacteria. A CLSM analysis is commonly used with vital staining techniques to determine the viability profile, architecture and spatial distribution in microbial biofilms (123). Nagayoshi et al (124) examined the live and dead oral microorganisms after treating with ozonated water; but the drawback would be the high background that originates from the out-of-focus planes and decreases the contrast. This makes it impossible to distinguish between live and dead cells, stained dentin and artifacts. For these reasons, conventional fluorescence microscopy is not the best method to visualize the bacterial distribution in dentinal tubules. In this regard, better methodologies for the identifying the live and
dead bacteria will be helpful to assess the quality of antibacterial agents. Zapata et al (64) have shown the ability of confocal laser scanning microscope to identify the live and dead *E. faecalis* in dentin. The viability of bacteria in infected dentin could be determined in situ in an effective way by CLSM. FDA/PI dyes and acridine orange dye are useful for this technique. After disinfection, the dentin specimens were stained with FDA dye which emitted green fluorescence in live cells and PI, which emitted red fluorescence in dead cells whose membranes were disrupted and bound to deoxyribonucleic acid (DNA). CLSM gives better image resolution, has the ability to eliminate scattered or out-of-focus light and shows individual bacterial cells inside dentinal tubules. Hence, CLSM was chosen to assess the viability of bacterial cells after treating with AgNps.

Percentage of live and dead *E. faecalis* after treating with saline, CHX and AgNps has been shown in Table 3 for 1 day and Table 4 for 3 days. At the end of 21 days, one sample was observed under CLSM and the presence of *E. faecalis* was confirmed inside the dentinal tubules. Figure 5 (A-E) shows the live and dead *E. faecalis*, CLSM images of Saline, CHX and AgNps. On day 1, percentage of dead bacteria for saline, CHX and AgNps was 7.15%, 64.54 and 75.96 % respectively. On day 3, percentage of dead bacteria for saline, CHX and AgNps was 6.95 %, 64.16 and 76.15 % respectively. Significant differences were observed within groups
(Saline, CHX and AgNps). From the results of the present study, comparing the percentage of dead bacteria present inside the dentinal tubules after treating with Saline, CHX and AgNps, Group III (AgNps) performed better followed by CHX and Saline. From the current investigation, it was shown that AgNps possessed better disinfecting potential compared to CHX. In Group III (AgNps), comparing the percentage of dead *E. faecalis* between day 1 (75.96 %) and day 3 (76.15 %), no statistical significant difference was observed. Hence, AgNps could be placed inside the root canal as intracanal medicament for 1 day (24 hrs). There was no need to place it for 3 days. Clinically, placing intracanal medicament for 1 day would be very useful to the clinician. From both the results, (Colony forming unit and Live/Dead bacterial cells), the present study revealed that AgNps had antibacterial efficacy against *E. faecalis* and helped in better root canal disinfection.

**Increasing the efficacy**

From the CLSM results, AgNps showed 24 % of remaining bacteria (live cells) present in the infected dentin. The next research question raised was: Was there any other way to further reduce the remaining bacteria (24 %) with AgNps. Hence we tried the following methods: i) replenishing ii) AgNps mixed with methyl cellulose iii) AgNps mixed with thermosensitive agent iii) AgNps mixed with thermosensitive agent followed by ultrasonic agitation.
Following are the details of the above mentioned methods:

i) Replenishing mode of application: AgNps mixed with methylcellulose, placed inside the canal. At the end of 24 hrs medicament (AgNps mixed with methylcellulose) was removed and irrigated with saline. Then, freshly mixed medicament (AgNps mixed with methylcellulose) was placed inside the canal and kept for another 24 hrs.

ii) AgNps mixed with methyl cellulose: Methyl cellulose has been used as a thickening agent. Various studies have been done to check the antibacterial efficacy of medicaments in tooth model where methylcellulose had been used as a thickening agent (63, 65).

iii) AgNps mixed with thermosensitive agent: Lutrol is poloxamer. Lutrol has thermosensitive property (sol-gel transition ability) - Ability to change from solution stage at low temperature to viscous gel at higher temperature. It can be used as thickening agent (78). Hence, AgNps mixed with lutrol was then injected into canal (solution state) so that it would shift to gel state later. It would remain inside the canal as a medicament.

iv) AgNps mixed with thermosensitive agent followed by ultrasonic agitation: AgNps mixed with lutrol, injected into canal, followed by the ultrasonic agitation. Later it would get converted into gel state. Table 5 shows the percentage of Live and Dead E faecalis for Replenishing with AgNps, AgNps with methyl cellulose, AgNps with thermosensitive gel, and AgNps with
thermosensitive gel followed by ultrasonic agitation. Statistically significant differences were observed between the groups. The percentage of dead cells was significantly increased in AgNps with thermosensitive gel followed by ultrasonic agitation (92.57 %) compared to Group I, Group II and Group III. Comparing Group III and Group IV, Group IV performed better and this might be attributed due to ultrasonic agitation. The ultrasonic activation, insertion of an ultrasonic tip into a canal filled with solution (AgNps and thermosensitive agent) was used to increase bacterial elimination. The passive ultrasonic activation enables the ultrasonic tip to oscillate freely in the root canal (79, 80, 81, 82). Under these conditions, acoustic streaming occurs and creates shear forces on the walls that can cause physical disruption of the bacterial cells (79, 80, 81, 82). Phaechamud et al (125) has studied the antibacterial efficacy of various concentrations of zinc oxide with thermosensitive gel against *Staphylococcus aureus, Escherichia coli* and *Candida albicans*. Zinc oxide gel was prepared by mixing it with lutrol (thermosensitive agent). They concluded that there was no zone of inhibition, in the Lutrol gel group without ZnO BP indicating that Lutrol had no antimicrobial activity. Shen et al (126) investigated whether the ultrasonic agitation increased the effect of CHX against biofilm. They showed that mechanical agitation was effective in enhancing the efficacy of the chlorhexidine products, whereas ultrasonics and
sonics alone did not influence bacterial viability in the biofilms. Hence in the present study, CLSM showed more dead cells. The probable reason might be the effect of AgNps while ultrasonic and thermosensitive agents had no influence.

**Mechanism behind the antibacterial efficacy of AgNps**

The bacterial cell wall plays a key role to protect the cell from mechanical damage. Especially in gram positive bacteria (*E. faecalis*), thickness of cell wall will be more (20 – 50 nm) and made up of peptidoglycan, which is attached to teichoic acids. SEM microscopy was used to evaluate the surface morphology of *E. faecalis* after treating with CHX and AgNps. Figure 7 (A) shows the presence of *E. faecalis* with long chain and well defined morphology. Figure 7 (B & C) shows the bacterial cells with significant changes and showed major damage, which was characterized by the formation of “pits” in their cell membrane. A similar effect was described by Klabunde et al (127) when *E. coli* bacteria were treated with highly reactive metal oxide nanoparticles. From our SEM experiment, we proved that AgNps altered the morphology of bacteria. *E. faecalis* attracted AgNps and this might be because: i) the cell wall of bacteria contain many sulphur compounds. AgNps have higher affinity to react with phosphorous and sulphur compounds ii) Electrostatic attraction: Nanosilver owing to its large surface area and positive charge, has been hypothesized to interact with the negatively charged cell wall.
(128). The AgNps somehow interact with “building elements” of the bacterial membrane, which leads to change in structure and degradation and finally leads to cell lysis (71).

FACS based membrane permeabilization of AgNps and CHX on *E. faecalis* cells was studied using the DNA binding dye, propidium iodide. PI is a membrane-impermeable red fluorophore dye. *E. faecalis* cells showed membrane damage when treated with both AgNps and CHX at their MIC for 15 min (Fig 8). The membrane damaged cells only allowed the PI to enter into the cells, which leads to cell lysis. The untreated cells showed no fluorescence. This could be the probable reason for better antibacterial mechanism of AgNps in the present study against *E. faecalis*. Results of the present study were in concurrence with the recent study reported by Wu et al (70). They have reported that duration of interaction between AgNps and bacteria was an important parameter to have antibiofilm efficacy of AgNps.

Few authors have proposed that the formation of ROS is suggested to be one of the bactericidal mechanisms of action of nanoparticles. The generated reactive oxygen species (ROS) lead to damage in intracellular macromolecules like DNA and proteins which in turn leads to cell lysis (46, 129, 130, 131). Hence, the ROS generation was examined when treated with AgNps. In order to determine if the mechanism of killing by AgNps and CHX was ROS
mediated, fluorescence based assay was used in combination with a well-known antioxidant ascorbic acid to monitor the generation of ROS in the *E. faecalis* after incubation with AgNps and CHX. Generation of ROS by medicaments was monitored by incubation of medicaments at their respective MIC with *E. faecalis* cells for 1h. The ROS generation was measured by flow cytometry. *E. faecalis* cells were cultured, harvested and treated with AgNps and CHX. Figure 9 shows the reactive oxygen species generated by A-untreated, B - H₂O₂, C-- AgNps and D – CHX. The amount of ROS generated was higher in H₂O₂ compared to AgNps and CHX. In the untreated group, no ROS was generated. No fluorescence was observed when the cells were incubated with both AgNps and CHX along with a well-known antioxidant ascorbic acid. From the results of the present study, it has been shown that reactive oxygen species was not generated and it cannot be considered as one of the mechanisms behind the AgNps.

**Hemolytic assay**

Hemolytic assay was used to determine the acute toxicity of AgNps on human red blood cells. In the present study, AgNps erythrocyte membrane interaction was evaluated in which the extent of disruption of erythrocyte membrane was a direct measure of AgNps toxicity. The mechanisms of disruption of red blood cells depends on the physical and chemical mature of the silver nanoparticles. Hemolysis is a fast process; the compound binds to
negatively charged surfaces of the erythrocyte membrane and can disturb the membrane integrity. The percentage of hemolysis of RBC when treated with AgNps and CHX has been showed in Figure 10. In the present study, red blood cells were treated with silver nanoparticles (8mM and 10mM), CHX, triton X 100 (positive control) and untreated (negative control). Triton X 100 showed higher hemolysis upto 100µm. From the results of the present study, CHX showed higher hemolysis compared to AgNps. As the concentration of AgNps is reduced, the hemolysis has been shown to be reduced. AgNps interacts with erythrocyte and causes abnormal membrane proteins and lipids that lead to destruction of erythrocyte integrity and hemolysis. Although the hemolysis of AgNps is about 40µm, it is less compared to CHX and hence it can be safely used as an intracanal medicament.

Microhardness

The other important parameter for any intracanal medicament is that it should not affect the micro hardness of the tooth. The relationship exists between microhardness and the structural changes of dentin associated with placement of intracanal medicament. Yoldas et al (88) evaluated the effect of a calcium hydroxide and glycerine mix and a calcium hydroxide and water mix on the micro hardness of human root dentin. They have shown that use of Ca(OH)2 combinations for intracanal medicament softens dentin. As micro hardness of dentin may vary considerably within
teeth (132), comparison of dentin hardness values were performed before and after treatment with medicament, within the same root dentin sample. This was performed to minimize the effect of structural variations of different teeth and to establish a reasonable baseline evaluation as suggested by Saleh & Ettman (133). Hence, we followed the same methodology, to assess the effect of AgNPs on micro hardness of dentin. Table 6 showed the micro hardness values of root dentin, before and after (1 day) placement of CHX and AgNps. The mean hardness values at baseline, CHX and AgNps for dentin were 50.63, 48.63 and 48.94. Comparing the baseline value and AgNps after 1 day, statistically no significant difference was observed. Hence from this investigation, it was shown that AgNps had no influence on reducing the microhardness of root dentin.