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In vitro Infection and Disinfection of Dentinal Tubules in Human Teeth

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DECLARATION

I Nichola Warren, declare that this dissertation entitled, *In vitro* Infection and Disinfection of Dentinal Tubules in Human Teeth, which I herewith submit to the University of Pretoria in partial fulfilment of the requirements for the degree MSc (Odont) is my own original work, and has never been submitted for any academic award to any other institution of higher learning.

_____ _____

SIGNATURE **DATE**

Great things are not done by impulse,

but by a series of small things

brought together.

- Vincent van Gogh

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ABSTRACT

Introduction: Bacteria are the most common pathogens responsible for pulpal necrosis and periapical disease conditions. The importance of eradicating bacteria and their endotoxic by-products by effective root canal irrigation has been highlighted in numerous studies. **Aim:** The aim of this *in vitro* study was to establish the efficacy of six different endodontic disinfection protocols in eradicating *Enterococcus faecalis* from single root canals of human teeth. **Materials and Methods:** Endodontic access cavities were prepared on 86, extracted, single rooted, human teeth. Root canal preparation was done using nickel titanium rotary files. Each tooth was sterilised, inoculated with *E. faecalis* and then randomly allocated to one of seven groups (n = 12). Irrigation solutions were either used alone, in combination with one another or in combination with photo-activated disinfection (PAD). The six disinfection protocols were 3% sodium hypochlorite solution (NaOCl), 2% chlorhexidine digluconate solution (CHX), Chlor-XTRA solution (6% NaOCl), 3% NaOCl combined with 2% CHX, 3% NaOCl followed by PAD and PAD applied alone. The first six groups were subjected to one of the disinfection protocols while the seventh group was irrigated with sterile water (control). The roots of the treated teeth were fractured longitudinally. The first half of each root was divided into three sections: coronal, middle and apical. Samples of the dentine from each of these sections were taken and plated onto brain heart infusion (BHI) agar plates. These were incubated at 37^0C for five days in facultative anaerobic conditions. The colony-forming units (cfu) were counted. The second half of each root was prepared for Scanning Electron Microscopy (SEM) according to standard methods. The Pairwise Wilcoxon Rank Sum test and the Kruskal-Wallis test were used to compare the cfu counts of the seven groups to one another. **Results:** Two per cent chlorhexidine digluconate (CHX), Chlor-XTRA, combination of sodium hypochlorite (NaOCl) and 2% CHX and the group where irrigation with 3% NaOCl was followed by Photo-activated disinfection (PAD) were able to completely eradicate *E. faecalis* from the coronal levels of the root canals. The group using a combination of 3% NaOCl and 2% CHX and the group where irrigation with 3% NaOCl was followed by PAD were able to completely eradicate *E. faecalis* from the middle levels of the root canals. None of the disinfection groups were able to completely eradicate the test organism from the apical levels of the root canals. The regimen of 3% NaOCl used in combination with 2% CHX was slightly more efficient in eradicating *E. faecalis* from

the root canals than the five other disinfection regimens that were tested. Erosion of the intertubular and peritubular dentine and precipitae formation were two incidental observations in some of the samples upon SEM examination.

LIST OF ABBREVIATIONS

g	-	gram/s
h	-	hour/s
L	-	Litre
mg	-	milligram/s
min	-	minute/s
ml	-	millilitre/s
mm	-	millimetre/s
mM	-	millimolar/s
n	-	number/s
nm	-	nanometre/s

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Overview of Endodontic Treatment

Endodontic pathosis of an infective nature can be largely attributed to microbial infection of the dental pulp, the associated periradicular space and the subsequent inflammatory immune response of the host to these microbes (Ingle and Bakland, 2002). Bacteria, viruses and fungi are all potential pathogens that can be implicated in infective endodontic disease; however, the majority of persistent endodontic infections are strongly associated with facultative anaerobic bacteria (Ingle and Bakland, 2002; Wang *et al*., 2012).

If one takes these factors into consideration, two phases of endodontic treatment can be identified: root canal preparation and obturation. Chemo-mechanical root canal preparation is carried out with the intent of getting rid of the residual vital and non-vital pulp tissue and the reducing and eradicating of pathogenic organisms (Berutti and Castellucci, 2005). After this phase has been initiated, an inter-visit medicament is usually placed inside the root canal in an attempt to further eliminate bacteria and discourage growth of microorganisms (Ingle and Bakland, 2002; Brändle *et al*., 2008). The ideal end result is complete resolution of the components of the inflammatory response and the restoring of periradicular tissues to a biologically healthy condition (Ingle and Bakland, 2002; Berutti and Castellucci, 2005). The prevention of microbial re-infection of the root canal via the apical and coronal routes is achieved by a three-dimensional obturation of the root canal and a sound coronal seal (Ray and Trope, 1995).

The bacteriolytic and bacteriostatic efficacy of solutions used for root canal irrigation during the preparatory stage of endodontic treatment is determined by several factors of which the following are generally considered to be the most significant (Ingle and Bakland, 2002; Zehnder, 2006; Sedigheh and Shokouhinejad, 2008):

- The configuration of the root(s) and the macro-anatomy of the associated root canal system;

- The anatomical complexity of the root canal system at a microscopic level;
- The degree of efficiency with which all pulp tissue and infective components are removed from the root canal;
- The level of efficiency with which the multi-faceted smear layer, formed during root canal preparation, is cleared away.

Biomechanical and chemical irrigation procedures are able to significantly reduce microorganisms within the root canal system (Cheung and Stock, 1993; Gomes, Lilley and Drucker, 1996; Lin, Lin and Rosenberg, 2007; Brändle *et al*., 2008). Irrigation during endodontic cleaning and shaping is crucial to the removal of intracanal components from the root canal system (van der Sluis *et al*., 2006). The chemical dissolution of these organic and inorganic components is equally important for the physical flushing action of irrigation solutions (Heling and Chandler, 1998; Siqueira *et al*., 2000; Peters *et al*., 2002; Ferraz *et al*., 2007; Vianna *et al*., 2007; Brändle *et al*., 2008). Another important function of irrigation solutions is the killing of micro-organisms (Safavi, Spångberg and Langeland, 1990). The aforementioned actions of endodontic irrigation solutions in combination with the cytotoxic effects to the bacterial cell ensure that the majority of microorganisms are eradicated (Spångberg, Engström and Langeland, 1973; Safavi, Spångberg and Langeland, 1990). Some bacteria are able to evade these processes and they remain dormant, sheltered within the dentinal tubules of the root canal wall. Removal of the dental pulp results in an environment void of organic tissue. The bacteria survive but they are deprived of nutrients and cannot proliferate (Weine, 1982). Should a source of nutrition become available via the coronal or the periodontal route, these dormant bacteria will be re-activated and will thrive once more (Ray and Trope, 1995; Heling *et al*., 2002; Kurtzman, 2006; Machtou, 2006).

1.2 Objectives of Endodontic Treatment

Endodontology is the speciality of dentistry that encompasses the prevention, diagnosis, and treatment of diseases of the dental pulp and the related pathology of the surrounding periradicular tissues (Berutti and Castellucci, 2005). In order to allow the diseased periradicular tissues time to re-establish a biologically healthy state (pulpal and periradicular), the irreversibly inflamed and/or infected dental pulp tissue and pathogens must be removed from the root canal system of the affected

tooth. To maintain this newly established healthy condition, three-dimensional endodontic obturation and a sound coronal seal must be accomplished so that recontamination via the apical and coronal routes can be prevented (Kurtzman, 2006; Torabinejad and Walton, 2009).

1.3 Microbial Infection of Root Canals

The root canals of all teeth presenting with signs of periradicular disease contain bacteria (Ramachandran Nair, 1987). The presence of these bacteria triggers an immune system response in the host. An inflammatory reaction ensues and results in the destruction of periodontal tissue, including a loss of periradicular bone, which manifests as a radiopaque periradicular lesion on a radiograph (Torabinejad and Walton, 2009). The size of the periradicular lesion is directly proportional to the diversity of the bacterial species and strains within the bacterial community and the bacterial load present in the root canal. The larger the periradicular lesion is, the greater the bacterial variance and the higher the total number of bacterial organisms within the root canal (Chugal, Clive and Spångberg, 2001).

1.3.1 Microbial Invasion of Dentinal Tubules

In general the structure of human dentine is tubular and for this reason it is permeable. Bacteria are somewhat impeded from passively entering dental tubule orifices by the hydrodynamic movement of fluid within the dentinal tubules of vital human teeth. The movement of the fluid is generally in a direction away from the pulp towards the relatively acidic environment on the outer surface of the tooth. This helps to slow down the ingress of bacteria into the dentinal tubules. In non-vital teeth, the pulp tissue is not intact and no live odontoblasts are present. For this reason not much tubular fluid exists within the dentinal tubules or any mechanism by which the fluid can move to offer resistance to bacterial ingress via this route (Nagaoka *et al.*, 1995; Torabinejad and Walton, 2009).

On the root surface of a tooth cementum prevents the dentinal tubules forming channels of communication between the periodontium and the pulp space. If cementum is damaged or absent in an area along the length of the root, this area can serve as a path for bacterial migration between these structures. In this way the

dental pulp can become infected by bacteria involved in disease of the periodontium and vice versa (Berutti and Castellucci, 2005).

1.3.2 Bacterial Adherence

Several studies have been undertaken to investigate the mechanisms that enable bacterial organisms to invade and adhere to dentine (Love, 1996; Love, McMillan and Jenkinson, 1997; Love, 2001; Love and Jenkinson, 2002). In one of these studies it was concluded that *E. faecalis* was so versatile that it was even able to invade dentinal tubules in the presence of human serum (Love, 2001). *Streptococcus* species can adhere to type I collagen when invading dentinal tubules (Love, McMillan and Jenkinson, 1997). The findings of a subsequent study by the same author not only confirmed this fact but also proved that the mechanism by which *E. Faecalis* adheres to the non-mineralised collagen within the dentinal tubules was much the same as for *Streptococci* (Love, 2001).

In the absence of a smear layer on the surface of the root canal wall, *E. faecalis* is able to enter dentinal tubules with ease. If a smear layer exists, *E. faecalis* cannot circumvent this physical barrier, which results in bacterial cells accumulating in vast numbers and adhering to the smear layer instead. In comparison to its adherence to smooth, intact dentine, bacterial adherence to rough, fractured dentine is superior. The strength of bacterial adherence to the dentinal walls is equal, irrespective of its vertical locality in the root canal; i.e. apical, middle or coronal region (Chivatxaranukul, Dashper and Messer, 2008).

1.3.3 Bacterial Colonies in Infected Root Canals

Endodontic flora includes a mixture of several different species of bacteria (Torabinejad and Walton, 2009). When light and electron microscopy was used to examine the root canals of teeth exhibiting periradicular pathology and that had been extracted without having undergone any endodontic treatment, all of these root canals contained bacterial colonies. These included the following species: cocci, rods, filamentous forms and spirochetes (Ramachandran Nair, 1987).

Microbes on the dentinal surface of the canal wall arrange themselves into a biofilm (Costerson, Stewart and Greenberg, 1999; Williamson, Cardon and Drake, 2009). A biofilm is a complex layer containing microbes, microbial extracellular by-products, and other organic and inorganic matter (originating from the pulp and dentine) in the root canal. This layer forms a matrix that attaches to the intertubular dentine of the root canal wall and to the dentinal tubules walls. Many bacterial cells are embedded within this matrix. In this way microorganisms can be shielded from: components of the body's immune system; endodontic irrigation solutions; and intracanal medicaments (Costerton et al., 1987; Costerson, Stewart and Greenberg, 1999).

1.4 *Enterococcus faecalis*

Enterococcus faecalis forms a part of the normal micro-flora of the human body but relatively low counts are found in the mouth (Kishen, George and Kumar, 2006). This bacterium is classified as a gram-positive, facultative, anaerobic microorganism. Facultative anaerobes, as opposed to aerobes, are able to survive in an environment that is void of oxygen (Torabinejad and Walton, 2009). The robust nature of *E. faecalis* is demonstrated by the fact that it is frequently implicated in failed endodontically treated teeth presenting with persistent periapical infection (Siqueira, 2001; Sundqvist and Figdor, 2003; Wang *et al*., 2012).

1.4.1 Chemical Eradication of *Enterococcus faecalis*

In addition to its ability to survive anaerobically, *E. faecalis* has been shown to endure chemo-mechanical endodontic procedures (Sundqvist and Figdor, 1998; Chavez de Paz *et al*., 2003; Sundqvist and Figdor, 2003). This microorganism is subsequently able to remain entombed in a dormant state within the root canal system for extensive periods of time (Sundqvist *et al.,* 1998). The survival ability of *E. faecalis* is greatly enhanced by its interaction with the root canal dentine which occurs in three stages. During the first stage, *E. faecalis* forms a biofilm on the dentine surface. In stage two, the bacteria dissolves mineral components of the dentine, and in stage three, carbonated-apatite is deposited in the biofilm. In this way *E. faecalis* is able to induce carbonated-apatite precipitation on a mature biofilm on the dentinal walls of the root canal. This plays an integral role in its

persistence after the endodontic treatment has been concluded (Kishen, George and Kumar, 2006).

Enterococcus faecalis has demonstrated resistance to several intracanal medicaments such as: calcium hydroxide (Gulabivala and Stock, 1995); hydrogen peroxide, acid and ethanol (Giard *et al*., 1996) and sodium hypochlorite (Laplace *et al*., 1997). It has been shown that *E. faecalis* is able to survive despite adverse conditions that render several other oral microorganisms non-viable (Giard *et al*., 1996; Hill *et al*., 1997; Hartke *et al*., 1998).

1.4.2 Persistent Endodontic Infection with *Enterococcus faecalis*

In accordance with several clinical studies *E. faecalis* is closely linked to unrelenting endodontic infections where previous endodontic treatment has been carried out but failed to resolve the associated periodontal pathology (Gomes, Lilley and Drucker, 1996; Molander *et al*., 1998; Sundqvist and Figdor, 1998; Gomes *et al*., 2004). The correlation between *E. faecalis* and endodontic treatment failure is clearly illustrated in the clinical study undertaken by Sundqvist and Figdor in 1998. The most common bacterium found to be present in the canals of 54 root canal filled teeth with persisting periapical lesions was *E. faecalis.* A subsequent investigation was carried out by Sundqvist and colleagues to demonstrate the effectiveness of non-surgical endodontic retreatment of failed root canal filled teeth with persisting periapical lesions. The five year follow-up success rate specific to the teeth that had been contaminated with *E. faecalis*, was lower (66%) as compared to an overall success rate of 73% for the group in its entirety (Sundqvist *et al*., 1998).

In another study, the presence of yeasts, gram-negative enteric rods and *Enterococcus* species in obturated root canals with chronic apical periodontitis was investigated. In 18% of cases, *Candida albicans* was present in the root canal. In all cases, bacteria were present in all the root canals, 50% of which were specifically contaminated with *E. faecalis* (Peciuliene *et al*., 2001).

1.5 Mechanical Debridement: Cleaning and Shaping

Mechanical cleaning in endodontics involves the physical removal of the pulp tissue, irritants and infected dentine from the root canal wall. Elimination of these components is imperative for endodontic treatment to be a success (Sjögren *et al.*, 1997). Endodontic shaping entails a methodical enlargement of the diameter of the root canal in a smooth, tapered fashion, while attempting to maintain the original spatial position of this canal in relation to the root as far as possible. A properly shaped root canal greatly improves the chances for success of root canal treatment (Patel and Barnes, 2011). Shaping is imperative for endodontic treatment to be a success for the following two reasons (Torabinejad and Walton, 2009):

- Increasing the diameter of the root canal facilitates the introduction of the irrigation needle. The irrigation solution can then be dispensed deep into the root canal so as to facilitate proper chemical cleaning while a passive irrigation technique is still employed.
- The enlarged diameter and coronal flare create space for the operator to introduce materials and instruments into the canal during the obturation procedure.

A smooth, tapered root canal wall with a conical shape, essentially identical to that of the corresponding master gutta percha (GP) cone should be the end result to well executed endodontic shaping. This allows the master GP cone to slide unhindered in the prepared canal to the predetermined working length. Should there be a need for lateral compaction, a finger spreader and the corresponding accessory GP points can also slide along the root canal wall without difficulty. This factor not only significantly reduces the time and effort needed for the obturation process but it also reduces the likelihood of voids being created in or alongside the master GP cone by the lateral spreader (Patel and Barnes, 2011).

1.5.1 Hand Instrumentation

Endodontic hand files are made of nickel titanium (NiTi) or stainless steel. The so-called "K-files" are produced by milling a round stainless steel wire so as the resulting shape in cross section is a uniform diamond shape along the length of the instrument

and a uniform apical taper of two per cent. This wire is then twisted by machine in an anti-clockwise direction so as to render a file with cutting blades in a spiral arrangement (Torabinejad and Walton, 2009).

There are two techniques that can be applied when using hand files. The operator can either employ a filing technique that is a simple rasping action applying pressure against the sides of the canal or a cutting action known as reaming. Reaming is achieved by inserting the file into the canal, twisting it in a clockwise direction until the file's blades engage the first layer of dentine of the root canal wall and then pulling out the file. Upon withdrawal of the file this dentine is removed (Torabinejad and Walton, 2009).

1.5.2 Rotary Instrumentation

The first article exploring the potential application of nickel-titanium (NiTi) in endodontics was published in 1988 (Walia, Brantley and Gerstein, 1988). Nickel-titanium is extremely elastic, being able to return to its original shape after exposure to a stress of up to 10%, whereas stainless steel undergoes deformation and retains permanent change in shape if exposed to stress of only one per cent. This property renders NiTi endodontic files superior to stainless steel files in that they are far more flexible, have the ability to replicate the natural curvature of the canal during preparation more precisely and are far less susceptible to separation (fracture) during use (Walia Brantley and Gerstein, 1988; Patel and Barnes, 2011).

1.6 Dentinal Smear Layer

Mechanical root canal cleaning and shaping, particularly the use of rotary files, leads to the deposition of a loose, amorphous, irregular deposit on the dentinal walls of the root canal (Peters and Barbakow, 2000). This deposit, referred to as a "smear layer", occludes the entrances of the dentinal tubules (McComb and Smith, 1975; Peters and Barbakow, 2000). A smear layer is formed regardless of whether the file is made of stainless steel or NiTi and regardless of the filing technique used (Torabinejad and Walton, 2009).

The smear layer is significant to all aspects of endodontic treatment. It acts as a physical barrier that hampers the penetration of endodontic irrigation solutions and intracanal medicaments into both the dentinal tubules of the root canal and the micro-anatomical portions (lateral canals, fins and deltas) of the root canal system. The smear layer separates the dentine of the root canal wall from the endodontic sealer, preventing proper adaptation of the root canal sealer to the prepared surface of the root canal wall. Subsequently the gutta percha (GP) is fixed to the smear layer instead of directly to the dentine and the resulting seal is suboptimal (Torabinejad *et al*., 2002).

1.6.1 Composition of the Dentinal Smear Layer

This smear layer has both organic and inorganic constituents. The organic substances include: fragmented processes of odontoblasts, collagen fibrils, inflammatory cells, micro-organisms and vital and necrotic dental pulp tissue. The inorganic component can include: intertubular dentine, calcified matter from pulp stones and denticles, remnants of restorative materials and intracanal medicaments (McComb and Smith, 1975; Torabinejad and Walton, 2009).

1.6.2 The Need for Removal of the Dentinal Smear Layer

The smear layer is a physical barrier that blocks the penetration of the dentinal tubules by endodontic irrigants. In this way the irrigation solution is unable to carry out its intended actions beyond the limit of this barrier. The most important actions are flushing the area to remove debris, killing bacteria and dissolving organic or inorganic matter (Ørstavic and Haapasalo, 1990; Torabinejad *et al*., 2002).

The smear layer also compromises the integrity of the endodontic obturation. This barrier at the dentine-sealer interface prevents close adaptation of the endodontic sealer to the prepared root canal surface and restricts its penetration of the dentinal tubules. This results in a lack of adhesion of the obturation material directly to the root canal surface (Pashley *et al.*, 1984; Gutmann, 1992). Other than the dormant bacteria themselves, the organic components of the smear layer are a source of nutrients for these latent organisms. In areas where these two organic components come into contact, the bacteria are able to make use of this nutritional source and

proliferate (Berutti and Castellucci, 2005). In time, the smear layer is liable to disintegrate, leaving voids alongside the obturation material. Such voids are potential weak spots whereby bacteria can infiltrate the root canal and can lead to failure of the endodontic treatment (Delivanis *et al.*, 1983). Even if the smear layer is free of bacteria, it has been shown that 70% of teeth with an intact smear layer can be penetrated by bacteria as opposed to only 30% of teeth that have had the smear layer removed (Kurtzman, 2006).

1.6.3 Methods of Removal of the Dentinal Smear Layer

Removing the smear layer is accomplished by rinsing the root canal with NaOCl to dissolve and flush out the organic remnants, followed by rinsing with a liquid chelating agent to dissolve inorganic components (Baumgartner and Mader, 1987).

Ethylene diamine tetra-acetic acid (EDTA) is a chelating agent that is widely used in endodontics and is commercially available in two forms for dental treatment: liquid and paste. In endodontic treatment it is used in concentrations of between 15% and 17% (Hülsmann, Heckendorff and Lennon, 2003).

1.7 Endodontic Irrigants

Root canals have complex micro-anatomy, making it impossible to reach the entire root canal system by mechanical means of endodontic preparation alone (Spångberg, Engström and Langeland, 1973; Safavi, Spångberg and Langeland, 1990). In order to access these areas, various solutions are used as endodontic irrigants for rinsing the root canal system. Endodontic irrigants must be contained within the limits of the root canal. Many irrigating solutions are corrosive and tissue-toxic, the introduction of which into the periradicular space can lead to post-operative complications (Weine, 1982).

The most important properties of an endodontic irrigant include: microbial killing and prevention of microbial growth; the ability to dissolve organic matter (protein and necrotic tissues); lubrication of endodontic instruments, and low toxicity or irritation to the periradicular tissues, intraoral and extraoral tissues (Safavi, Spångberg and Langeland, 1990; Berutti and Castellucci, 2005). The pursuit of new irrigating solutions

that possess all of these is ongoing. Experimental irrigants have included hydrogen peroxide (Heling and Chandler, 1998), castor oil (Leonardo *et al*., 2001); "BioPure MTAD" (Davis, Maki and Bahcall, 2007) and morinda citrifolia juice (Murray *et al*., 2008).

At present, there is no single endodontic irrigant that possesses the full spectrum of desired properties (Torabinejad and Walton, 2009). A variety of different solutions and irrigation regimens, each one with its own unique combination of properties, have been tested in the quest to discover the irrigation regimen that yields the best results (Bystrom and Sundqvist, 1983; Bystrom, Claeson and Sundqvist, 1985; Baumgartner and Cuenin, 1992; Almyroudi *et al*., 2002; Berber *et al*., 2006; Edgar, Marshall and Baumgartner, 2006).

1.7.1 Sodium Hypochlorite

Sodium hypochlorite (NaOCl) is a good solvent of organic matter, with an alkaline pH varying between 11 and 12 (Berutti and Castellucci, 2005). Worldwide it is the most popular choice of endodontic irrigation solution, used in concentrations between 0.25% and 5.25% (Pataky *et al*., 2002; Torabinejad and Walton, 2009). Its popularity may be explained by the free availability of this very cost-effective solution as a household detergent and the high number of criteria considered as properties of an ideal irrigant, with which NaOCl complies (Pataky *et al*., 2002; Berutti and Castellucci, 2005).

Sodium hypochlorite's low surface tension and liquid state facilitate the effective flushing out of the contents of the root canal (Siqueira *et al*., 2000). Chemically it is capable of dissolving both vital and necrotic organic matter very quickly and efficiently (Goldman *et al*., 1982; Berutti and Castellucci, 2005; Stojicic et al., 2010). Free chlorine ions break down the proteins of the organic-tissue component of the dental pulp into amino acids and bacterial cell membranes (Torabinejad and Walton, 2009).

The primary constituent of bacteria is largely organic in nature, and NaOCl is therefore highly effective in destroying bacterial cells with which it makes contact in the root canal system (Goldman *et al*., 1982). A considerable number of bacterial

cells are eliminated from the main root canal via two sorts of mechanical force: the physical action of endodontic files and the flushing action of irrigation solutions. The rate of bacterial elimination is greatly improved when NaOCl is used as the endodontic irrigant (Siqueira *et al.*, 2000).

Sodium hypochlorite has been shown to have antibacterial activity against *E. faecalis*. The bacterial killing potential of NaOCl within the root canal improves in direct relation to an increase in the concentration of the NaOCl used for endodontic irrigation (Goldman *et al.*, 1982; Baumgartner and Cuenin, 1992; Siqueira *et al.*, 2000; Retamozo *et al.*, 2010; Stojicic *et al.*, 2010).

Sodium hypochlorite has several disadvantages that have a bearing on its application in chemical endodontic cleaning. Indiscriminate organic tissue dissolution accounts for the most significant disadvantage (Pashley *et al.*, 1985; Brown *et al.*, 1995; Barnhart *et al.*, 2005). Sodium hypochlorite is a non-specific toxic solvent of organic matter (Barnhart *et al.*, 2005). It is not able to differentiate between the organic components that are intended to be dissolved (necrotic and vital pulp remnants and bacteria), and those that are meant to be preserved (periradicular tissues and the periodontal ligament). The periradicular tissue beyond the limits of the root canal system itself and the soft tissues of the oral cavity are susceptible to the corrosive effects of NaOCl. Great care must thus be exercised during root canal irrigation with this hazardous chemical (Pashley *et al.*, 1985; Brown *et al.*, 1995; Hülsmann and Hahn, 2000).

1.7.2 Chlorhexidine Gluconate

Chlorhexidine gluconate (CHX) is a cationic bisguanide considered to be a broad-spectrum antimicrobial agent (Lin, Mickel and Chogle, 2003; Ferraz *et al.*, 2007). Both bacterial cells and CHX molecules are positively charged and these two entities compete for attachment at binding sites on hydroxyapatite crystals and soft tissues. Binding leads to the reversal of the electrical field at the binding site and the CHX molecule and bacterial cell compete to attach at the site of binding. Another mechanism of cytotoxic action of CHX can be explained as follows: because a large proportion of the inner membrane of the bacterial cell wall consists of phosphate groups, it is negatively charged. When cationic CHX molecules bond to

this membrane, the charge is neutralised. This undermines the integrity of the cell wall and intracellular fluid leaks out of the cell. These unique binding abilities of CHX are extraordinarily long lasting and equate to a residual bacteriostatic phenomenon known as substantivity (Lindskog, Pierce and Blomhöf, 1998; Leonardo *et al*., 1999; Gomes *et al*., 2003; Lin, Mickel and Chogle, 2003).

The two most important advantages of CHX over NaOCl as an endodontic irrigant are its very low level of toxicity to periapical tissue and that it exhibits the unique property of substantivity. The only shortcoming of CHX is that it is not able to break down the organic matter of the dental pulp (Jeansonne and White, 1994).

1.7.3 A Combination of Chlorhexidine Gluconate and Sodium Hypochlorite

Chlorhexidine gluconate would in all probability be the ideal endodontic irrigant if it were not for its incapacity to dissolve organic matter (Jeansonne and White, 1994). The most widely used endodontic irrigant, NaOCl, is a highly efficient solvent of organic tissue. An endodontic irrigation regime that includes both CHX and NaOCl for endodontic irrigation is beneficial in that the two solutions complement each other, each making up for the shortcomings of the other (Kuruvilla and Kamath, 1998; Baca *et al*., 2011).

When compared to irrigation with both 2.5% NaOCl and 0.2% CHX alone, the combined use of 2.5% NaOCl and 0.2% CHX for endodontic irrigation results in the greatest decrease in intracanal bacteria (Kuruvilla and Kamath, 1998).

1.7.4 Ethylene-diamine-tetra-acetic Acid

The chelating agent, Ethylene-diamine-tetra-acetic Acid (EDTA), is used to aid shaping of the root canal. It is an excellent lubricant and dissolves inorganic matter. When used during root canal preparation, it is effective in dissolving the inorganic components of the dentinal wall and smear layer (Sedigheh and Shokouhinejad, 2008).

Using EDTA in combination with NaOCl the entire smear layer can be successfully removed (Baumgartner and Mader, 1987). The most efficient method of smear layer

removal is: rinsing the root canal for one minute with 17% liquid EDTA following thorough rinsing with 5% NaOCl during the endodontic preparation of the root canal (Giovannone *et al*., 2006; Sedigheh and Shokouhinejad, 2008).

In an *in vitro* study comparing the cleaning efficiency of different regimes of root canal irrigation, the root canals of teeth were shaped with nickel titanium rotary files. Four different irrigation regimens were applied to the teeth and the walls of root canals were compared using a Scanning Electron Microscope (SEM). It was concluded that a combination of 5% NaOCl and 17% EDTA liquid thoroughly cleans all walls of the root canal except for those in the apical third (Giovannone *et al.*, 2006).

1.7.5 Chlor-XTRA

A commercially available endodontic irrigant, Chlor-XTRA (Vista Dental Products, Racine, Wisconsin, USA), is a highly concentrated NaOCl (5.25%) solution that has been adapted in order to improve its efficacy as an endodontic irrigant. In addition to the main active ingredient, NaOCl, Chlor-XTRA contains a wetting agent, special surface modifying agents and alkylating agents. Surface modifying agents lower the solution's surface tension and alkylating agents increase its electrical capacity (Williamson, Cardon and Drake, 2009). These additional agents significantly increase the organic tissue-dissolution efficiency of NaOCl. Chlor-XTRA is more effective in tissue dissolution than unaltered NaOCl at concentrations of up to 5.8% when the two solutions are heated, ultrasonically activated or sonically activated (Stojicic *et al*., 2010).

1.7.6 BioPure MTAD

The endodontic irrigant, BioPure MTAD (Tulsa/Dentsply, Tulsa, USA), is a combination of tetracycline, citric acid and a detergent (Torabinejad and Walton, 2009). Davis, Maki and Bahcall undertook a study in 2007 in which the bacteriostatic ability of BioPure MTAD was weighed up against that of several other endodontic irrigation solutions. BioPure MTAD demonstrated a larger inhibitory zone when tested against aerobes and anaerobes than the inhibitory zone of both 2% CHX and 5.25% NaOCl.

1.8 Photo-activated Disinfection

The principal mechanism of action of photo-activated disinfection (PAD) in root canal therapy is as follows: A non-toxic photo-activating agent (dye) is placed into the prepared root canal. The molecules within the dye attach to any bacterial cells with which it makes contact and act as a marker. A light source is applied inside the canal and a chemical reaction, during which the dye becomes excited, is initiated. Highly reactive oxygen ions released from the dye have a toxic effect upon bacterial cells, damaging their protoplasm, cell membrane and DNA. Ultimately this results in bacterial cell lysis and death (Wainwright, 1998; Garcez *et al*., 2006).

The dyes most commonly used for PAD are Toluidine blue (TB) (also known as Tolonium Chloride) or Methylene blue (MB). Two different light sources have been developed for application in the dental field: laser diode and Light emitting diode (LED).

1.8.1 The Application of Photo-activated Disinfection in Endodontics

Photo-activated disinfection (PAD) is not a suitable replacement for traditional root canal irrigation solutions and regimes. However, when PAD with the use of a laser diode as the light source is used in addition to the standard chemical and mechanical root canal cleaning techniques and materials, the decrease in bacteria is significantly improved (Bonsor *et al*., 2006; Williams, Pearson and Colles, 2006; Bergmans *et al*., 2008; Garcez *et al*., 2008).

In 2008 Fonseca and co-workers conducted an experiment where the root canals of 46 teeth were endodontically cleaned and shaped, sterilised and infected with *E. faecalis*. One half of this sample was treated with PAD (TB and a diode laser) and the other half of this sample was kept aside as a control group. The reduction of the bacterial load after PAD was 99.9% (Fonseca *et al*., 2008).

The intracanal efficacy of PAD varies against different bacterial species (Bergmans *et al*., 2008). The bacterial killing potential of PAD is not affected by an increased concentration of the dye marker, but this potential is increased when the dose of energy emitted by the light source is increased (Williams, Pearson and Colles, 2006).

Although PAD has a broad spectrum of bacterial killing, some endodontic pathogens are able to grow in mono-species biofilms and evade such attempts at eradication (Bergmans *et al*., 2008). It is recommended that PAD be used as a complementary treatment to traditional methods of cleaning and shaping of root canals rather than as the sole method of root canal disinfection in endodontic treatment (Soukos *et al*., 2006; Bergmans *et al*., 2008; Pinheiro *et al*., 2009). The application of PAD in endodontic cleaning is advantageous in that it has the ability to reach bacterial cells that are sheltered in the deeper recesses of the dentine. In contrast, most attempts at using PAD to eradicate bacteria that are arranged in a biofilm have proven unsuccessful (Soukos *et al.,* 2006; Bergmans *et al*., 2008; Fimple *et al*., 2008; Garcez *et al*., 2008).

In 2009, Lim and co-workers conducted an *in vitro* study to the compare the efficacy to which chemo-mechanical root canal preparation, standard diode laser PAD, improved diode laser PAD and NaOCl were able to reduce bacteria (in biofilm). These different preparation techniques were tested on several biofilms of different levels of maturity. A combination of NaOCl and PAD were able to significantly inactivate a four-day-old (immature) biofilm of *E. faecalis*. The improved PAD in combination with conventional mechanical and chemical cleaning was able to significantly inactivate four-week-old biofilm of *E. faecalis* (Lim *et al*., 2009*)*.

The heat generated by a diode laser is a limiting factor for the application of PAD in dentistry. In order to keep the laser from causing damage to the biological tissues of the mouth the energy used for endodontic treatment must be low (Bahcall *et al*., 1992; Ramskold, Fong and Stromberg, 1997). The lower energy calls for an increase in the exposure time in order to illicit the desired bacteriocidal effect (Ramskold, Fong and Stromberg, 1997). In order to decrease the prolonged exposure time needed for bacterial eradication, alternative sources of light have been sought leading to the development of light emitting diode (LED) devices for use in medicine and dentistry light source instead (Schlafer *et al*., 2010).
In 2010 Schlafer and colleagues conducted an *in vitro* experiment to evaluate the bacterial eradication by PAD using a conventional LED as the light source and TB as the dye. The experiment consisted of two parts. The first part tested the bacterial eradication by PAD of bacteria in planktonic suspension in the root canals and the

second part tested the eradication of bacteria that had been given time to adhere to the root canals walls. In both parts of the experiment the bacteria were significantly reduced. The reduction of the bacteria in suspension was 99.7% and the reduction of the bacteria adhered to the root canal wall was 95.82%.

1.8.2 The Aseptim Plus Device in Photo-activated Disinfection

The photo-active dye marker in Aseptim fluid (SciCan, Toronto, Canada) is tolonium chloride. This fluid is placed into the root canal prior to introduction of the light source. The tolonium chloride is given time to disperse within the root canal and mark the target bacterial cells. The device is activated and the tip is inserted into the root canal. A chemical reaction between the light and the dye leads to the release of activated oxygen ions on those bacterial cells that have been targeted by the dye. The action of these ions on the marked bacterial cells leads to the rupture of their cell membranes and consequential cell death. The peak light absorption of tolonium chloride is 633 nm. The tip of the Aseptim Plus device (SciCan, Toronto, Canada) is an LED with an illumination of 635 nm (SciCan Homepage).

1.9 The Importance of Coronal Seal

In an obturated root canal, bacteria can be present for one of the following three reasons: insufficient cleaning of the canal, recontamination of the obturated canal and viable bacteria entombed within the root canal system (Peters, Wesslink and Moorer, 1995; Machtou, 2006). Ray and Trope (1995) undertook the first clinical study to evaluate the association between the quality of the coronal seal and periapical pathology in endodontically treated teeth. The study involved the assessment of a population of patients with a high incidence of periradicular pathology. They concluded that significantly fewer periapical radiolucencies were present in teeth that had a good coronal seal.

Compromise of the coronal seal plays a greater role in the failure of endodontic treatment than bacterial reinfection via the apical route (Davalou, Gutmann and Nunn, 1999; Heling *et al*., 2002; Kurtzman, 2006). Hommez and co-workers (2004) investigated the relationship between the state of the coronal restoration and the make-up of the bacterial community in teeth that presented with periapical

periodontitis. The teeth either had a necrotic pulp or had undergone previous endodontic treatment. In the cases where the coronal restoration was defective the mean count of bacteria in the group with endodontic fillings was increased while the bacterial count in the group with pulp necrosis that had never been endodontically treated was not significantly increased.

1.10 Determination of Successful Endodontic Treatment

Postoperative success in endodontics has traditionally been defined in one of two ways: clinical success or complete healing. Clinical success takes into consideration the resolution of clinical signs and symptoms alone, while complete healing is considered to be a combination of both clinical success and a return to health of the periradicular tissues as evident clinically and radiographically (Friedman, 1997; Friedman, 2002).

Friedman and co-workers (1995) undertook a study according to which first-time (primary) endodontic treatment cases and endodontic retreatment (failed) cases were evaluated retrospectively. Complete healing was found in 78% of cases as compared to clinical success in 16% of cases. This clinical success was indicated, despite a lack of supportive radiographic evidence of periradicular healing. In this study, a high total clinical success rate of 94% was observed.

CHAPTER 2: AIM AND OBJECTIVES

2.1 Aim

The aim of this *in vitro* study was to compare the intracanal disinfection properties of four different endodontic irrigation regimens and two techniques of photo activated disinfection (PAD) to a control of distilled water.

2.2 Objectives

The broad objectives in this study were to:

i) Prepare root canals of extracted human maxillary anteriors or premolars, with straight roots and single root canals.
ii) Infect these prepared root canals with *Enterococcus faecalis.*
iii) compare the effectiveness of 3% sodium hypochlorite (NaOCl), 2% chlorhexidine gluconate (CHX), ChlorXTRA, 3% NaOCl in combination with 2% CHX, PAD used subsequent to irrigation with 3% NaOCl and PAD applied alone in eliminating *E. faecalis* from the dentinal tubules in the coronal, middle and apical thirds of human teeth when used for endodontic disinfection.

2.3 Hypothesis

The test groups: 3% NaOCl, 2% CHX, ChlorXTRA solution, 3% NaOCl used in combination with 2% CHX, PAD used subsequent to irrigation with 3% NaOCl and PAD applied alone, will be more effective than the control group (distilled water) in the eradication of *E. faecalis.*

2.4 Statistical Null/Zero Hypothesis:

Neither 3% NaOCl, 2% CHX, ChlorXTRA solution, 3% NaOCl used in combination with 2% CHX, PAD used subsequent to irrigation with 3% NaOCl nor PAD applied alone, will show more effective eradication of *E. faecalis* than the control group (distilled water).

CHAPTER 3: MATERIALS AND METHODS

3.1 Method Description

The method that was used for this *in vitro* study is a modified version of the model used in the study conducted by Haapasalo and Ørstavic in 1987. The model has since been applied successfully in many other experiments (Almyroudi *et al*., 2002; Heling and Chandler, 1998; Mohammadi and Shahriari, 2008).

3.2 Collection of Material

The teeth collected for the purpose of this experiment were extracted for reasons unrelated to the objectives of this *in vitro* study. Teeth were collected from the out-patient dental extraction clinic of the Oral and Dental Hospital, School of Dentistry, University of Pretoria. Each patient or the patient's legal guardian, in the case of a minor, that attends this facility for dental extraction is asked to complete an informed consent form (Addendum A). Patients that give this written consent grant permission for their extracted teeth to be used for the purposes of scientific research.

Every aspect of this research project was conducted in line with the ethical and safety guidelines for handling human tissues and conducting laboratory research, as prescribed by South African law: the Health Professions Act 56 of 1974 (South African National Health Bill, 2003).

3.3 Selection of Teeth

From the teeth collected, 86 were selected based on the following criteria: absence of caries, maxillary, central or lateral incisors with straight roots and the length of the root (CEJ to anatomical apex) between 15mm and 18mm (Fig. 3.1).

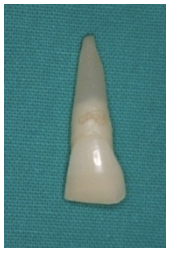

Fig. 3.1: A representative sample of a non-carious, human central maxillary incisor with a single straight root

3.4 Radiographs

Periapical radiographs were taken of the collected teeth. The radiographs were carefully examined in order to confirm that each tooth that was selected had a single root canal and that the canal was patent (Fig. 3.2).

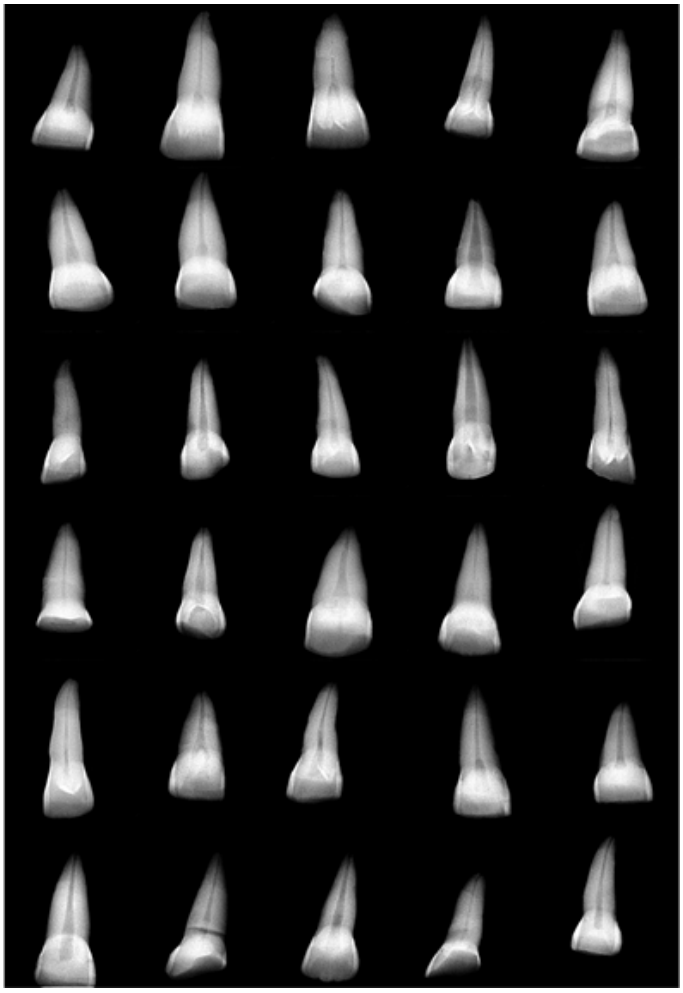

Fig. 3.2: A representative image of the periapical radiographs of central and lateral maxillary incisors that were collected for this study

3.5 Preparation of Teeth

Directly after extraction, the teeth were rinsed under cold running tap water for one minute. They were then placed in distilled water in an ultrasonic water bath and sonificated for several cycles of 15 minutes each. Fresh distilled water was used for each new cycle and sonification was repeated until all evident soft tissue had been removed from the root surfaces. In order to avoid dehydration, the teeth were stored in glass jars of distilled water at 4 °C.

The crowns of the teeth were removed using a diamond wafering blade in an Isomet 11 - 1180 low speed saw (Buehler Ltd., Lake Bluff, Illinois, USA) (Fig. 3.3). Each tooth was decoronated at a level so as to standardise the root canal to a length of 15mm (Fig. 3.4).

Fig. 3.3: A representative photograph of a central maxillary incisor being decoronated by a diamond wafering blade in an Isomet 11 – 1180 low speed saw

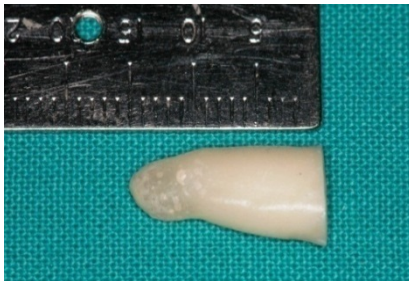

Fig. 3.4: A decoronated root with a standardised root length of 15mm

3.6 Root Canal Preparation

The working length of each root canal was determined by placing a size 10 K-file (Dentstply/Maillefer, Ballaigues, Switzerland) into the root canal, visualising the tip of the file exiting at the anatomical apical foramen, recording the measurement and subtracting 1mm to give a final working length 1mm short of the apical foramen.

The root canal preparations were done using Nickel Titanium (NiTi) rotary instruments, according to the prescribed method in the manufacturers' instructions. The files were driven by a NSK Endomate DT rotary motor (Nakanishi Inc., Kanuma, Tochigi, Japan) (Fig. 3.5).

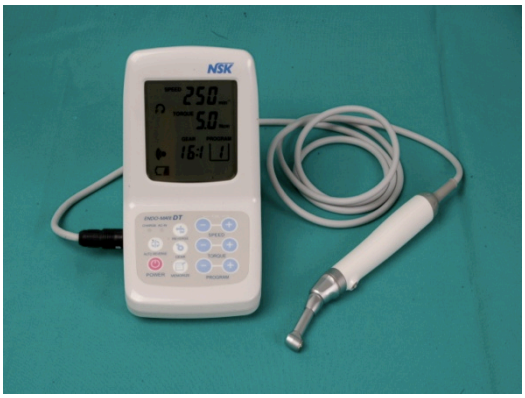

Fig. 3.5: NSK Endomate DT rotary motor

Each canal was prepared using ProTaper Universal (Dentstply/Maillefer) NiTi rotary endodontic files (Fig. 3.6). The endodontic preparation was refined with a size 45, 6% taper ProFile (Dentstply/Maillefer) rotary file (Fig. 3.7) to produce a standardised taper for all root canals.

Fig. 3.6: ProTaper Universal rotary files

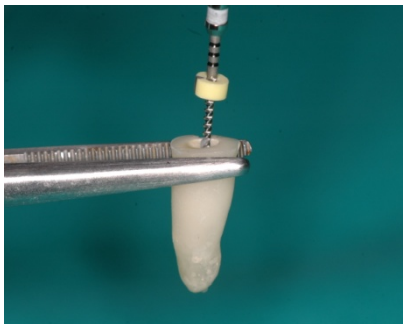

Fig. 3.7: Canal refinement with a size 45 6% taper ProFile rotary file

During root canal preparation, copious amounts of 3% sodium hypochlorite (NaOCl) (Rekitt Benckiser, South Africa (Pty) Ltd., Elandsfontein, Gauteng, South Africa) (Fig. 3.8) were used for root canal irrigation. Once preparation was complete, each root canal was irrigated with 3% NaOCl for five minutes followed by irrigation with distilled water for two minutes.

Fig. 3.8: Jik (3% sodium hypochlorite) used for root canal irrigation during root canal preparation

3.7 Removal of Smear Layer

In order to remove the inorganic components of the smear layer, the canals were irrigated with 17% ethylene diamine tetra-acetic acid (EDTA) (Vista Dental Products, Toronto, Canada) (Fig. 3.9) for one minute. Each root canal was filled with fresh 17% EDTA liquid, which was left in the canal for one minute before it was thoroughly rinsed with distilled water for two minutes.

Fig. 3.9: 17% EDTA liquid

3.8 Laboratory Procedure

3.8.1 Microbial Infection

The teeth were placed in sterile Ringer's solution (Merck SA (Pty) Ltd.) and sterilised by autoclave (Hung-Lin Medical Instruments Co. Ltd.) (Fig. 3.10) at 121°C for 15 minutes.

From this point onwards a sterile positive airflow laminar flow (Fig. 3.10), sterile instruments, gloves and masks were used so as to ensure that sterile conditions were maintained during every step of the experiment.

Fig. 3.10: A sterile positive airflow laminar flow

Before the inoculation procedure, sterile paper points were inserted into the root canals sampling for sterility. The paper points were placed onto Tryptone blood agar (TBA) plates (Onderstepoort Biological Products Ltd.) that were incubated under facultative anaerobic conditions using Anaerocult A® (Merck SA (Pty) Ltd.) at 37˚C for three days. All cultures were negative, confirming the absence of facultative anaerobic bacteria and verifying that all the canals were sterile. Two teeth were prepared for SEM as a negative control.

The prepared teeth were aseptically removed from the Ringers solution and randomly allocated to one of seven groups of twelve teeth each and placed into seven sterile glass containers containing sterile Brain Heart Infusion (BHI) broth (Merck SA (Pty) Ltd.).

3.8.2 Preparation of *Enterococcus faecalis* suspensions

A one per cent MacFarland standard-1 suspension (8 x 10^8 colony-forming units) (MacFarland, 1907) in Brain Heart Infusion (BHI) broth (Merck SA (Pty) Ltd.) were prepared from 48-hour cultures of *Enterococcus faecalis* (ATCC 49474). A 1% inoculum of this was added to the teeth and the inoculated samples were incubated in a Vortex platform (shake) incubator (Ika-Works Inc.) (Fig. 3.11) for 48 hours.

Fig. 3.11 Vortex platform incubator

3.8.3 Irrigation of Root Canals

The test tubes containing the infected specimens were randomly divided into seven groups. In each group the root canals of the teeth were irrigated according to the specific disinfection protocol of that group. The irrigation times and disinfection regimens used were based upon literature. Irrigation protocols were approved by the Research Committee of the School of Dentistry, University of Pretoria.

In the first group, the root canals were irrigated with 12ml of 3% NaOCl (Fig. 3.8) for four minutes and then rinsed with 3ml of distilled water for one minute.

The root canals of teeth allocated to group two were irrigated with 12ml of 2% chlorhexidine gluconate (CHX) (Dismed Pharmaceutical (Pty) Ltd.) (Fig. 3.12) for four minutes and then rinsed with 3ml of distilled water for one minute.

For the third group of root canals, 9ml of Chlor-XTRA (Vista Dental Products) (Fig. 3.13) was used for irrigation of the root canals over a period of three minutes and then the canals were rinsed with 3ml of distilled water for one minute.

In group four the root canals were irrigated with 6ml of 3% NaOCl for the first two minutes, rinsed with 3ml of distilled water for one minute, irrigated with 3ml of 2% CHX for one minute and then rinsed again with 3ml of distilled water for one minute.

The root canals of the teeth in the fifth group were irrigated with 9ml of 3% NaOCl for the first three minutes and rinsed with 3ml of distilled water for one minute. The

canals were filled with toludine chloride and this was activated with the Aseptim plus LED device (SciCan, Toronto, Canada) (Fig. 3.14) for two intervals of two minutes each. The canals were then rinsed again with 3ml of distilled water for one minute.

The root canals in the sixth group were rinsed with 3ml of distilled water for one minute. The canals were filled with toludine chloride and this fluid was activated with the Aseptim plus light emitting diode (LED) device for two intervals of two minutes each, and then rinsed again with 3ml of distilled water for one minute.

The seventh group was the control group. The root canals of the teeth in this group were rinsed with 15ml of distilled water over a total treatment period of five minutes.

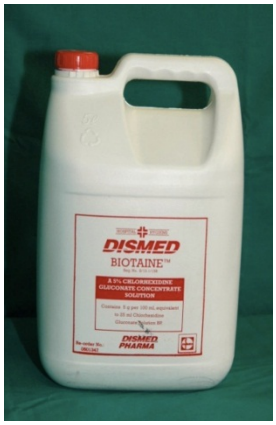

Fig.3.12: Chlorhexidine gluconate solution

Fig. 3.13: Chlor-XTRA irrigation solution

Fig. 3.14: Aseptim plus LED device

3.8.4 Dentine Extraction

Two guide grooves of 0.5mm in depth were cut (directly opposite to one another) into the external surface along the length of each of the samples with a diamond disc. Great care was taken to avoid penetrating the pulp space. The samples were then fractured longitudinally by placing a thin sterile stainless steel chisel in one of the guide grooves and striking the back of the handle with a sterile stainless steel hammer (Fig. 3.15) while the tooth was held in place with a pair of sterile artery forceps (Fig. 3.16). In this way the samples were split in two parts of approximately the same size.

Fig. 3.15: Sterile chisel and hammer

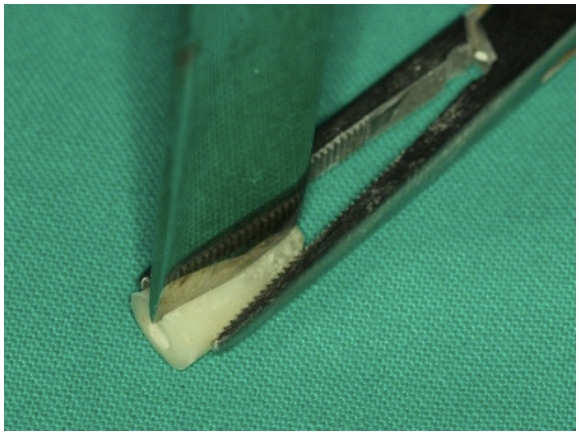

Fig. 3.16: A sterile chisel fracturing a tooth longitudinally

One of the two halves of each sample was horizontally fractured into three sections: coronal (C), middle (M) and apical (A). Each section was placed directly into a separate, sterile petri dish which was clearly labelled with a permanent marker indicating: the tooth number and group from which it had originated and the initial position (C, M or A) of the section before the tooth was divided.

A pair of sterile forceps was used to hold each section in place while extraction of dentine samples was performed using a sterile round tungsten carbide bur (Dentsply/Maillefer) (Fig. 3.17) mounted in an NSK Endomate DT rotary motor (Fig. 3.5).

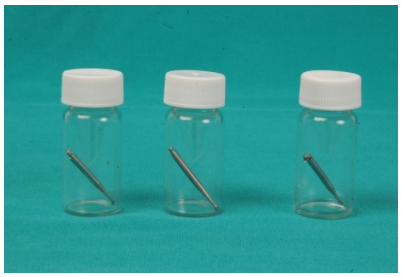

Fig. 3.17: Sterile tungsten carbide burs in sterile Bijou bottles

The dentine chips were collected over separate sterile pre-weighed Bijou bottles (Merck SA (Pty) Ltd.) (Fig. 3.18). The weight of the dentine chips was determined and the weight/volume concentration of each sample was calculated. This concentration was used to quantify the amount of viable *E. faecalis* that survived in each root canal after having undergone the irrigation treatment. An amount of 1ml of saline was added to the dentine in the Bijou bottles after they had been weighed.

The colony-forming units (cfu) were determined as follows: ten-fold dilutions were made in sterile quarter-strength Ringers solution. A quantity of 1ml of 10^{-3} to 10^{-7} of these dilutions was plated in duplicate onto BHI agar plates (Gerhardt *et al.*, 1981). All plates were incubated at 37^{0}C for five days in facultative anaerobic conditions by the use of Anaerocult A®. Daily inspection and cfu counting was carried out for a period of five days.

Fig. 3.18: A representative photograph of dentine retrieval with a sterile tungsten carbide bur; collection over a sterile Bijou bottle

Fig. 3.19: A representative photograph of a Bijou bottle containing a dentine sample in 1 ml of Ringers solution

3.8.5 Preparation for Scanning Electron Microscopy (SEM)

The second half of each sample (tooth) was treated according to standard preparation methods for Scanning Electron Microscopy (Brown and Brenn, 1931; Glauert, 1975; Hayat, 1981) as follows:

- Each half was fixed in 2% gluteraldehyde (Merck (Pty) Ltd, South Africa) for one hour.
- The gluteraldehyde was sucked off with a pipette and the samples were rinsed three times in a phosphate buffer (Whittaker MA Bioproducts Inc., Walkersville, Md) for five minutes each time.
- Samples were then fixed in 0,25% osmium tetroxide (OsO_4) (Merck (Pty) Ltd, South Africa) for 30 minutes. They were then rinsed with phosphate buffer (PBS) three times for five minutes each time.
- The samples were rinsed for five minutes each time in increasing concentrations of ethanol (Merck (Pty) Ltd, South Africa), namely 30%, 50%, 70% and 90%.
- Samples were placed into test tubes containing 100% ethanol for storage until such time as they could be dried in a critical point dryer (Polaron, Oxford, England) until dry (approximately eight hours).
- Once dried, each sample was mounted (onto a small aluminium plate using conductive carbon cement) with the fracture plane facing upwards in order to make viewing of the dentine of the prepared and treated root canal easier.
- All the prepared samples were then coated with gold in a Polaron E5200 sputter-coater (Polaron Equipment Ltd, Hertfordshire, England) before they were examined in a JEOL JSM 840 Scanning Electron Microscope (JEOL Ltd, Akishima Tokyo, Japan).

3.9 Statistical Analysis

A pairwise comparison of the cfu counts of all seven treatment groups was done using the Pairwise Wilcoxon Rank Sum test and a non-parametric analogue of the one-way ANOVA test, the Kruskal Wallis test.

CHAPTER 4: RESULTS

4.1 Confirmation of Sterility of the Teeth

All of the specimens were sterilised in an autoclave. Samples of the endodontically prepared root canals of two of the teeth (negative control) were taken with sterile paper points and placed onto brain heart infusion (BHI) plates. These were incubated under anaerobic conditions for 72 hours. No colony forming units (cfu) could be observed (Fig. 4.1), which indicated that all pre-existing bacteria had been successfully eradicated from the root canals.

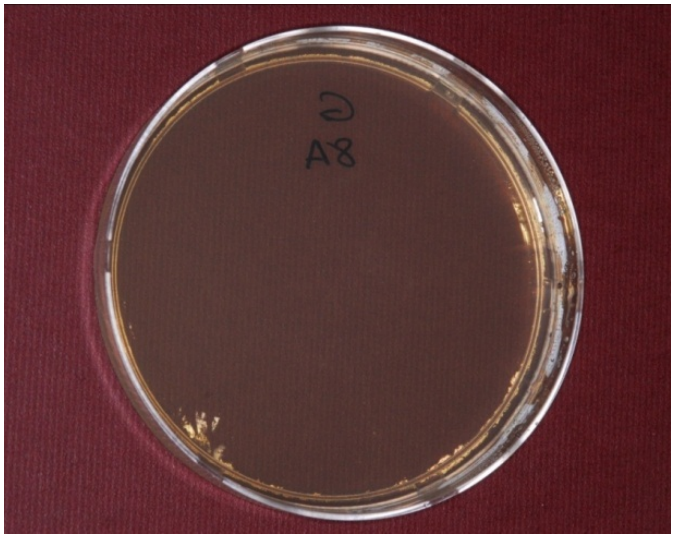

Fig. 4.1: A representative photograph of a post-sterilisation sample (negative control) on a BHI plate with no cfu present

The same two samples were then fractured longitudinally under sterile conditions and one half of each was prepared for SEM analysis according to standard methods for microscopy. Upon SEM examination, no micro-organisms could be seen on the peritubular dentine, intertubular dentine or inside the orifices of the dentinal tubules, confirming that the root canals were sterile. The dentine was also clear of smear layer and no erosion or interruption of the intertubular dentine could be observed (Fig. 4.2).

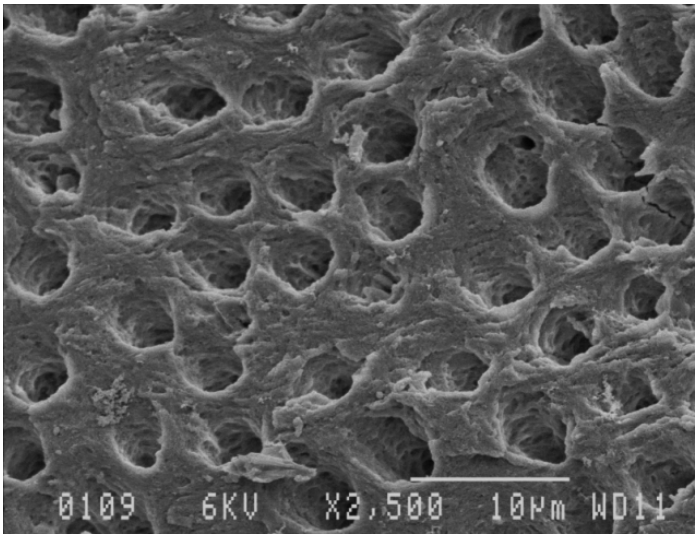

Fig. 4.2: A representative SEM image of the dentine surface of the root canal of the negative control. Note the absence of smear layer and microorganisms (magnification 2500X)

4.2 Confirmation of *Enterococcus faecalis* Infection in the Root Canals

After the sterilised specimens had been inoculated with *E. faecalis,* random samples of the prepared root canals were taken, placed onto BHI plates and incubated under anaerobic conditions (positive control). After 72 hours, numerous cfu of the test organism could be observed on the BHI plates (Fig. 4.3). Two samples were prepared for SEM examination. Numerous *E. faecalis* cells could be observed on the intertubular, peritubular and intratubular dentine (Fig. 4.4). This served as confirmation that the root canals had been successfully inoculated with *E. faecalis*.

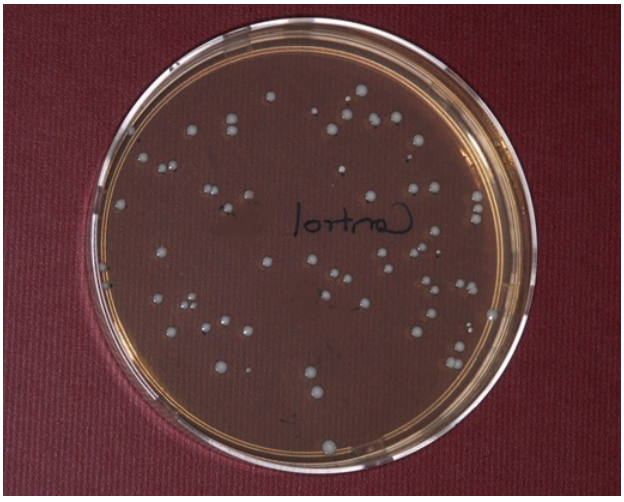

Fig. 4.3: A representative photograph of a post-inoculation sample (positive control) on a BHI plate, showing numerous colony forming units (cfu)

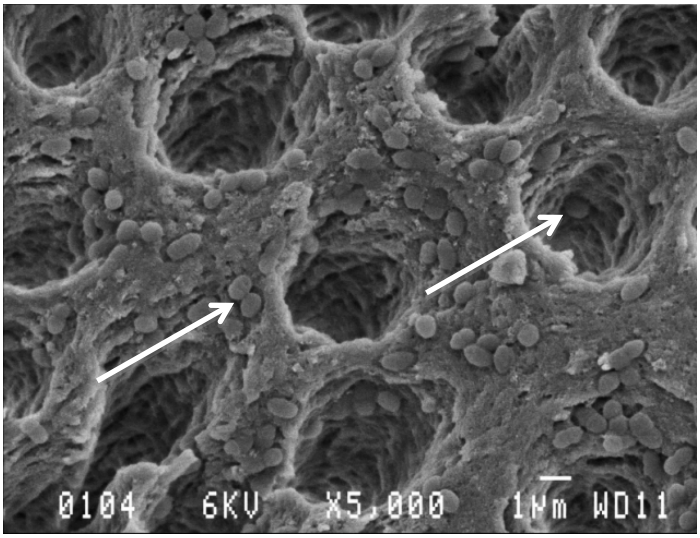

Fig. 4.4: A representative SEM image of the dentine wall after inoculation with *E. faecalis*. Viable bacterial cells could be seen attached to the peritubular and intertubular dentine and inside the dentinal tubules (arrows) (magnification 5000X)

4.3 Colony Forming Unit Counts from Dentine Extracted from Root Canals in All Groups

The measurements of the cfu counts (three areas of each of the 12 root canals) of the six different treatment groups, the total cfu counts for each canal and the mean of each of three areas in each canal are presented in tables 4.1 to 4.6. The cfu measurements for the control group are presented in table 4.7.

4.3.1 3% Sodium Hypochlorite

No areas of any of the 12 samples were free of bacteria. The mean values of the cfu measurements in the three areas were: coronal, 13.08 x 10^8 cfu, middle, 106.5 x 10^8 cfu and apical, 512.75 x 10^8 cfu. The mean of the cfu counts for the 12 root canals in their entirety was 633 x 10^8 cfu.

4.3.2 2% Chlorhexidine Digluconate

The dentine samples from the coronal area of the 12 root canals irrigated with 2% chlorhexidine digluconate rendered no cfu. In both the middle and the apical areas, half of the root canals were free of bacteria. In the six samples with positive bacterial cultures the mean of the cfu counts in the middle area was 3.83 x 10^8 cfu and the mean of the cfu counts for the apical area was 47.92x 10^8 cfu. The mean of the cfu counts for the 12 samples in this group was 51.75 x 10^8 cfu.

4.3.3 Chlor-XTRA

No cfu were cultivated from any dentine samples from the coronal areas of any of the 12 root canals irrigated with this solution. The mean values of the cfu counts for the middle and apical areas were 0.17 x 10^8 cfu and 0.58 x 10^8 cfu respectively. The mean of the cfu counts for all three areas of the canal of the 12 samples was 0.75 x 10^8 cfu.

4.3.4 Combination of 3% Sodium Hypochlorite and 2% Chlorhexidine Digluconate

The samples from both the coronal and the middle areas of all 12 samples in this group did not yield any cfu. The apical areas of eight of the 12 samples were also free of bacteria, with the mean of the cfu counts for the apical area being equivalent to the mean of the cfu counts of the root canals, 0.75 x 10^8 cfu.

4.3.5 3% Sodium Hypochlorite and Photo-Activated Disinfection (PAD)

Both the coronal and middle areas of all 12 samples in this group were free of bacteria. The apical areas of half of the root canals were also free of bacteria and the mean of the cfu count of all the apical areas was equivalent to the mean of the cfu counts of the 12 full root canals: 1.83 x 10^8 cfu.

4.3.6 Photo-activated Disinfection (PAD)

The concentration of cfu increased in an apical direction with the means of the cfu counts of 324 x 10^8 cfu (coronal), 291.25 x 10^8 cfu (middle) and 3372.83 x 10^8 cfu (apical). The total sum of cfu counts for the 12 samples was 3952.67 x 10^8 cfu.

4.3.7 Distilled Water (Control)

All 12 samples of the control group had high cfu counts in all three areas of the root canals. The mean total cfu measurement for the entire root canal of the 12 samples was 4150 x 10^8 cfu. The concentration of cfu increased in an apical direction – with mean cfu measurements of 372.42 x 10^8 cfu (coronal), 1161.50 x 10^8 cfu (middle) and 2626.58 x 10^8 cfu (apical).

4.4 Ranks of Colony Forming Unit (cfu) Count Values of all Seven Treatment Groups

Some of the measurement observations of the cfu counts for the six different treatment groups did not meet the normality assumptions of a one-way ANOVA test. It was therefore necessary to make use of a non-parametric analogue of a one-way ANOVA, the Kruskal-Wallis test. As this test is performed on ranked data, the cfu measurements were converted to their ranks in the overall data set (i.e. the smallest value is assigned a rank of 1, the next smallest is assigned a rank of 2, and so on). The Kruskal Wallis test was used to compare the total number of bacteria in the entire canal of the 12 samples from all seven treatment groups to one another. The resulting Pr value was < 0.0001, which indicated that there were statistically significant differences somewhere between these groups. Table 4.8 shows this comparison. Of the six test groups, the irrigation regimen of 3% sodium hypochlorite combined with 2% chlorhexidine digluconate was the most effective irrigation protocol in eradicating *E. faecalis* from the root canals. In descending order of efficacy, this protocol is followed by: 3% sodium hypochlorite in combination with PAD, Chlor-XTRA and 2% chlorhexidine digluconate. The irrigation protocols that performed the worst in this *in vitro* study were 3% sodium hypochlorite and PAD.

The seven treatment groups rendered 21 possible pair combinations for comparison. The 21 pair combinations were applied in order to use the Pairwise Wilcoxon Rank Sum test and the Kruskal-Wallis test to compare the cfu counts of the seven groups to one another. These comparisons are presented in tables 4.9 to 4.32.

4.4.1 3% Sodium Hypochlorite

The total rank sum of cfu counts for the 12 samples irrigated with 3% sodium hypochlorite was 6123. The Pairwise Wilcoxon Rank Sum test showed that only two irrigation protocols performed significantly less effective in removing *E. faecalis* from the root canals: the PAD-alone group ($p < 0.0001$) and distilled water ($p < 0.0001$). The other three disinfection regimes were all significantly ($p < 0.0001$) more effective at removing *E. faecalis* from the root canals compared to 3% sodium hypochlorite.

4.4.2 2% Chlorhexidine Digluconate

The total rank sum of cfu counts for the 12 samples irrigated with 2% chlorhexidine digluconate was 3113. The Pairwise Wilcoxon Rank Sum test showed that this solution was significantly more effective than both PAD and distilled water ($p < 0.0001$). Although this solution did perform slightly better than: Chlor-XTRA ($p = 0.1638$), the regime using a combination of 3% sodium hypochlorite/2% chlorhexidine digluconate ($p = 0.0194$) and 3% sodium hypochlorite/PAD ($p = 0.0589$), these differences were not statistically significant.

4.4.3 Chlor-XTRA

The total rank sum of cfu counts for the 12 samples irrigated with Chlor-XTRA was 2619. The Pairwise Wilcoxon Rank Sum test showed that this solution was significantly more effective at eradicating *E. faecalis* from the root canals as compared to 3% sodium hypochlorite ($p < 0.0001$). It performed slightly better than the 2% chlorhexidine digluconate solution ($p = 0.1638$), but the difference in efficacy between these two irrigants was not statistically significant. Chlor-XTRA was significantly more effective ($p < 0.0001$) in eradicating *E. faecalis* from the root canals than PAD, 3% sodium hypochlorite and distilled water.

4.4.4 Combination of 3% Sodium Hypochlorite and 2% Chlorhexidine Digluconate

The combination of these two irrigation solutions resulted in a total rank sum of 2370. The Pairwise Wilcoxon Rank Sum test showed that this irrigation protocol was more efficient at eradicating *E. faecalis* from the root canals than all five other disinfection regimens that were tested. There were, however, no significant differences between the efficacy of this protocol and 2% chlorhexidine digluconate ($p = 0.0194$), Chlor-XTRA ($p = 0.2256$) or 3% sodium hypochlorite/PAD ($p = 0.5677$). This protocol was significantly more effective ($p < 0.0001$) than PAD, 3% sodium hypochlorite and distilled water.

4.4.5 3% Sodium Hypochlorite and Photo-activated Disinfection (PAD)

The total rank sum of cfu counts for the 12 samples irrigated with 3% sodium hypochlorite was 2485. The Pairwise Wilcoxon Rank Sum test indicated that Photo-activated Disinfection (PAD) worked significantly better when the root canals were irrigated with 3% sodium hypochlorite before the application of PAD ($p < 0.0001$) than when PAD was applied to the root canals on its own. This technique performed marginally better than Chlor-XTRA ($p = 0.5665$) and 2% chlorhexidine digluconate ($p = 0.0589$) but these differences were not statistically significant. The improvement in *E. faecalis* eradication demonstrated by this protocol was significant ($p < 0.0001$) as compared to PAD, 3% sodium hypochlorite and distilled water (control).

4.4.6 Photo-activated Disinfection (PAD)

The total rank sum of cfu counts for the 12 samples irrigated with 3% sodium hypochlorite was 7343. Photo-activated disinfection performed poorly in eradication of *E. faecalis* from the root canals. The Pairwise Wilcoxon Rank Sum test indicated that this protocol performed slightly better than the control but the difference was not significant (0.0487). All five other disinfection protocols were significantly more efficient ($p < 0.0001$).

4.4.7 Distilled Water (Control)

The 12 samples of the control group where distilled water was used resulted in a total rank sum of cfu counts of 7824. As was expected all test groups were better able to remove *E. faecalis* from the root canals than distilled water. This difference was significant in all test groups except for the group where PAD was applied alone; i.e. without any pre-irrigation ($p = 0.0487$).

4.5 Scanning Electron Microscope (SEM) Analysis

The figures in this part of the results (Fig. 4.5 to Fig. 4.22) are representative photographs of the SEM images taken of prepared root canals treated with EDTA to remove the smear layer and inoculated with *E. faecalis*. Thereafter, each sample was randomly assigned to one of the seven experimental groups and treated according to the disinfection protocol of the group.

4.5.1 3% Sodium Hypochlorite

No *E. faecalis* cells could be observed at the coronal or middle level of the root canals in this group. Very few *E. faecalis* cells were detected in the apical level of the root canal. Erosion of the peritubular dentine was evident at the middle and apical levels of the root canal (Fig. 4.5 to Fig. 4.7).

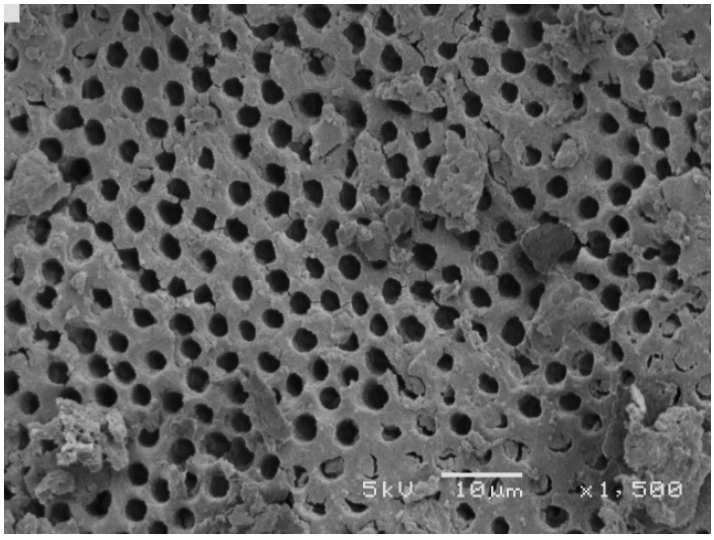

Fig. 4.5: An SEM photograph of a coronal section of a root canal irrigated with 3% NaOCl. No *E. faecalis* cells could be observed (magnification: 1500X)

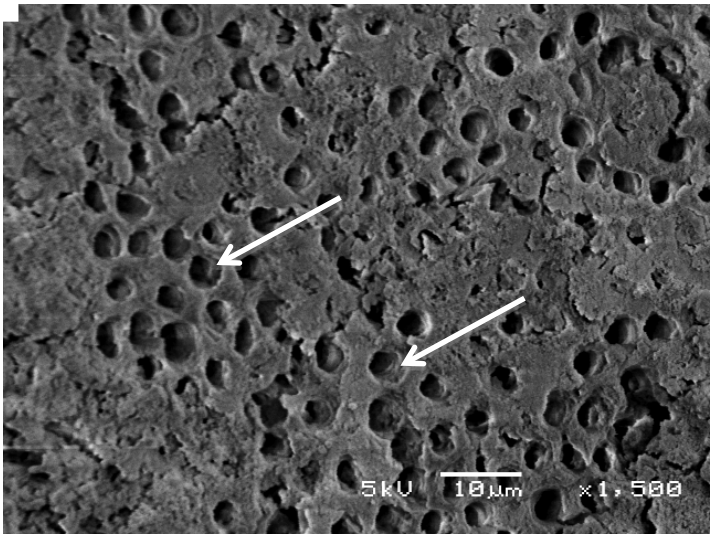

Fig. 4.6: An SEM photograph of the middle region of a root canal irrigated with 3% NaOCl. No *E. faecalis* cells could be visualised and much of the peritubular dentine had been eroded (arrows) (magnification: 1500X)

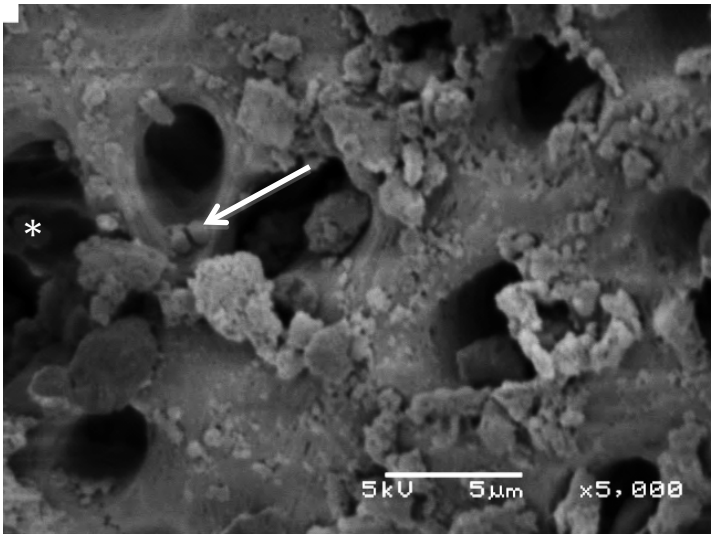

Fig. 4.7: An SEM photograph of the apical section of a root canal irrigated with 3% NaOCl. Few *E. faecalis* cells could be observed on the peritubular and intertubular dentine (arrow) and there was evidence of erosion of the peritubular dentine (asterisk) (magnification: 5000X)

4.5.2 2% Chlorhexidine Digluconate

At all levels of the root canals, a precipitate could be observed on the peritubular and intertubular dentine (Fig. 4.8 to Fig. 4.10). In the coronal, middle and apical sections a few *E. faecalis* cells could be observed on the intertubular dentine (Fig. 4.8 to Fig. 4.9).

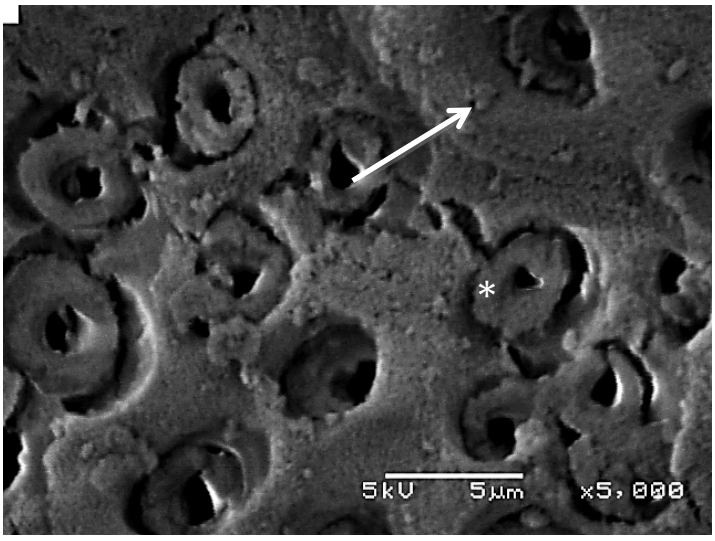

Fig. 4.8: An SEM photograph of the coronal section of a root canal irrigated with 2% CHX. A precipitate could be observed in the dentinal tubule orifices and on the intertubular and peritubular dentine (asterisk). A few deformed *E. faecalis* cells could be observed on the peritubular dentine (arrow) (magnification: 5000X)

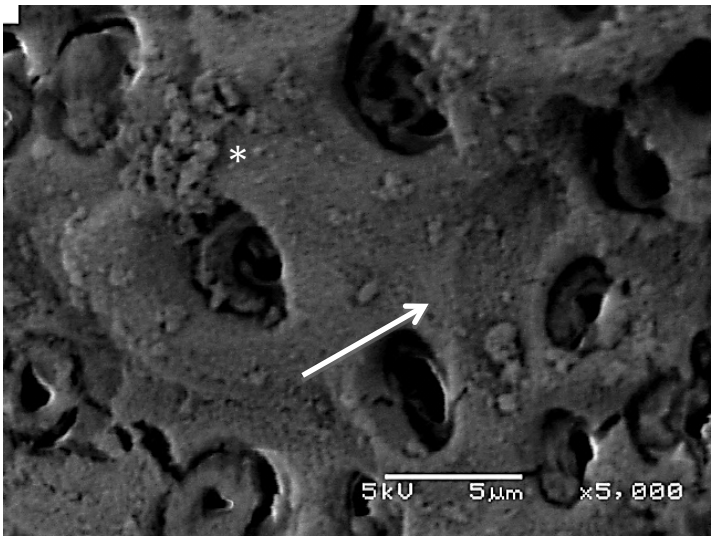

Fig. 4.9: An SEM photograph of the middle section of a root canal irrigated with 2% CHX. A precipitate could be observed in the dentinal tubule orifices and on the intertubular and peritubular dentine (asterisk). A few *E. faecalis* cells could be observed on the intertubular dentine (arrow) (magnification: 5000X)

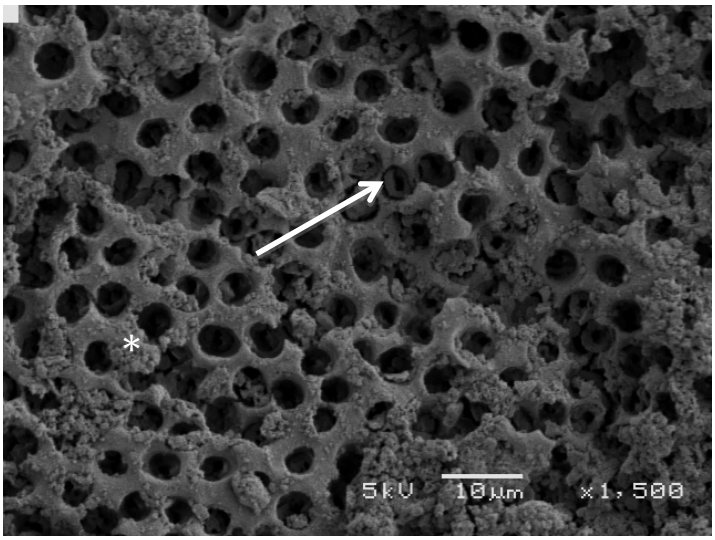

Fig. 4.10: An SEM photograph of the apical region of a root canal irrigated with 2% CHX. The peritubular and intertubular dentine was covered by a precipitate (asterisk), which was also visible inside some of the dentinal tubules (arrow). No *E. faecalis* cells could be observed (magnification: 1500X)

4.5.3 Chlor-XTRA

No *E. faecalis* cells could be observed at any level of the root canals. Severe erosion of the intertubular dentine could be observed at the coronal level (Fig. 4.11). The

peritubular dentine was eroded at the middle and apical levels of the root canal (Fig. 4.12 and Fig. 4.13).

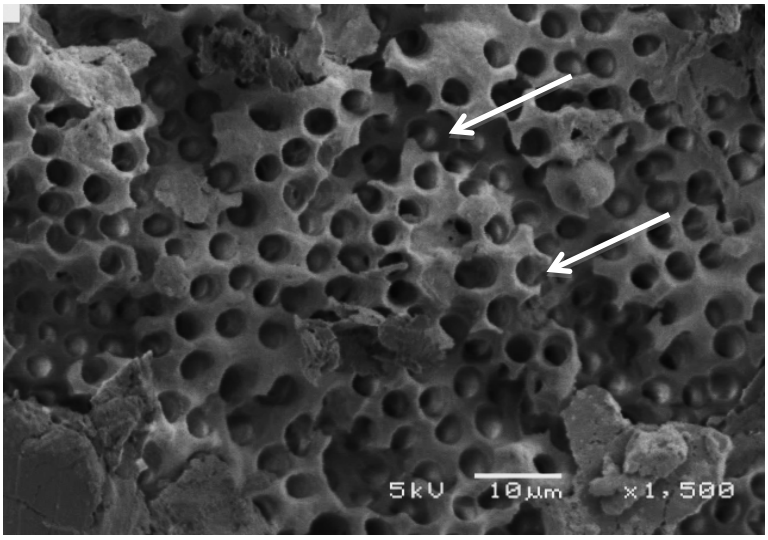

Fig. 4.11: An SEM photograph of a coronal section of a root canal irrigated with Chlor-XTRA. No *E. faecalis* cells could be observed. The intertubular dentine was severely eroded (arrows) (magnification: 1500X)

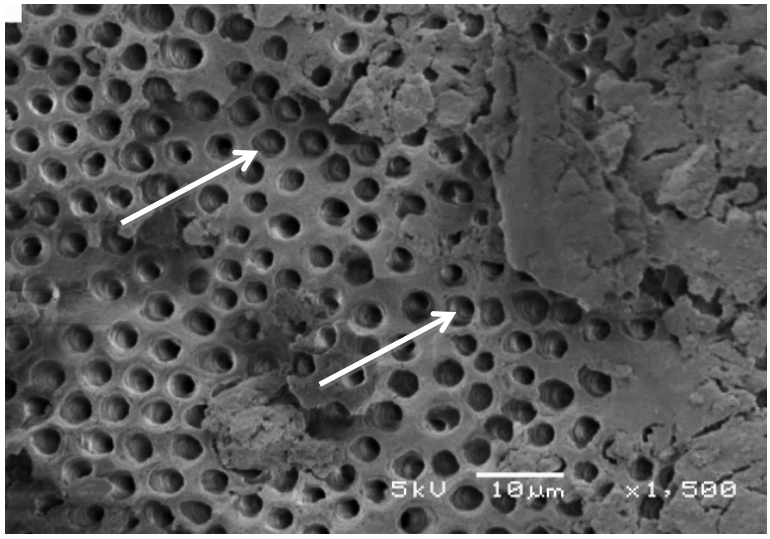

Fig. 4.12: An SEM photograph of a middle area of a root canal irrigated with Chlor-XTRA. No *E. faecalis* cells could be observed. The peritubular dentine was eroded (arrows) (magnification: 1500X)

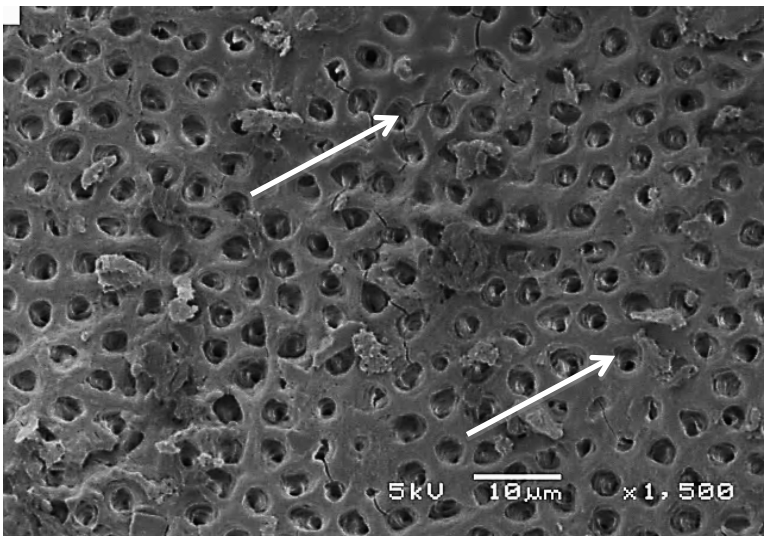

Fig. 4.13: An SEM photograph of the apical section of a root canal irrigated with Chlor-XTRA. No *E. faecalis* cells could be observed. The peritubular dentine was moderately eroded (arrows) (magnification: 1500X)

4.5.4 Combination of 3% Sodium Hypochlorite and 2% Chlorhexidine Digluconate

No *E. faecalis* cells were detected at any of the three levels of the canals in this group. A precipitate was detected upon the peritubular/intertubular dentine at all three levels (Fig. 4.14 to Fig. 4.16). In comparison to that observed in the coronal section, the precipitate was more abundant at the middle level (Fig. 4.15) and it was thickest at the apical level (Fig. 4.16). Several of the dentinal tubule orifices of the middle and apical sections of the root canal could not be discerned as they were occluded by this precipitate (Fig. 4.15 & Fig. 4.16).

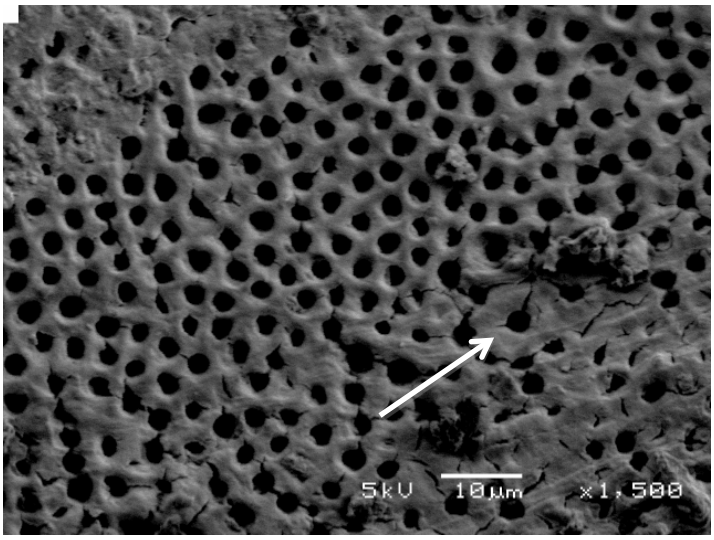

Fig. 4.14: An SEM photograph of a coronal section of a root canal irrigated with 3% NaOCl and 2% CHX. A precipitate could be observed covering the peritubular and intertubular dentine in some areas (arrow). No *E. faecalis* cells could be observed (magnification: 1500X)

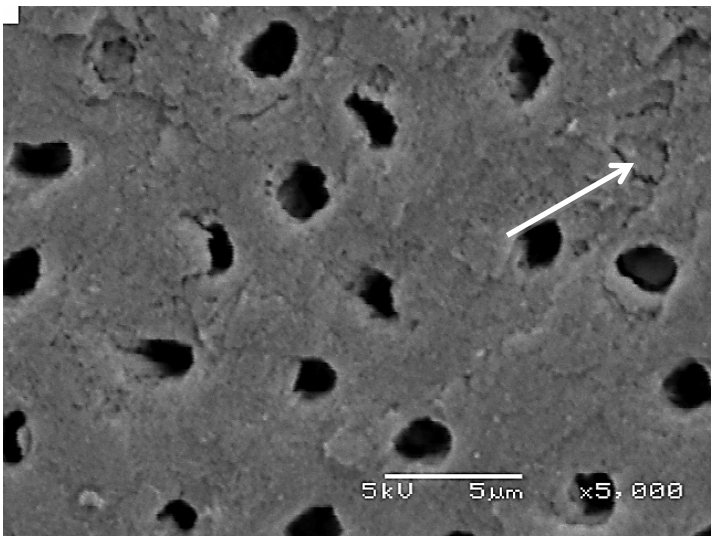

Fig. 4.15: An SEM photograph of a middle section of a root canal irrigated with 3% NaOCl and 2% CHX. A precipitate could be observed covering the peritubular and intertubular dentine. This layer occluded some of the dentinal tubule orifices (arrow). No *E. faecalis* cells could be observed (magnification: 5000X)

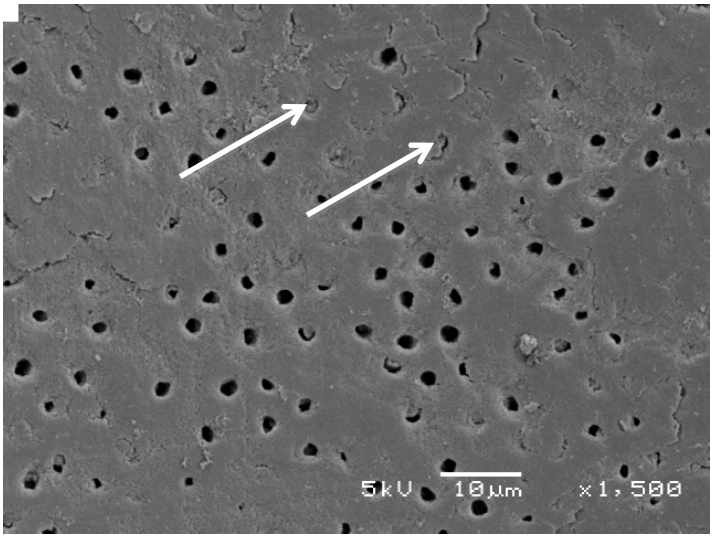

Fig. 4.16: An SEM photograph of an apical section of a root canal irrigated with 3% NaOCl followed by 2% CHX. A precipitate was observed upon the peritubular and intertubular dentine. Some dentinal tubule orifices had been occluded by this layer (arrows). No *E. faecalis* cells could be observed (magnification: 1500X)

4.5.5 3% Sodium Hypochlorite and Photo-Activated Disinfection (PAD)

No *E. faecalis* cells could be observed in any of the three areas of the root canals (Fig. 4.17, Fig. 4.18, and Fig. 4.19). Erosion of the intertubular dentine of the dentinal tubules of the coronal sections could be observed (Fig. 4.17). In the middle section peritubular erosion was signified by enlargement of the diameter of the dentinal tubule orifices (Fig. 4.18).

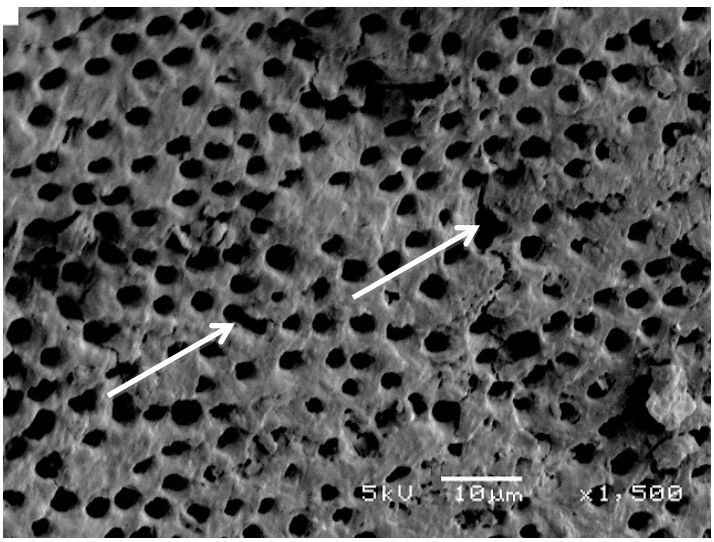

Fig. 4.17: An SEM photograph of the coronal part of a root canal irrigated with 3% NaOCl followed treatment with PAD. Enlargement of the dentinal tubule orifices and destruction of the intertubular dentine (arrows) could be observed. No *E. faecalis* cells could be observed (magnification: 1500X)

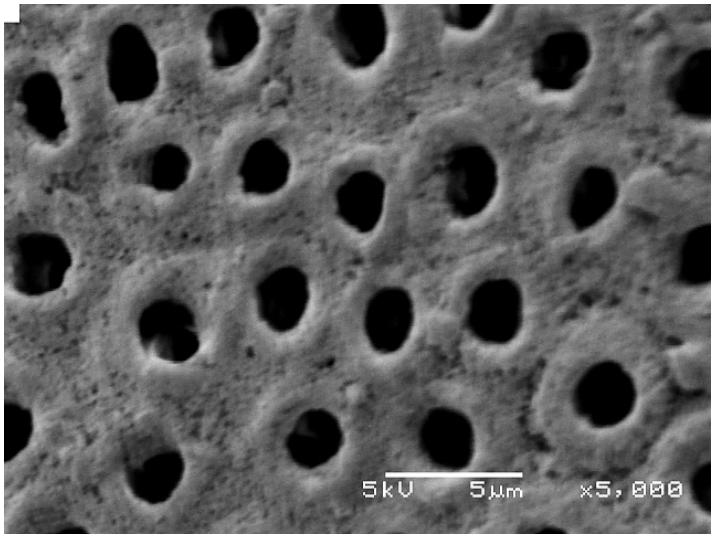

Fig. 4.18: An SEM photograph of a middle section of a root canal irrigated with 3% NaOCl followed by PAD. Enlargement of the dentinal tubule orifices could be observed. No intertubular dentine had been destroyed. No *E. faecalis* cells could be observed (magnification: 5000X)

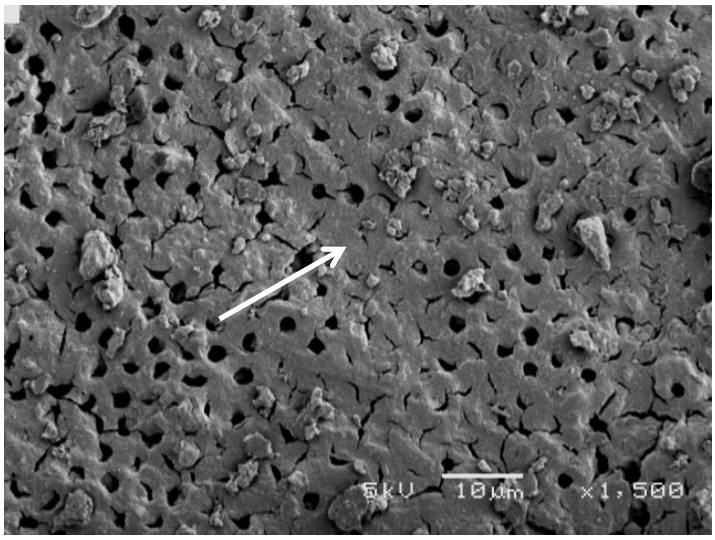

Fig. 4.19: An SEM photograph of an apical section of a root canal irrigated with 3% NaOCl followed by PAD. Some smear layer could be observed covering the intertubular dentine (arrow). No *E. faecalis* cells could be observed (magnification: 1500X)

4.5.6 Photo-activated Disinfection (PAD)

In the coronal section, several *E. faecalis* cells could be observed on the intertubular dentine between the dentinal tubule orifices (Fig. 4.20). The middle layer had numerous *E. faecalis* cells distributed on the intertubular dentine (Fig. 4.21). In the apical section abundant *E. faecalis* cells could be observed clustered in groups on the peritubular and intertubular dentine (Fig. 4.22).

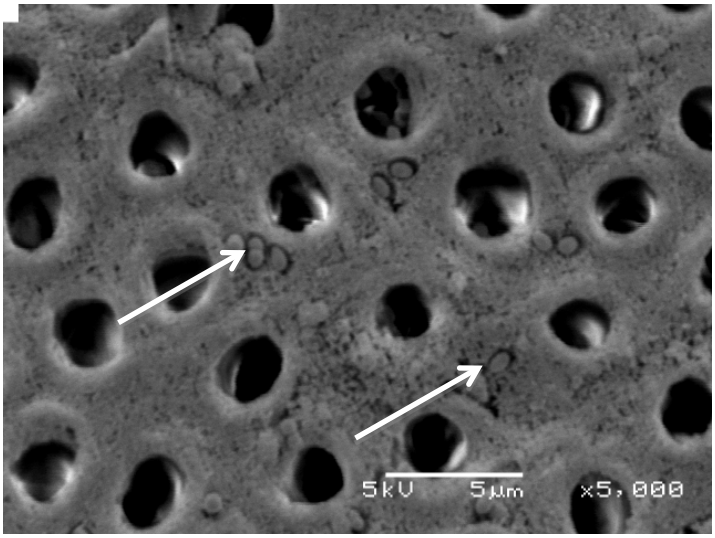

Fig. 4.20: An SEM photograph of the coronal section of a root canal treated with PAD. Several *E. faecalis* cells could be observed on the intertubular dentine (arrows) (magnification: 5000X)

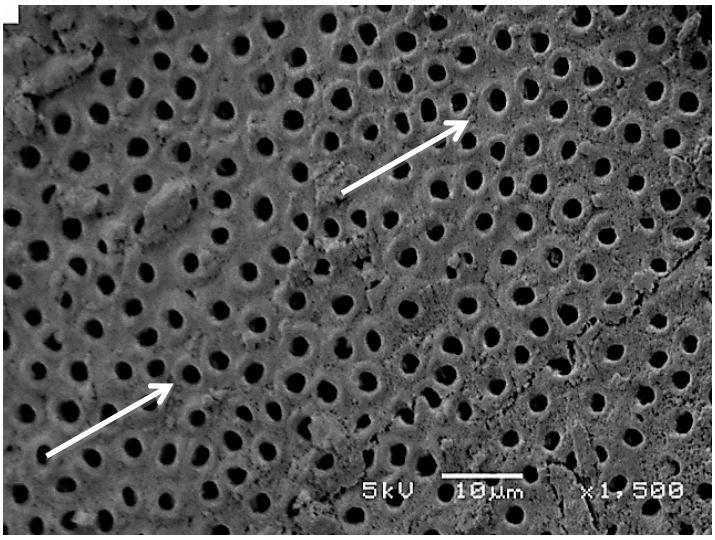

Fig. 4.21: An SEM photograph of the middle section of a root canal treated with PAD. *Enterococcus faecalis* cells could be observed on the intertubular dentine (arrows) (magnification: 1500X)

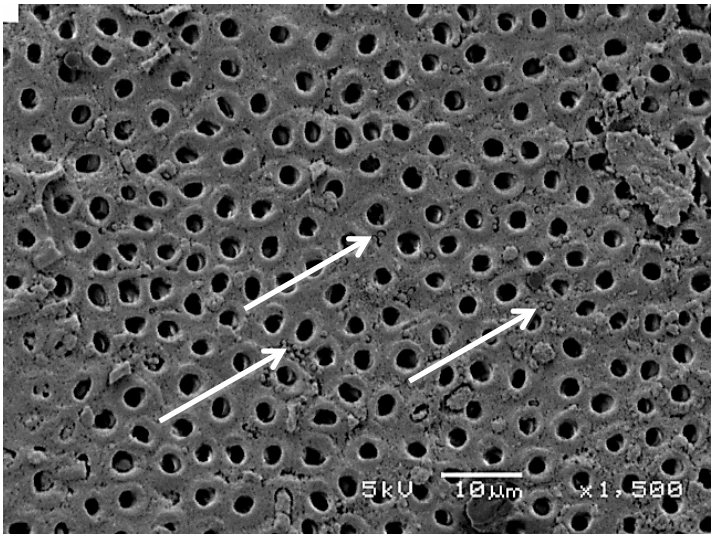

Fig. 4.22: An SEM photograph of the apical section of a root canal treated with PAD. Abundant *E. faecalis* cells could be observed on the intertubular dentine (arrows) (magnification: 1500X)

4.5.7 Distilled Water (Control)

As was observed in the SEM images of the positive control (Fig. 4.4), numerous *E. faecalis* cells could be observed on the peritubular, intertubular and intratubular dentine at all three levels of the root canals of this group.

Table 4.1: The cfu counts of group 1: 3% Sodium Hypochlorite (NaOCl)

Sample	Coronal cfu x 10^1	Middle cfu x 10^1	Apical cfu x 10^1	Total Canal cfu x 10^1
1	5	156	295	456
2	19	35	483	537
3	4	41	612	657
4	12	69	516	597
5	9	132	798	939
6	31	48	588	667
7	21	32	229	282
8	13	248	624	893
9	14	56	608	678
10	6	139	302	447
11	18	147	696	861
12	5	175	402	582
Mean	13.08	106.5	512.75	633

Table 4.2: The cfu counts of group 2: 2% Chlorhexidine Digluconate (CHX)

Sample	Coronal cfu x 10^1	Middle cfu x 10^1	Apical cfu x 10^1	Total Canal cfu x 10^1
1	0	0	0	0
2	0	1	19	20
3	0	3	79	82
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	1	2	3
8	0	12	321	333
9	0	22	38	60
10	0	0	0	0
11	0	0	0	0
12	0	7	116	123
Mean	0	3.83	47.92	51.75

Table 4.3: The cfu counts of group 3: Chlor-XTRA

Sample	Coronal cfu x 10^1	Middle cfu x 10^1	Apical cfu x 10^1	Total Canal cfu x 10^1
1	0	0	0	0
2	0	0	0	0
3	0	0	1	1
4	0	1	1	2
5	0	0	0	0
6	0	0	0	0
7	0	0	1	1
8	0	0	0	0
9	0	0	1	1
10	0	0	1	1
11	0	0	1	1
12	0	1	1	2
Mean	0	0.17	0.58	0.75

Table 4.4: The cfu counts of group 4: combination of 3% Sodium Hypochlorite (NaOCl) followed by 2% Chlorhexidine Digluconate (CHX)

Sample	Coronal cfu x 10^1	Middle cfu x 10^1	Apical cfu x 10^1	Total Canal cfu x 10^1
1	0	0	0	0
2	0	0	11	11
3	0	0	6	6
4	0	0	0	0
5	0	0	0	0
6	0	0	0	0
7	0	0	0	0
8	0	0	0	0
9	0	0	1	1
10	0	0	0	0
11	0	0	0	0
12	0	0	1	1
Mean	0	0	1.58	1.58

Table 4.5: The cfu counts of group 5: 3% Sodium Hypochlorite (NaOCl) followed by Photo-activated Disinfection (PAD)

Sample	Coronal cfu x 10^1	Middle cfu x 10^1	Apical cfu x 10^1	Total Canal cfu x 10^1
1	0	0	1	1
2	0	0	9	9
3	0	0	0	0
4	0	0	0	0
5	0	0	1	1
6	0	0	7	7
7	0	0	0	0
8	0	0	0	0
9	0	0	0	0
10	0	0	2	2
11	0	0	0	0
12	0	0	2	2
Mean	0	0	1.83	1.83

Table 4.6: The cfu counts of group 6: Photo-activated Disinfection (PAD)

Sample	Coronal cfu x 10^1	Middle cfu x 10^1	Apical cfu x 10^1	Total Canal cfu x 10^1
1	457	438	4296	5191
2	292	91	4248	4631
3	241	295	561	1061
4	177	187	1309	1673
5	434	406	3516	4356
6	283	167	4266	4716
7	242	198	4194	4634
8	352	432	3444	4228
9	443	420	4128	4991
10	389	376	3720	4096
11	351	272	3384	4007
12	227	213	3408	3848
Mean	324	291.25	3372.83	3952.67

Table 4.7: The cfu counts of group 7: Distilled Water (control)

Sample	Coronal cfu x 10^1	Middle cfu x 10^1	Apical Cfu x 10^1	Total Canal cfu x 10^1
1	234	454	2389	3077
2	469	1845	3474	5788
3	454	1116	2076	3646
4	302	516	1788	2606
5	448	1308	2964	4720
6	321	1356	2376	4053
7	461	1446	2712	4619
8	212	481	3624	4317
9	206	526	1848	2580
10	487	1746	2376	4609
11	453	1632	2616	4701
12	422	1512	3276	5210
Mean	372.42	1161.50	2626.58	4160.50

Table 4.8: Kruskal Wallis test comparison of the variable cfu counts of all seven treatment groups

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl	36	6123.00	4554.0	386.185301	170.08333
2% CHX	36	3113.00	4554.0	386.185301	86.472222
Chlor-XTRA	36	2619.00	4554.0	386.185301	72.750000
3% NaOCl + CHX	36	2370.50	4554.0	386.185301	65.847222
3% NaOCl + PAD	36	2485.50	4554.0	386.185301	69.041667
PAD	36	7342.50	4554.0	386.185301	203.958333
Control	36	7824.50	4554.0	386.185301	217.347222

Average scores were used for ties.

Kruskal–Wallis Test

Chi-Square	205.7565
DF	6
Pr > Chi-Square	<0.0001

Table 4.9: Comparison of the variable cfu counts of the 3% Sodium Hypochlorite (NaOCl) treatment group to those of the 2% Chlorhexidine Digluconate (CHX) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl	36	1855.0	1314.0	87.131760	51.527778
2% CHX	36	773.0	1314.0	87.131760	21.472222

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1855.0000
Normal Approximation	
Z	6.2032
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	38.5515
DF	1
Pr > Chi-Square	<0.0001

Table 4.10: Comparison of the variable cfu counts of the 3% Sodium Hypochlorite (NaOCl) treatment group to those of the Chlor-XTRA treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl	36	1962.0	1314.0	86.333043	54.50
Chlor-XTRA	36	666.0	1314.0	86.333043	18.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1962.0000
Normal Approximation	
Z	7.5000
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	56.3373
DF	1
Pr > Chi-Square	<0.0001

Table 4.11: Comparison of the variable cfu counts of the 3% Sodium Hypochlorite (NaOCl) treatment group to those of the combination (3% NaOCl and 2% CHX) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl	36	1948.0	1314.0	84.805743	54.111111
3% NaOCl + 2% CHX	36	680.0	1314.0	84.805743	18.888889

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1948.0000
Normal Approximation	
Z	7.4700
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal –Wallis Test

Chi-Square	55.8892
DF	1
Pr > Chi-Square	<0.0001

Table 4.12: Comparison of the variable cfu counts of the 3% Sodium Hypochlorite (NaOCl) treatment group to those of the 3% Sodium Hypochlorite (NaOCl)/Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl	36	1953.50	1314.0	85.520198	54.263889
3% NaOCl + PAD	36	674.50	1314.0	85.520198	18.736111

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1953.5000
Normal Approximation	
Z	7.4719
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	55.9170
DF	1
Pr > Chi-Square	<0.0001

Table 4.13: Comparison of the variable cfu counts of the 3% Sodium Hypochlorite (NaOCl) treatment group to those of the Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl	36	928.50	1314.0	88.790464	25.791667
PAD	36	1699.50	1314.0	88.790464	47.208333

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	928.5000
Normal Approximation	
Z	-4.3361
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal –Wallis Test

Chi-Square	18.8502
DF	1
Pr > Chi-Square	<0.0001

Table 4.14: Comparison of the variable cfu counts of the 3% Sodium Hypochlorite (NaOCl) treatment group to those of the Distilled Water (control) group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl	36	806.0	1314.0	88.788322	22.388889
Control	36	1822.0	1314.0	88.788322	50.611111

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	806.0000
Normal Approximation	
Z	-5.7158
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	32.7353
DF	1
Pr > Chi-Square	<0.0001

Table 4.15: Comparison of the variable cfu counts of the 2% Chlorhexidine Digluconate (CHX) treatment group to those of the Chlor-XTRA treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
2% CHX	36	1413.0	1314.0	71.096443	39.250
Chlor-XTRA	36	1215.0	1314.0	71.096443	33.750

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1413.0000
Normal Approximation	
Z	1.3854
One-sided Pr > Z	0.0830
Two-sided Pr > |Z|	0.1659
t Approximation	
One-sided Pr > Z	0.0851
Two-sided Pr > |Z|	0.1703

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	1.9390
DF	1
Pr > Chi-Square	0.1638

Table 4.16: Comparison of the variable cfu counts of the 2% Chlorhexidine Digluconate (CHX) treatment group as compared to those of the combination (3% NaOCl and 2% CHX) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
2% CHX	36	1465.0	1314.0	64.611188	40.694444
3% NaOCl + 2% CHX	36	1163.0	1314.0	64.611188	32.305556

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1465.0000
Normal Approximation	
Z	2.3293
One-sided Pr > Z	0.0099
Two-sided Pr > |Z|	0.0198
t Approximation	
One-sided Pr > Z	0.0113
Two-sided Pr > |Z|	0.0227

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	5.4618
DF	1
Pr > Chi-Square	0.0194

Table 4.17: Comparison of the variable cfu counts of the 2% Chlorhexidine Digluconate (CHX) treatment group to those of the 3% Sodium Hypochlorite (NaOCl)/Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
2% CHX	36	1441.50	1314.0	67.502113	40.041667
3% NaOCl + PAD	36	1186.50	1314.0	67.502113	32.958333

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1441.5000
Normal Approximation	
Z	1.8814
One-sided Pr > Z	0.0300
Two-sided Pr > |Z|	0.0599
t Approximation	
One-sided Pr > Z	0.0320
Two-sided Pr > |Z|	0.0640

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	3.5677
DF	1
Pr > Chi-Square	0.0589

Table 4.18: Comparison of the variable cfu counts of the 2% Chlorhexidine Digluconate (CHX) treatment group to those of the Photo-activated disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
2% CHX	36	680.0	1314.0	87.133943	18.888889
PAD	36	1948.0	1314.0	87.133943	54.111111

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	680.0000
Normal Approximation	
Z	-7.2704
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	52.9424
DF	1
Pr > Chi-Square	<0.0001

Table 4.19: Comparison of the variable cfu counts of the 2% Chlorhexidine Digluconate (CHX) treatment group to those of the Distilled Water (control) group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
2% CHX	36	670.50	1314.0	87.131760	18.6250
Control	36	1957.50	1314.0	87.131760	54.3750

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	670.5000
Normal Approximation	
Z	-7.3796
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	54.5436
DF	1
Pr > Chi-Square	<0.0001

Table 4.20: Comparison of the variable cfu counts of the combination (3% NaOCl and 2% CHX) treatment group to those of the Chlor-XTRA treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl + 2% CHX	36	1386.0	1314.0	59.422574	38.50
Chlor-XTRA	36	1242.0	1314.0	59.422574	34.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1386.0000
Normal Approximation	
Z	1.2032
One-sided Pr > Z	0.1144
Two-sided Pr > |Z|	0.2289
t Approximation	
One-sided Pr > Z	0.1164
Two-sided Pr > |Z|	0.2329

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	1.4681
DF	1
Pr > Chi-Square	0.2256

Table 4.21: Comparison of the variable cfu counts of the Chlor-XTRA treatment group as compared to those of the 3% Sodium Hypochlorite (NaOCl)/ Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
Chlor-XTRA	36	1350.0	1314.0	62.806544	37.50
3% NaOCl + PAD	36	1278.0	1314.0	62.806544	35.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1350.0000
Normal Approximation	
Z	0.5652
One-sided Pr > Z	0.2860
Two-sided Pr > |Z|	0.5719
t Approximation	
One-sided Pr > Z	0.2869
Two-sided Pr > |Z|	0.5737

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	0.3285
DF	1
Pr > Chi-Square	0.5665

Table 4.22: Comparison of the variable cfu counts of the Chlor-XTRA group as compared to those of the Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
Chlor-XTRA	36	666.0	1314.0	86.333777	18.50
PAD	36	1962.0	1314.0	86.333777	54.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	666.0000
Normal Approximation	
Z	-7.5000
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	56.3363
DF	1
Pr > Chi-Square	<0.0001

Table 4.23: Comparison of the variable cfu counts of the Chlor-XTRA treatment group as compared to those of the Distilled Water (control) group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
Chlor-XTRA	36	666.0	1314.0	86.332309	18.50
Control	36	1962.0	1314.0	86.332309	54.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	666.0000
Normal Approximation	
Z	-7.5001
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	56.3382
DF	1
Pr > Chi-Square	<0.0001

Table 4.24: Comparison of the variable cfu counts of the combination (3% NaOCl and 2% CHX) treatment group to those of the 3% Sodium Hypochlorite (NaOCl)/ Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl + 2% CHX	36	1283.50	1314.0	53.380246	35.652778
3% NaOCl + PAD	36	1344.50	1314.0	53.380246	37.347222

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1283.5000
Normal Approximation	
Z	-0.5620
One-sided Pr > Z	0.2871
Two-sided Pr > |Z|	0.5741
t Approximation	
One-sided Pr > Z	0.2879
Two-sided Pr > |Z|	0.5759

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	0.3265
DF	1
Pr > Chi-Square	0.5677

Table 4.25: Comparison of the variable cfu counts of the combination (3% NaOCl and 2% CHX) treatment group to those of the Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl + 2% CHX	36	666.0	1314.0	84.807985	18.50
PAD	36	1962.0	1314.0	84.807985	54.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	666.0000
Normal Approximation	
Z	-7.6349
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	58.3817
DF	1
Pr > Chi-Square	<0.0001

Table 4.26: Comparison of the variable cfu counts of the combination (3% NaOCl and 2% CHX) treatment group to those of the Distilled Water (control) group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl + 2% CHX	36	666.0	1314.0	84.807985	18.50
Control	36	1962.0	1314.0	84.807985	54.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	666.0000
Normal Approximation	
Z	-7.6350
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	58.3837
DF	1
Pr > Chi-Square	<0.0001

Table 4.27: Comparison of the variable cfu counts of the combination (3% NaOCl and 2% CHX) treatment group to those of the Photo-activated Disinfection (PAD) treatment group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl + PAD	36	666.0	1314.0	84.807985	18.50
PAD	36	1962.0	1314.0	84.807985	54.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	666.0000
Normal Approximation	
Z	-7.5712
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	57.4113
DF	1
Pr > Chi-Square	<0.0001

Table 4.28: Comparison of the variable cfu counts of the combination (3% NaOCl and 2% CHX) treatment group to those of the Distilled Water (control) group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
3% NaOCl + PAD	36	666.0	1314.0	84.807985	18.50
Control	36	1962.0	1314.0	84.807985	54.50

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	666.0000
Normal Approximation	
Z	-7.5713
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001
t Approximation	
One-sided Pr > Z	<0.0001
Two-sided Pr > |Z|	<0.0001

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	57.4133
DF	1
Pr > Chi-Square	<0.0001

Table 4.29: Comparison of the variable cfu counts of Photo-activated Disinfection (PAD) treatment group to those of the Distilled Water (control) group

Treatment Group	N	Sum of Scores	Expected Under HO	Std Deviation Under HO	Mean Score
PAD	36	1139.0	1314.0	88.790464	31.638889
Control	36	1489.0	1314.0	88.790464	41.361111

Average scores were used for ties.

Wilcoxon Two-Sample Test

Statistic	1139.0000
Normal Approximation	
Z	-1.9653
One-sided Pr > Z	0.0247
Two-sided Pr > |Z|	0.0494
t Approximation	
One-sided Pr > Z	0.0266
Two-sided Pr > |Z|	0.0533

Z includes a continuity correction of 0.5

Kruskal–Wallis Test

Chi-Square	3.8846
DF	1
Pr > Chi-Square	0.0487

CHAPTER 5: DISCUSSION

In this *in vitro* study the ability of six different root canal disinfection regimens to eradicate the anaerobic bacteria *E. faecalis* from the root canals of human maxillary incisors was investigated. The disinfection efficacy was compared by microbiological culture and scanning electron microscopy. The extracted tooth model has been shown to be a reliable method for evaluating the bacterial eradication efficacy of root canal irrigants (Hope *et al*., 2011).

In order to attempt to remove the smear layer, which is formed during root canal preparation, 3% sodium hypochlorite (NaOCl) was used for irrigation during preparation of the samples and 17% ethylene diamine tetra-acetic acid (EDTA) as the final rinse (continuous passive irrigation) for one minute. This method of smear layer removal is advocated by the authors of several articles (Tinaz *et al*., 2006; Arruda *et al*., 2007; da Silva *et al*., 2008; Saito *et al*., 2008). The present study showed that it was not possible to consistently remove the entire smear layer in the apical regions of the root canals using this method. Recent studies have shown ways in which smear layer removal can be improved. It has been demonstrated that increasing the time of continuous passive irrigation from one minute to three minutes while maintaining the volume of the 17% EDTA at 5ml ensures that little to no smear layer is left behind (Mello *et al.,* 2010). The smear layer is also far more effectively removed when the 17% EDTA is activated by apical negative pressure (EndoVac) or manual dynamic activation as compared to passive irrigation or passive ultrasonic irrigation (Saber and Hashem, 2011).

Enterococcus faecalis was chosen as the test organism in this study for its resilient, resistant nature (Ferrari, Cai and Bombana, 2005; Appelbe and Sedgley, 2007; Bryce *et al*., 2009). A microorganism found in the oral cavity of humans, *E. faecalis* is the single bacterial pathogen most commonly implicated in mono-infective post-endodontic cases of recurring diseases of the dental pulp and periapical tissues (Hancock *et al*., 2001; Schirrmeister *et al*., 2009; Wang *et al*., 2012). The close association of this bacterial species with failing endodontic treatment can be attributed to its ability to survive under facultative anaerobic conditions (Figdor, 2002; Liu *et al*., 2010).

5.1 *Enterococcus faecalis* Elimination

The differences between the performance of the regimen of 3% NaOCl in combination with 2% CHX, 2% CHX, Chlor-XTRA (5.25% NaOCl) or 3% NaOCl/PAD-combination were not statistically significant. Despite this finding, the regimen of 3% NaOCl in combination with 2% CHX was slightly more efficient at eradicating *E. faecalis* from the root canals than the five other disinfection regimens that were tested.

One hundred per cent of the coronal and middle sections and 67% of the apical sections of the root canals of the combination group of 3% NaOCl and 2% CHX rendered no colony forming units (cfu). Scanning Electron Microscopy (SEM) examination revealed no *E. faecalis* cells at any of the three levels of the canals in this group. In contrast to these findings, Vianna and colleagues found no enhancement of the bacterial eradication ability of CHX by using it in combination with NaOCl (Vianna and Gomes, 2009). More recently a study by Baca and colleagues showed that although the initial residual activity of 2.5% NaOCl was low (18.10%) the bacterial kill rate was increased to 100% when this same 2.5% NaOCl irrigation protocol culminated in a final rinse with 2% CHX (Baca *et al*., 2011).

In the present study CHX was used at a concentration of 2%. The dentine samples from the coronal area of the 12 root canals irrigated with 2% CHX rendered no cfu and at the middle and the apical levels half of the root canals were also bacteria-free. Low numbers of bacteria could, however, be observed by SEM examination at all three levels in some samples. The ability of CHX to reduce bacterial numbers in root canals is supported by several studies (Baca *et al*., 2011; Rôças and Siqueira, 2011). Shahani and Subba Reddy have also advocated the use of 2% CHX as a final rinse because of its excellent substantivity in the first 72 hours after irrigation (Shahani and Subba Reddy, 2013).

A disadvantage of CHX is the inability of this solution to dissolve the organic component of bacterial cells. This is the reason why CHX is less effective at disrupting and removing bacterial biofilms compared to NaOCl (Clegg *et al*., 2006; del Carpio-Perochena *et al*., 2011). One study by Arias-Moliz and colleagues used *E. faecalis*-

infected dentine powder to evaluate the disinfection potential of several irigation solutions. This study showed that under ideal conditions 2% CHX was able to destroy bacterial biofilm in two minutes (Arias-Moliz *et al*., 2009).

In the samples from the root canals of the 3% NaOCl irrigation group significantly fewer numbers of *E. faecalis* cfu were cultured than from the control group ($p < 0.0001$). These findings are in agreement with the findings of several other studies that showed 2.5% NaOCl to be effective in decreasing the number of bacteria in infected root canals (Rôças and Siqueira, 2011). In agreement with the work of Retamozo and colleagues, the most likely reason for the lack of root canal disinfection seems to be that a concentration of 3% NaOCl is not potent enough to completely eradicate *E. faecalis* from infected dentine within the duration of irrigant exposure in our study (Retamozo *et al*., 2010).

There are not very many studies specifically investigating Chlor-XTRA as an irrigation solution. A recent study by Jungbluth and colleagues compared Chlor-XTRA to several brands of household bleach (NaOCl) (Jungbluth *et al*., 2012). The findings of this study were that compared to commercially available NaOCl, this solution had no unique characteristics. It was also determined that the suggested advantage of reduced surface tension did not significantly improve the soft tissue dissolution by Chlor-XTRA (Jungbluth *et al*., 2012). Consequently, the higher cost of this solution seems to be the only concrete difference between Chlor-XTRA and NaOCl of the same concentration and studies comparing 5.25% to 6% NaOCl have been referred to in this discussion.

The work of Retamazo and colleagues showed that the bacteria-elimination ability of NaOCl is dependent upon the concentration of the solution and the duration for which it is used in the root canal (Retamozo *et al*., 2010). The findings of several other studies support the findings of the present study in showing that Chlor-XTRA (5.25% NaOCl) is significantly better at eradicating the test organism than 3% NaOCl (Ayhan *et al*., 1999; Oliveira *et al*., 2007; Retamozo *et al*., 2010).

Despite the positive cfu count for the 3% NaOCl group, no *E. faecalis* cells could be observed at the coronal or middle level of the root canals on SEM examination. Very

few cells were detected in the apical levels of the root canals (Fig. 5.7). The most likely explanation for this is that the bacterial cells were situated more deeply in the dentinal tubules than could be visualised on the SEM. It has also been shown that *E. faecalis* is more resistant to NaOCl. However, by using a relatively low concentration of 0.5% NaOCl for an increased duration of time (30 min) it has been shown that cfu counts of zero are attainable (Radcliffe *et al*., 2004).

The results of the present study with regard to the efficacy of PAD both as a supplementary disinfection method to conventional irrigation with 3% NaOCl or when used alone are in agreement with the findings of the study by Souza and colleagues. They found that PAD applied with either methylene blue (MB) or toluidine blue (TB) did not significantly enhance root canal disinfection secondary to after chemo-mechanical preparation using NaOCl as an irrigant (Souza *et al*., 2010).

At all levels of the root canals to which only PAD had been applied, high numbers of *E. faecalis* cells were found both with culture and upon SEM examination. The results of the bacterial reduction ability of this disinfection method were not significantly different from those obtained with irrigation with distilled water.

Contrary to the findings of he present study, Soukos and colleagues showed PAD to be very effective when applied alone for root canal disinfection. These authors' results showed the *E. faecalis* biofilm was reduced by 97% with the use of methylene blue and a fibre optic diode laser, which emitted light through a radius of 360° (Soukos *et al*., 2006). Foschi and colleagues also observed good results with PAD, finding that PAD with a diode laser achieved a bacterial (*E. faecalis*) reduction of 77,5% (Foschi et al., 2007).

The results of the study by Schlafer and colleagues in 2010 were also contradictory to the findings of the present study. Using a conventional LED as the light source and TB as the dye they had similar success to the abovementioned diode laser studies. The two-tiered experiment carried out by Schlafer and coleagues in 2010, showed a 99.7% reduction of the bacteria in suspension and a 95.82% reduction of bacteria that had been adhered to the root canal wall.

The findings of the present study with regard to PAD alone or when used for the augmentation of 3% NaOCl as a root canal disinfectant are in contrast to the findings of Rios and colleagues. This study showed that when root canals were treated with PAD (using LED as a light source) for 30 seconds alone the number of viable *E. faecalis* cells in the root canal was reduced to 2.9%. This number dropped to only 0.1% when PAD was used secondary to NaOCl irrigation (Rios *et al.*, 2011).

As expected, irrigation with distilled water had no significant effect on the number of bacteria in the root canals. The cfu counts were high at all levels of the root canal and abundant *E. faecalis* cells could be observed on the peritubular, intertubular and intratubular dentine at all three levels of the root canals of this group.

5.2 Erosion of the Root Canal Wall Dentine

Before the present study's experiment commenced, all the root canals had been prepared with rotary files using copious amounts of 3% NaOCl intermittently and a final rinse of 17% EDTA. The prepared root canals that were subsequently irrigated according to the 3% NaOCl irrigation regimen, revealed mild erosion of the peritubular dentine at the middle- and apical levels of the root canal on SEM examination. This finding is in agreement with the study by Sayin and colleagues that showed that 2.5% NaOCl caused erosion of the root canal dentine when it was used in association not only with EDTA but also with EDTAC and tetracyclin-HCl (Sayin *et al.*, 2007). A more recent study by Kaya and colleagues described the images obtained from dentine irrigated with 2.6% NaOCl as having a "wormhole appearance" on SEM examination as a result of the enlarged diameter of the dentinal tubules caused by mild erosion (Kaya, It-Özer and Adigüzel, 2011).

The mechanism by which NaOCl has led to this pronounced dentine erosion is related to the state of the dentine structure as it was at the beginning of the irrigation application in the present study. Dentine consists of an organic network of collagen covered by an inorganic hydroxyl apatite layer (Di Renzo *et al.*, 2001). In the present study, the root canal samples were all prepared with rotary instruments and 3% NaOCl was used for inter-file irrigation. After this, EDTA was used in the root canals as a final irrigant for one minute. When NaOCl comes into contact with the

organic component of the dentine (collagen), an oxidative reaction occurs and the amino acids of the collagen disintegrate (McDonnel and Russel, 1999). If NaOCl is used before EDTA, the inorganic hydroxyapatite component is present to shield the organic collagen fibres. When NaOCl is used after the application of EDTA the collagen is exposed, as EDTA has removed the inorganic hydroxyapatite crystals. The NaOCl comes into direct contact with the collagen, resulting in further disintegration of the organic component of the dentine and subsequent exposure of the next layer of inorganic material (Dogan and Qalt, 2001).

The severity of root canal dentine erosion is directly related to the strength of the concentration of the NaOCl used for irrigation. The study by Zhang and colleagues showed that at a concentration of 1.3%, NaOCl is able to extract organic material from mineralised dentine (Zhang *et al*., 2010). The increased severity of intertubular and peritubular erosion that was observed in the root canals of the samples of the Chlor-XTRA group in the present study is in agreement with the findings of the study by Kaya and colleagues, where 60% of samples irrigated with 5.25% NaOCl exhibited severe dentinal erosion (Kaya, It-Özer and Adigüzel, 2011).

5.3 Microscopic Observation of Precipitate Formation

A unique observation was made in the SEM images of the root canals in the 2% CHX group. A distinctive pattern of precipitation in a ring-like pattern had formed within the dentinal tubules. Precipitate could also be observed on the peritubular and intertubular dentine at all levels of the root canal (Fig. 5.8 to Fig 5.10). A possible explanation for this phenomenon is the manner in which CHX binds to the organic and inorganic components of dentine. These CHX-molecules can bind to type I collagen, forming quite a stable monolayer of CHX retained within the dentine structure (Breschi *et al*., 2010; Baca *et al*., 2011; Kim *et al*., 2011; Singh *et al*., 2011).

This CHX-rich layer can act as a smear layer and block access to dentinal tubules. This has been shown to interfere with the bonding ability of root canal sealer to the root canal wall, negatively affecting apical seal (Bodrumlu, Parlak and Bodrumlu, 2010). Two positive consequences of the CHX-rich layer formation are that it inhibits dentine proteases and it has been found to strengthen the scaffold at the dentine-

resin bonding interface. Advantage can be taken of these factors by electing to use resin-based root canal sealers and resin-bonded post and composite resin cores (Carrilho *et al*., 2009).

The excellent long-term antibacterial action of CHX facilitated by it's binding to bacteria and dentine should be kept in mind (Carrilho *et al*., 2009; Kim *et al*., 2010; Souza *et al.,* 2010; Singh *et al*., 2011). In the present study the duration of irrigation with this irrigant was four minutes. The root canals were then rinsed with sterile water and the samples were fixed in gluteraldehyde, which would deactivate the CHX molecules. The substantivity of CHX has been shown to give lasting antibacterial effects for 90 days after irrigation (Souza *et al*., 2010).

Another group in which a precipitate was observed was the combination regime of 3% NaOCl followed by 2% CHX. This precipitate could be seen on the peritubular and intertubular dentine at all three levels (Fig. 5.14 to Fig. 5.16). Precipitate was not situated inside the dentinal tubules in the same pattern observed in the CHX group. The precipitate became incrementally denser in a coronal to apical direction, obscuring several of the dentinal tubule orifices at the middle and apical levels.

A likely origin of this precipitate is the acid-base reaction that occurs when NaOCl and CHX come into contact (Basrani *et al*., 2007; Marchesan *et al*., 2007; Bui, Baumgartner and Mitchell, 2008; Akisue *et al*., 2010; Krishnamurthy and Sudhakoran, 2010). Several studies have shown that formation of this precipitate, commonly known as a "flocculate", can occur when the NaOCl concentration is between 0.023% and 5.25% and that of CHX is from 0.16% to 2% (Marchesan *et al*., 2007; Bui, Baumgartner and Mitchell, 2008; Akisue *et al*., 2010; Prado *et al*., 2013). Flocculate forms immediately when the two solutions come into contact with one another. The flocculate has a brown-orange colour and it adheres firmly to the dentine of the root canal wall (Krishnamurthy and Sudhakoran, 2010; Prado *et al*., 2013). The chemical constituents of this flocculate are two molecules: parachlorophenylurea (PCU) and parachlorophenylguanidyl-1,6 diguanidyl-hexane (PCGH) (Nowicki and Sem, 2011).

This precipitate can present a clinical problem in that it acts as a chemical smear layer that has been shown to affect the bond strength between the root canal sealer and the dentine wall of the root canal (Gupta *et al*., 2013).

In the present study, distilled water was clearly not effective as an inter-irrigant root canal flush to inhibit the NaOCl-CHX reaction. It has been shown that water can merely minimise the amount of precipitate formed, while rinsing the canal with absolute alcohol between irrigants can prevent precipitate formation (Krishnamurthy and Sudhakoran, 2010).

5.4 Future Research

Further studies should be carried out to determine the best method of application of the NaOCl/CHX solution combination regimen for endodontic irrigation. Additional studies need to be carried out to determine whether the additional time, effort and expense needed to apply PAD as a supplementary method of root canal disinfection is justifiable or not.

CHAPTER 6: CONCLUSIONS

The following conclusions can be drawn from this *in vitro* study:

6.1 At the coronal levels of the root canals, four groups: 2% chlorhexidine digluconate (CHX), Chlor-XTRA, 3% sodium hypochlorite (NaOCl) combined with 2% CHX and the group where irrigation with 3% NaOCl was followed by Photo-activated disinfection (PAD) were able to completely eradicate the test organism, *Enterococcus faecalis*.

6.2 At the middle levels of the root canals, two groups: 3% NaOCl combined with 2% CHX and the group where irrigation with 3% NaOCl was followed by Photo-activated disinfection (PAD) were able to completely eradicate *E. faecalis*.

6.3 At the apical levels of the root canals none of the disinfection groups were able to completely eradicate *E. faecalis*.

6.4 The group using 3% NaOCl in combination with 2% CHX showed superior performance over all the other disinfection protocols tested. However, there were no significant differences between the efficacy of this protocol compared to 2% CHX ($p = 0.0194$), Chlor-XTRA ($p = 0.2256$) or 3% NaOCl/PAD ($p = 0.5677$).

6.5 When the root canals were assessed as complete units, disinfection was significantly better in the following four groups: 3% NaOCl in combination with 2% CHX, 2% CHX, Chlor-XTRA and 3% NaOCl/PAD, than in the PAD group, the 3% NaOCl irrigation group and the control group.

6.6 An incidental observation in this study was the presence of mild peritubular dentine erosion in root canals irrigated with the 3% NaOCl irrigation regimen after the initial preparation and irrigation of the samples. Even more severe intertubular and peritubular dentine erosion was observed in the root canals of the samples of the Chlor-XTRA group.

6.7 Another incidental observation in this study was the presence of precipitate deposition. In the group irrigated with 3% NaOCl followed by 2% CHX precipitate could be observed on the peritubular and intertubular dentine at all levels of the root canal. In the CHX group a precipitate had formed in a ring-like pattern within the dentinal tubules.

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ADDENDUM A

Pro Forma – Patient information leaflet and informed consent.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

School of Dentistry

ORAL AND DENTAL HOSPITAL

Dear Patient,

The personnel and students of the Oral and Dental Teaching Hospital of the University of Pretoria appreciate your confidence in us for your dental treatment.

Although we strive to complete treatment as speedily as possible, our primary task is the training of students. For this reason, treatment of patients will necessarily be more time-consuming than in private sector.

The student who is responsible for your treatment is dependent on your promptness, co-operation and availability. While we strive to train students to treat you, as patient, in the best possible manner, we earnestly requested your indulgence should be situation from time to time not always be ideal. We would, however, appreciate you informing us should any circumstances not be to your liking. In this way we shall be able to improve our service to you.
Refusal by a patient to be treated by a particular student/dentist to whom he/she is allocated, is not acceptable. In such circumstances, further routine treatment for a patient will be refused. We appreciate your co-operation in this regard. If you have any enquiries, please discuss it with a lecturer on duty.

The Oral and Dental Hospital is a service rendering unit which is part of the University of Pretoria. Research, apart from teaching, is aimed at the continuous improvement of dental treatment and dental materials. The teaching and research that is done, is partly dependent on obtaining suitable material from our patients. We kindly request you to study the consent form and if you approve, please complete it. If you should have any enquiries, please feel free to discuss it with a lecturer on duty.

CONSENT

Herewith I (full names and surname) _____ (patient/parent/guardian) grant permission to be treated by the Oral and Dental Teaching Hospital and that material and related information obtained during dental procedures at the Oral and Dental Teaching Hospital, University of Pretoria, may be used for dental training an/or research and or the advancement of dentistry in the School of Dentistry, University of Pretoria. The permission is granted with the understanding that my identity will in all circumstances remain anonymous and that all my personal details will be treated with strict confidentiality.

Patient No: _____
Date of Birth: _____

May our association with the Oral and Dental Hospital be a positive and rewarding experience.

Signature: _____

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SUMMARY

Bacteria are the most common pathogens responsible for pulpal necrosis and periapical disease conditions. The importance of eradicating bacteria and their endotoxic by-products by effective root canal irrigation has been highlighted in numerous studies. The aim of this *in vitro* study was to compare the efficacy of six different endodontic disinfection protocols in eradicating *Enterococcus faecalis* from single root canals of human teeth.

Endodontic access cavities were prepared on 86, extracted, single rooted, human teeth. Root canal preparation was done using nickel titanium rotary files. Each tooth was sterilised, inoculated with *E. faecalis* and then randomly allocated to one of seven groups (n = 12). Irrigation solutions were either used alone, in combination with one another or in combination with photo-activated disinfection (PAD). The six disinfection protocols were 3% sodium hypochlorite solution (NaOCl), 2% chlorhexidine digluconate solution (CHX), Chlor-XTRA solution (6% NaOCl), 3% NaOCl combined with 2% CHX, 3% NaOCl followed by PAD and PAD applied alone. The first six groups were subjected to one of the disinfection protocols while the seventh group was irrigated with sterile water (control). The roots of the treated teeth were fractured longitudinally. The first half of each root was divided into three sections: coronal, middle and apical. Samples of the dentine from each of these sections were taken and plated onto brain heart infusion (BHI) agar plates. These were incubated at 370C for five days in facultative anaerobic conditions. The colony-forming units (cfu) were counted. The second half of each root was prepared for Scanning Electron Microscopy (SEM) according to standard methods. The Pairwise Wilcoxon Rank Sum test and the Kruskal-Wallis test were used to compare the cfu counts of the seven groups to one another. The results showed that 2% CHX, Chlor-XTRA, combination of 3% NaOCl and 2% CHX and the group where irrigation with 3% NaOCl was followed by PAD were able to completely eradicate *E. faecalis* from the coronal levels of the root canals. The group using a combination of 3% NaOCl and 2% CHX and the group where irrigation with 3% NaOCl was followed by PAD were able to

completely eradicate *E. faecalis* from the middle levels of the root canals. None of the disinfection groups were able to completely eradicate the test organism from the apical levels of the root canals. The regimen of 3% NaOCl used in combination with 2% CHX was slightly more efficient in eradicating *E. faecalis* from the root canals than the five other disinfection regimens that were tested. Erosion of the intertubular and peritubular dentine and precipitae formation were two incidental observations in some of the samples upon SEM examination.

Key Words: root canal irrigation, disinfection, *Enterococcus faecalis* eradication, Photo-activated Disinfection (PAD)