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Analysis of the Substantivity of Chlorhexidine in the Root Canal System

Sidney Rosenthal

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Analysis of the Substantivity of Chlorhexidine in the Root Canal System

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B.S., Florida State University, 1992
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Master of Dental Science Thesis

Analysis of the Substantivity of Chlorhexidine in the Root Canal System

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Introduction

Microorganisms left behind after root canal preparation and disinfection have been shown to be a significant contributor to failure in endodontic therapy (Sjögren et al 1997). Methods to reduce the number of these microbes have been described in numerous studies and include proper instrumentation, the use of an effective irrigating solution, and intracanal medicaments. Coronal microleakage through temporary and permanent restorations is also suggested to be of great importance among factors related to failure (Madison and Wilcox 1988). After root canal obturation and during final coronal restoration, when no antimicrobial agent is present in the canal space, there are circumstances in which this space may become re-infected. Of significance is the period of time when the pulp space is only temporarily closed. IRM and other zinc oxide and eugenol products are popular temporary restorations for endodontically treated teeth. However, there are many studies using dye, bacteria and radioisotopes which have shown these restorations to provide relatively poor seals (Marosky et al 1977, Keller et al 1981, Blaney et al 1981, Anderson et al 1988, Chohayeb et al 1985). Cavit, another popular temporary restorative material, provides an acceptable seal but it is still prone to leakage and it is mechanically weak.
(Barthel et al 1999, Deveaux et al 1999, Kazemi et al 1994). In general, coronal leakage has received a great deal of attention in the endodontic literature. It has been stated that the coronal seal of endodontically treated teeth may be as important as the apical seal in the ultimate success or failure of root canal therapy (Madison and Wilcox 1988). In a study by Madison et al, extracted teeth were obturated with laterally condensed gutta-percha and three different sealers and evaluated by dye penetration (Madison et al 1987). All three sealers demonstrated significant microleakage with AH26 (De Trey) leaking more than Sealapex (Kerr) or Roth’s sealer (Roth Drug Co.). In another study in vitro by Swanson and Madison root canals obturated with laterally condensed gutta-percha and sealer were exposed to artificial saliva for various time periods and evaluated by dye penetration (Swanson and Madison 1987). Penetration of the dye was extensive and was observed as early as 3 days following exposure to the artificial saliva. Torabinejad et al evaluated the penetration of bacteria into obturated and coronally unsealed root canals (Torabinejad et al 1990). Over 50% of the specimens were completely contaminated after a 19-day exposure to Staphylococcus epidermidis and after a 42-day exposure to Proteus vulgaris. Wu and Wesselink observed in a review that in much of the literature concerning dye leakage in teeth with laterally condensed gutta-percha, the
results varied widely while the methodology was very similar (Wu and Wesselink 1993). Although the methods used to perform many of these leakage studies have been criticized it is generally accepted that root canal fillings with available materials will leak to some degree (Spångberg et al 1989, Wu and Wesselink 1993). There is no method known to predictably reduce or eliminate such leakage or kill bacteria that penetrates the obturated pulp space. The inclusion of a long acting antimicrobial agent to eliminate or reduce the effect of leakage would seem logical in light of this provided, of course, this could be accomplished without injury to periradicular tissues or compromising the integrity of the root filling material.

Antimicrobial agents used in endodontic treatment are either irrigation fluids or deposit antiseptics. The current standard irrigant, sodium hypochlorite, possesses many desirable properties including the ability to dissolve necrotic debris as well as a potent antimicrobial action (Byström et al 1985, Byström and Sundqvist 1983, Spångberg et al 1973, Grossman and Meiman 1941). However, it is not known to exhibit any degree of prolonged substantive antimicrobial activity. Intracanal medicaments such as the phenolic compounds, while somewhat effective as antiseptics, are known to dissipate rapidly from the canal space leaving no delayed antimicrobial effect (Messer and Feigal 1984). Calcium hydroxide has
become a standard intracanal medicament due to its ability to predictably disinfect the root canal space (Byström et al 1985, Sjögren et al 1991). After mechanically removing the calcium hydroxide there is no residual antimicrobial effect, however. Furthermore, other studies have suggested that calcium hydroxide is ineffective against Enterococcus faecalis as well as Candida albicans (Waltimo et al 1999, Haapasalo and Ørstavik 1987). This fact is important because infected root canals and endodontically treated teeth with persistent apical periodontitis are frequently found to be infected with Enterococcus faecalis (Sundqvist et al 1998, Molander et al 1998, Winkler and Van Amerongen 1959). Candida albicans has also been suggested as a possible etiology for endodontic failure (Nair et al 1990). Chlorhexidine digluconate (CHX) is a broad-spectrum antibacterial agent that has many attractive characteristics as an antiseptic and it has been shown to be particularly effective against many strains of bacteria found in infected root canals (Ohara et al 1993). Studies in vitro have shown CHX to exhibit sustained antimicrobial activity in the root canal when used as an endodontic irrigant (White et al 1997). CHX has been suggested as a root canal irrigant due to its unique ability to bind to dentin, its effectiveness as an antimicrobial agent, and its apparent substantivity in the root canal

Review of the Literature

Historically, CHX was developed in the late 1940’s in a search for water-soluble polymeric substances which might possess anti-viral activity (Foulkes 1973). Most of those compounds examined were polysaccharides and other non-ionic compounds together with a few anionic compounds. However, none of the compounds tested exhibited anti-viral properties yet were found to be very effective against a wide range of bacteria (Foulkes 1973). CHX was among the best of a range of biguanides found to possess antibacterial qualities and was given the trade name “Hibitane®” (Davies et al 1954).

Chemistry-

CHX is a symmetric cationic molecule consisting of two 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain (Figure II). It is a strong base and is most stable in the form of its salts, normally carrying two protons uniformly distributed over all its ten nitrogen atoms; it contains relatively small lipophilic groups and is
most frequently used as the digluconate salt (Fardal and Turnbull 1986, Davies 1973). The hydrophilic/lipophilic balance appears to be a major factor in the influence in the antibacterial activity of a series of related biguanides (Cutler et al 1966). Specifically, this is related to CHX being a positively charged molecule and its attraction to negatively charged bacteria. CHX is weakly surface active, reducing the surface tension of water to about 50 dynes/cm at 0.59% at which concentration it forms micelles (Heard and Ashworth 1968). In addition, at this concentration, the molecule can fold up on itself so as to have one hydrophilic and one hydrophobic face (Davies 1973).

**Antimicrobial Effects**

CHX, being cationic, interacts with bacteria because they carry negative charges on their surface at physiologic pH values (Davies 1973). Its action is the result of the adsorption of CHX onto the cell wall of the microorganism, resulting in leakage of intracellular components (Gjermo 1974). CHX does not cause lysis of cells as is found with agents such as penicillin and antiseptics such as hypochlorite (Hennessey 1977). At low concentrations of CHX, small molecular weight substances, such as potassium and phosphorus, will leach out of the bacterial cells. This effect on the bacterial cell is bacteriostatic and can be reversed upon removal of
CHX (Fardal and Turnbull 1986). However, the bactericidal effect of CHX is most likely related to the extensive intracellular damage rather than cell membrane leakage (Hennessey 1977). Damage resulting in "leakiness" is reversible and moreover, it is known that some non-ionic detergents can cause extensive leakage without being bactericidal (Hennessey 1977, Davies and Field 1969). In one study, the ability of CHX to increase permeability did not correlate well with bactericidal potency (Davies 1973, Davies and Field 1969). Therefore the real significance of this increased permeability is that it may facilitate entry of CHX to the cytoplasmic constituents and precipitate the cell content (Davies 1973, Davies and Field 1969). Precipitation of cytoplasm has been demonstrated to correlate well with bactericidal activity in a series of biguanides, including CHX (Davies and Field 1969). This precipitation will prevent the repair of the cytoplasmic membranes thus, the bacteria will never recover their content of metabolic intermediates, co-factors, etc and will therefore be non-viable (Davies 1973).

In a study by Hennessey (1973), the wide bacteriostatic spectrum of CHX was evaluated. Exposure of suspensions of various bacterial species to 0.02% CHX for 10 minutes at room temperature reduced the viable organisms by about 99.99% in most cases. It was shown that gram-positive cocci were especially sensitive to CHX with a mean inhibitory concentration
(MIC) of 0.19 to 2.0 μg/ml. However, among the least susceptible bacterial species tested were *Pseudomonas aeruginosa* and *Proteus sp.* Seven of eleven strains of *Pseudomonas aeruginosa* were inhibited only at a concentration of 100μg/ml or more and where two of eleven species of *Proteus sp.* had MIC values of 100μg/ml or more. These tests were made in distilled water, however. Biological fluids have a marked inhibitory effect on the effectiveness of conventional antiseptics, as well as the effect of CHX (Hennessey 1973). Thus, when suspensions of *Pseudomonas aeruginosa* were exposed to CHX in the presence of 10% horse serum, about four times more CHX was required to produce the same antimicrobial effect (Hennessey 1973).

Many organisms affected by CHX have varying degrees of susceptibility. Emilson (1977) studied the susceptibility to CHX of bacteria in aerobic, facultatively anaerobic and anaerobic isolates from clinical specimens of wounds, urine, saliva, and dental plaque. There was a broad range of susceptibility to CHX among both Gram-positive and Gram-negative strains and low MIC values were noted for *Staphylococci sp.*, *S. mutans*, *S. salivarius* and *E. coli*, while strains of *Proteus sp.*, *Pseudomonas sp.* and *Klebsiella sp.* were less susceptible (Emilson 1977). Among the anaerobic isolates tested, the strains most susceptible to chlorhexidine were
Propionibacterium and Selenomonas, while the least susceptible strains were Gram-negative cocci resembling Veillonella (Emilson 1977). Evans et al (1977) performed a study using saliva-treated bovine enamel slabs for determining the potential of chemotherapeutic agents to adsorb to tooth surfaces and act against plaque-forming bacteria. They found that CHX inhibited the formation of plaque in vitro by the bacterial strains tested, A. viscosus, A. naeslundii, S. mutans, and S. sanguis (Evans et al 1977).

Susceptibility of bacterial strains during prolonged use may take place. Hennessey (1973) studied this phenomenon in experiments in vitro and in vivo. Strains of E. Coli and Streptococci sp. were passed in brain-heart infusion containing subinhibitory amounts of various agents including ampicillin, streptomycin, tetracycline, and CHX. Small changes in susceptibility were noted for CHX, however they were considered by the author to be insignificant. Ampicillin and streptomycin exhibited rapid selection for mutant strains of the bacteria tested, as expected (Hennessey 1973). Part of the in vivo portion of this study included treatment of four adults in a regimen of rinsing for one minute twice a day with 0.2% CHX after tooth brushing. After approximately seven weeks, only transitory decreases in susceptibility were noted and this actually improved as the study continued (Hennessey 1973). It is interesting to note that Ps.
aeruginosa was the least susceptible to CHX in that the study. Some other strains of Pseudomonas will actually survive and may even grow in the presence of CHX (Hennessey 1973). In a study by Bassett et al (1970), Pseudomonas multivorans was found to be a contaminant in various disinfecting solutions, including those containing chlorhexidine.

In another study by Hennessey (1977), mean inhibitory concentrations (MIC) were determined for 80 strains of bacteria as was the proportion of each bacterial suspension that was killed during a 10-minute exposure to 0.02% CHX at room temperature. Strains of E. Coli were inhibited at concentrations of CHX of about 1µg/ml whereas certain isolates of Proteus or Pseudomonas were unaffected by 100µg/ml (Hennessey 1977). Of the Gram-positive species, the single strain of S. mutans was exquisitely sensitive at MIC 0.19µg/ml (Hennessey 1977). The other streptococcal and staphylococcal strains were also susceptible to low concentrations of CHX. Streptococcus faecalis, for example, exhibited a range of MIC values from 0.39 to 1.56µg/ml with a mean value of 0.97µg/ml (Hennessey 1977). In general it was shown that bacteria that are highly susceptible to the bacteriostatic action of CHX are also killed relatively easily. However, this association is not complete as organisms with higher MIC values were not necessarily less susceptible to the cidal activity of CHX (Hennessey 1977).
In clinical studies by Rindom-Schiøtt et al (1970), CHX was shown to possess significant antimicrobial action with tooth surfaces remaining relatively free of bacteria. Yet, some common strains still remained especially around the marginal gingiva. Based on these findings, Rölla et al (1970) investigated the specific affinity of CHX for hydroxyapatite. Quantification of CHX was facilitated by the fact that it absorbs UV light and displays distinct maxima around 230nm and 253nm (Rölla et al 1970). Specifically, the concentration of CHX was determined spectrophotometrically by plotting against optical density at 253nm. Adsorption of CHX was determined by allowing it to remain in contact with fresh hydroxyapatite (Bio-Gel HT-6236) for varying time periods and at various temperatures (Rölla et al 1970). One gram of hydroxyapatite was shown to bind maximally about 18µmol of CHX (Rölla et al 1970). The maximum binding capacity varied between 15 and 20 mmol per gram hydroxyapatite among different series (Rölla et al 1970). The amount of CHX adsorbed after 5 minutes and after 30 minutes of contact with hydroxyapatite showed no variance, whereas one minute gave a 25% reduction in adsorption. In a series of pilot experiments it was found that the adsorptions at 22°C and at 37°C were identical, but about 20% lower at 4°C. Adsorption of CHX to tooth surfaces *in vitro* was also measured. Three
human molar teeth were submerged in 3ml of a 2% solution of CHX for 5 minutes. After rinsing with distilled water, the teeth were placed in 10ml of distilled water and the amount of CHX released from the tooth surfaces was measured. They found that each tooth adsorbed approximately 1mg of CHX. In conclusion, their studies showed that CHX adsorbs to hydroxyapatite and tooth surfaces and that it is released from these surfaces when the concentration in the environment decreases (Rölla et al 1970).

In a study by Emilson et al (1973), the uptake of CHX to hydroxyapatite was studied in vitro. Various concentrations of CHX were mixed with hydroxyapatite. In addition various amounts of hydroxyapatite were mixed with a solution of 2% CHX. Data obtained indicated a multilayer absorption at concentrations above 2% and a stable monolayer at lower concentrations (Emilson et al 1973). In other words, at concentrations greater than 2%, the substrate would be saturated and excess would be rapidly released. It was also determined that the uptake of CHX to hydroxyapatite is directly related to the concentration of CHX (Emilson et al 1973). They speculated that application of a low concentration, 0.005-0.01%, topically would give a relatively stable monolayer of CHX and that higher concentrations might give only an oversaturation with a rapid release.
of excess when the surrounding concentration was decreased (Emilson et al 1973).

**Safety**

Chlorhexidine has been widely used in medicine since the early 1950’s and has demonstrated to be safe with very few adverse effects. Adsorption of CHX from the GI tract is low when the compound is given orally to any species, which is reflected in extremely high LD$_{50}$ values (Case 1977). Toxicological evaluation has established a marked difference between intravenous and oral LD$_{50}$ at 22mg/kg and 1800mg/kg respectively (Foulkes 1973). In a teratology study, pregnant rats were dosed orally with aqueous solutions of CHX at dose levels of 10, 25, and 50 mg/kg (Case 1977). Litter size, fetal loss, embryonic and fetal development were unchanged after treatment with CHX as judged by control groups (Case 1977). Peri- and post-natal studies, reproduction and fertility studies at doses as high as 50mg/kg in the rat have similarly shown no effect to the oral administration of CHX (Case 1977). In a study by Magnusson and Heyden (1973), $^{14}$C-chlorhexidine was given orally and analyzed by apposition autoradiography and liquid emulsion microautoradiography. They found that radiolabeled CHX showed a significant affinity to the epithelial lining of the alimentary and respiratory tracts as well as appearing in the liver and
kidneys. While it could be demonstrated that the bulk of the CHX had left the body two weeks after its application, trace amounts were detected even two months in the respiratory tract, in the secretory mucosa of the stomach and in the kidneys (Magnusson and Heyden, 1973). However, it is speculated that the constant presence of $^{14}$C-chlorhexidine in the respiratory tract may be due to the method of administration resulting in the sneezing and coughing of the mice (Magnusson and Heyden, 1973). Additionally, they offer that the presence of $^{14}$C-chlorhexidine or its metabolites in the liver and kidneys merely indicates that it is metabolized and excreted via the usual pathways common to most ingested compounds (Magnusson and Heyden, 1973). To investigate the uptake and retention of $^{14}$C-chlorhexidine in the human mouth, a mouth rinse study using radiolabeled material was conducted (Winrow 1973). Sixty-seven percent of the radiolabeled CHX was removed in the ejected rinse and further analysis revealed that CHX was readily desorbed from sites in the mouth to saliva (Winrow 1973). In another study, systemic absorption of CHX was investigated using $^{14}$C-chlorhexidine as well as a non-radiolabeled form (Rushton 1977). In the $^{14}$C-chlorhexidine study, subjects were administered the dose and urine and feces were collected for up to 10 days. Venous blood samples were taken at frequent intervals during the first 24-hour period. Radioactivity was not
detected in blood samples and less than 0.3% of the dose was detected in the urine (Rushton 1977). In the second portion of the study using non-radiolabeled CHX, the subjects rinsed twice daily with 10ml of CHX mouthrinse for six weeks at which time venous blood samples were taken (Rushton 1977). This resulted in no detection at any time of CHX in the blood. Additionally, the excretion patterns of radioactive material after administration of the ring and chain labeled forms are very similar providing evidence that metabolic cleavage of the CHX molecule does not occur to a significant extent (Winrow 1973, Rushton 1977). The only serious adverse effect found was sensorineural deafness after direct application in the middle ear (Aurnsnes 1981). Aursnes (1981) identified damage to the organ of Corti and pathologic changes to the mucosal lining of the tympanic cavity in guinea pigs after exposure to CHX.

In a study comparing a number of root canal irrigants and antiseptics, all were found to be markedly toxic compared to their antimicrobial effect (Spångberg et al 1973). On a basis of a balance between cytotoxicity and antimicrobial effect, CHX was comparable to other endodontic irrigants. Another study reported good tissue recovery and no more toxicity or tissue irritating effect with CHX than with any other endodontic irrigants tested in vitro and in vivo (Wennberg 1980). A recent study in vivo involving
subcutaneous injections of endodontic irrigants into guinea pigs found CHX to be comparable to others tested and not excessively toxic (Yesiloy et al 1995). Irrigants tested in this last study were Peridex®, 0.12% CHX in water (aq.), NaOCl (5.25%, 2.5%, and 0.5%), 11.6% alcohol, Therasol®, and saline.

**Use of CHX in Endodontics**

With safety and antimicrobial efficacy of CHX having been well demonstrated, its use as an endodontic irrigant has been investigated. In a study *in vitro* by Parsons et al (1980), bovine dentin and pulp specimens were treated with either a 0.02% or 1.00% solution of CHX for either 20 or 40 minutes. Using the test organism *S. faecalis*, acquisition of antibacterial properties by the treated specimens was determined. They reported that both concentrations of CHX was adsorbed to both dentin and pulp tissues and imparted antibacterial properties which then inhibited the growth of *S. faecalis* at 24h and 1 week. In another study *in vitro*, various endodontic irrigants, including CHX, were tested on selected anaerobic bacteria (Ohara et al 1993). These bacteria included *P. magnus, P. acnes, V. parvula, L. fermentum, P. gingivalis*, and *F. nucleatum*. The organisms were mixed separately with dilutions of each irrigant and evaluated for growth. These
dilutions were the following: full strength, 1/5, 1/10, 1/20, and 1/40. The bacteria were allowed to remain in contact with the irrigants for the entire observation period of 1 week, with sampling times of 1 min., 15 min., 30 min., 60 min., and 1 week. Among the irrigants, including 5.25% NaOCl and 3% H₂O₂, 0.2% CHX was found to be the most effective. Specifically CHX was the only irrigant tested to support no growth of bacteria among all dilutions and time periods (Ohara et al 1993). In contrast, a study by Siqueira et al (1998), found 4%NaOCl and 2.5%NaOCl had more potent antimicrobial activity than 2% or 0.2% CHX when zones of inhibition were measured in an agar diffusion test. The tested bacteria included *P. endodontalis*, *P. gingivalis*, *P. nigrescens*, *P. intermedia*, *E. faecalis*, *S. mutans*, *S. sanguis*, and *S. sobrinus*.

A study *in vivo* by Barbosa et al (1997) evaluated the effectiveness of camphorated monochlorophenol (CMPC), CHX, and calcium hydroxide in teeth with necrotic pulps and radiographic evidence of periradicular lesions. All teeth were initially treated with CMPC and then sampled for bacteria at a second appointment. If the samples were negative for bacteria they were obturated, otherwise they were medicated with CMPC, 0.12% CHX, or calcium hydroxide for one week and then resampled. While a higher percentage of cases treated with CHX were negative for bacterial growth at
the third appointment the difference was not statistically significant (Barbosa et al 1997).

Another study *in vivo* was conducted by Ringel et al (1982) in an attempt to compare the effects of CHX and sodium hypochlorite on root canal flora when used as endodontic irrigants in teeth with necrotic pulps. Sixty asymptomatic, single rooted necrotic teeth were used. Thirty teeth were assigned to an experimental group where 0.2% CHX was used, while the other thirty were assigned to a control group using 2.5% sodium hypochlorite. Aerobic and anaerobic microbiologic samples were taken at the beginning and end of each appointment. The investigators concluded that sodium hypochlorite was more effective in yielding negative cultures by the third appointment. In addition they stated that when 0.2% CHX solution is used *in vivo* as an endodontic irrigant, it has no more lasting bactericidal effect than 2.5% sodium hypochlorite. In an attempt to reconcile their results with conflicting data from other studies (Parsons et al 1980), they state that monoculture studies *in vitro* do not accurately reflect the diversity of microorganisms encountered in the root canal system (Ringel et al 1982). In addition, they explain that intracanal microorganisms may exist in symbiotic relationships, which might confer additional resistance to chemical disinfection.
In contrast to Ringels' (1982) study *in vivo*, a study *in vitro* by Jeanssone and White (1994) found that 2.0% CHX and 5.25% sodium hypochlorite were equally effective as antimicrobial irrigants. In this study, three groups of necrotic, extracted teeth were instrumented and irrigated with either saline, 2.0% CHX, or 5.25% sodium hypochlorite. Pre-irrigant cultures were taken and confirmed all teeth to be infected. After biomechanical preparation of canals, post-irrigation cultures were taken. Teeth irrigated with saline all remained infected after preparation while those irrigated with either CHX or sodium hypochlorite had a 70% and 58% reduction in post-irrigant cultures respectively.

In a study by Delany et al (1982), CHX was used as an irrigant and an interappointment dressing. Forty “condemned”, necrotic teeth were extracted and stored in a moist environment for no longer than 24 hour prior to instrumentation. Twenty of the teeth were single-rooted while the other twenty were multi-rooted. Utilizing aseptic technique, root canals were accessed and canal contents were removed with barbed broaches and placed on sucrose agar and labeled “canal contents”. If no viable organisms were detected the specimen was eliminated from the study. Ten single-rooted and 10 multi-rooted teeth were irrigated with a 0.2% aqueous solution of CHX while another 10 single-rooted and 10 multi-rooted teeth were irrigated with
a 0.9% solution of sterile saline. A file was then placed into the canal to the apex and rotated to produce filings. The handle of the file was cut and the remaining portion was placed in reduced transport fluid which was then plated on sucrose agar. This was labeled “root canal culture 1”. The canals were then further enlarged to a size 35 K-file while being irrigated with either saline or a 0.2% CHX solution, sampled as above and then labeled “root canal sample 2”. At this point the apices were sealed with Cavit-G®. Teeth, which were treated with CHX during instrumentation, were irrigated with CHX, which was left in the canal, and the access sealed with Cavit-G®. Teeth, which were treated with saline during instrumentation, were irrigated with saline, which was left in the canal, and the access sealed with Cavit-G®. After 24 hours all teeth were irrigated with saline and dried with paper points. Dentin filings were obtained and sampled microbiologically as described and labeled as “root canal culture 3”. Culturing from “canal contents” sample revealed all teeth to contain viable organism, thus, none were eliminated from the study. In teeth treated with CHX, there was a highly significant reduction in microbes after “root canal culture 2”. Additionally, microbes were completely eliminated in 70% of single-rooted teeth and in 80% of multi-rooted teeth after “root canal culture 3”. In teeth treated with saline after the second culture, an increase in numbers of
microorganisms was found in 80% of single-rooted teeth and 50% of multi-rooted teeth. They concluded that 0.2% CHX is an effective antimicrobial agent when used as an endodontic irrigant. When used as an intracanal, interappointment dressing, CHX helps to further reduce the microbial population remaining in the canal following instrumentation (Delaney et al 1982).

While much of the focus on the ability of CHX to bind to oral tissues has been in the periodontal literature (Rölla et al 1970, Davies 1973, Gjermo 1974, Hennessey 1973, Gjermo et al 1977, Hennessey 1977), only a few studies on the substantivity of CHX in the root canal have been published in the endodontic literature (White et al 1997, Komorowski et al 2000, Leonardo et al 1999). A study by White et al (1997) attempted to demonstrate whether substantive antimicrobial activity could be induced with CHX as an endodontic irrigant. Extracted, human teeth were instrumented by means of step-back technique with Flex-R® files. Between each change in file size 23 teeth were irrigated with 1ml of 2.0% CHX(aq.) and 21 teeth with 0.12% CHX (Peridex®). After completion of file instrumentation a 0.050” Para-Post® drill was placed into the canal to make a reservoir for testing purposes. There is no indication in this study as to when instrumentation was considered complete or how far the Para-Post drill was
taken into canal. Following enlargement each tooth was again irrigated with 1ml of their respective CHX solutions, flushed with 3ml of sterile saline, and dried with paper points. Teeth were then filled with sterile water and stored in a humidifier for 6 hours after which time canal samples were taken. Canal samples were taken by placing the broad end of a #80 paper point into the canal for 2 minutes. Canals were then irrigated with three 1ml sterile water rinses, refilled with sterile water and returned to the humidifier. This procedure was repeated at 6, 12, 24, 48, and 72 hours after treatment. The paper points were assayed for antimicrobial activity by placing them on Mitis-salivarius agar plates containing bacitracin (200U/ml) and streptomycin (200mg/ml) and inoculated with Streptococcus mutans. These plates were incubated in an increased carbon dioxide atmosphere for 48 hours after which time zones of inhibition were measured perpendicular to the paper point. The authors explain that the presence of these inhibitors in the medium negated the need for sterilization of the specimens. Additionally, they state, Streptococcus mutans can be grown selectively in this medium in the presence of contaminant bacteria. Controls consisted of paper point immersed in a series of CHX dilutions and a negative control of paper points in sterile water. These control dilutions consisted of 2.0%, 0.2%, 0.02%, 0.002%, and 0.0002% CHX. All controls were plated and
analyzed as described above. Antimicrobial activity was found in all specimens, however zones from the 2% group were found to be significantly greater at all time periods when compared to the 0.12% group. Further, they found that their assay could detect as little as 0.002% CHX. They conclude that CHX does indeed instill substantive antimicrobial properties and continues to be released as long as 48 to 72 hours after application. Of importance is that in the studies of Ringel et al (1982) and Delaney et al (1982) viable organisms remained within root canals irrigated with 0.2% CHX. Noting the difference between those studies and the White et al (1997) study, it becomes clear that a higher concentration of CHX, such as 2% might be preferable. Further studies may confirm this.

In a similar study to that done by White et al (1997), Leonardo et al (1999) performed a study *in vivo* to evaluate the antimicrobial efficacy and substantivity of 2% CHX as a root canal irrigant. Twenty-two root canals of incisors and molars with confirmed pulp necrosis and radiographically visible periapical lesions were selected from 12 patients. Rubber dam isolation and disinfection with 0.3% iodoethanol was utilized. After access openings were made the operative field was again disinfected, however, no effort was made to neutralize the disinfectant. The first canal sample was taken at this time and submitted for microbiologic analysis as a “pre-
irrigation culture.” All root canals were then instrumented with K-files by a “step-down technique” and irrigated with 1.8ml of 2% CHX (aq.) between instrument size changes. There is no indication in this study as to when instrumentation was considered complete. When instrumentation was complete, root canals were dried with paper points, left empty, and temporarily restored with zinc-oxide-eugenol cement (IRM®). After 48 hours, canals were sampled by placing a paper point in the canal for approximately 30 seconds. This paper point was placed on an agar plate inoculated with *Micrococcus luteus* and incubated for 24 hours at 37°C after which time zones of inhibition were measured. Two additional paper points were used to sample the canal as a “post-irrigation culture”. These were then submitted for microbiologic analysis. Microbiologic analysis for pre- and post-irrigation consisted of anaerobic culturing and counting of colony-forming units (CFU’s). Mutans streptococci, which were present in 10 cases prior to treatment, were reduced 100% after instrumentation and irrigation with CHX while treatment showed an efficacy of 77.78% for anaerobic species. The diameters of the zone of inhibition were found to have an average of 5.70 mm with a range of 4.38 to 7.4 mm. While this study clearly shows the substantive effects and antimicrobial activity of CHX it is severely flawed. Not only were there no treatment controls but bacterial
growth inhibition analysis had no negative controls, no measurement of positive controls, no description of measurement technique, and no discussion of these results.

A recent study by Komorowski et al (2000) tested the substantivity of CHX in bovine root dentin in an attempt to determine if antimicrobial activity could be measured over a period of 21 days. Sixty bovine root sections were prepared by resecting the crown and apex, removing cementum, and standardizing the internal diameter with an ISO 033 bur. Each root section was 5mm on its long axis with an external diameter of 7mm. All sections were treated with 17% EDTA followed by 5.25% sodium hypochlorite for 5 minutes. All specimens were then placed in brain heart infusion (BHI) broth and steam autoclaved 3 times. Sterility was confirmed by incubating for 24 hours at 37°C. To enhance penetration of broth into dentinal tubules, all specimens were placed in an ultrasonic bath of fresh BHI broth. Specimens were then blotted dry, externally coated with a layer of nail polish, and mounted on sticky wax in Petri dishes. Sixty specimens were equally divided into three groups, each to be treated by either sterile saline (positive control), 2.5% sodium hypochlorite, or 0.2% CHX (aq.). Solutions were delivered by introducing a sterile irrigation needle into canal. Care was taken to assure that external surfaces were untouched by the
solution. Half of the specimens in each group were divided equally to be treated by the test solutions for either 5 minutes or 7 days. Specimens treated for 7 days were kept in an incubator at 37°C and the test solutions were “replenished daily”. After these respective time periods, the root canal of each specimen was filled with an “overnight suspension” _Enterococcus faecalis_ in BHI broth for 21 days. Fresh BHI broth was added each day and fresh inoculum was added every other day. Specimens were incubated at 37°C. After 21 days specimens were removed from culture and rinsed with sterile water and dried. Root canals from each of the specimens were sequentially enlarged with round burs and dentin shavings were removed at a depth ranging from 0.1mm to 0.45mm then incubated for 24 hours at 37°C in BHI broth. The culture was also examined for purity of _Enterococcus faecalis_. Optical density of this culture was measured in a spectrophotometer at 540nm. The optical density from specimens that were treated for 5 minutes by each of the test solutions did not differ significantly. However, specimens treated with CHX for 7 days demonstrated significantly lower optical density values than those treated with CHX for 5 minutes or those treated with the other solutions and were consistent at all depths (Komorowski et al 2000). They conclude that CHX should be used as an intracanal medicament for 7 days rather than a 5-minute period as an
irrigant. Not only does this study indicate the potential for CHX to be released for longer than 21 days but it inadvertently indicates that sodium hypochlorite does not possess any substantive qualities. At seven days, the sodium hypochlorite group did not differ significantly from the saline group in relation to optical density values.

In some studies investigating the effects of CHX in the root canal, results may have been affected by the lack of antimicrobial inactivation prior to culturing (Vahdaty et al 1993, Parsons et al 1980, Ringel et al 1982, Delaney et al 1982, Komorowski et al 2000, Leonardo et al 1999). For example, in the study by Delany et al (1982), infected teeth were instrumented, irrigated with CHX, and then sampled microbiologically by placing the dentin filings on agar plates. However, as CHX binds easily and rapidly to dentin, any remaining CHX from these dentin filings would continue to exert an antimicrobial effect on bacteria as they are incubated. This would tend to overestimate any effect CHX may have. Therefore, it would be essential to neutralize remaining amounts of CHX in the sample prior to culturing.
The purpose of this investigation is to evaluate the substantivity of CHX within the root canal system of obturated bovine root segments. Specifically to:

A. measure spectrophotometrically retained amounts of CHX after storage of obturated root segments in saline for 1 day, 3 weeks, 6 weeks, and 12 weeks

B. determine whether retained CHX exhibits antimicrobial properties by mixture with cultures of *Enterococcus hirae* (ATCC 9790, formerly *Enterococcus faecalis*)

The hypothesis to be tested is as follows: CHX will be retained in sufficient quantities in treated and obturated bovine root segments after extended storage in saline to be measured both quantitatively and qualitatively.

**Materials and Methods**

**A. Preparation of Root Segments**

Sixty bovine incisor roots were extracted from sectioned mandibles procured from a meat processing facility in Southington, CT, USA. Crowns and apices were sectioned off and then roots sections were standardized to 8mm using a water-cooled diamond circular saw. Root sections were then
instrumented utilizing Peeso reamers numbered 4 through 6 depending on size of canal space. An Aseptico® (Woodenville, WA, USA) electric motor running at 20,000 rpm was used with the Peeso Reamers. Instrumentation was occasionally supplemented by a size 120 K-file to remove any remaining pulp tissue. Canals were irrigated copiously with a solution of 1% sodium hypochlorite buffered with sodium bicarbonate. Instrumentation was considered complete when the canal wall was visually confirmed as smooth and free of debris. All specimens were then treated with a 1M solution of EDTA for 10 minutes, rinsed well with sterile saline, and then steam autoclaved.

Sixty standardized, 8mm bovine root sections were randomly assigned to two treatment groups: experimental (n=40) and control (n=20). Experimental sections were treated by immersion in a 2% solution of CHX for 10 minutes. This solution was prepared by dilution of a 20% stock CHX (Sigma Scientific, St. Louis, MO, USA) with sterile de-ionized water. All solutions of CHX were prepared fresh, stored in dark bottles, and used within approximately two weeks. Control sections were treated by immersion in sterile saline for 10 minutes. Both control and experimental sections were then blotted dry with sterile gauze and canals were dried with extra-large paper points. In preparation for obturation, AH26® (DeTrey,
Dentsply, Konstanz, Germany) root canal sealer was mixed according to manufacturers’ instructions. In addition, an Obtura® (Spartan USA, Fenton, MO) warm gutta percha device was loaded with pellets of gutta percha and temperature set to 200°C. The canal walls of each root section were evenly coated with a layer of sealer using a size 120 K-file. While held against a sterile glass slab, root sections were obturated with 2 separate aliquots of warm gutta percha and condensed with a #12 Schilder plugger (Dentsply Maillefer, Ballaigues, Switzerland). Aseptic technique was utilized for obturation procedures.

After experimental and control sections were obturated, they were further randomly divided into four separate “storage” groups to be placed in sterile saline for various time periods. These groups were as follows:

- **Experimental**: E1(n=10) storage for 1 day; E2(n=10) storage for 3 weeks; E3(n=10) storage for 6 weeks; E4(n=10) storage for 12 weeks

- **Control**: C1(n=5) storage for 1 day; C2(n=5) storage for 3 weeks; C3(n=5) storage for 6 weeks; C4(n=5) storage for 12 weeks

In preparation for storage, 400 ml dark glass storage jars were sterilized by ethylene oxide gas sterilization. Each jar was then filled with 150ml of sterile saline. Experimental and control root sections were placed in their
respective jars and refrigerated at 2°C to prevent bacterial growth. Storage saline was not replenished at any time.

**B. Processing of Specimens: Quantification of CHX**

After their respective storage periods root segments were removed from the saline and prepared for quantitative analysis of CHX. The sides of each root section were scored with a #2 round bur and then split in half with a pair of orthodontic wire cutters. Gutta percha remaining in one half of the specimen was removed by teasing it out with a dental explorer. Using a #6 Peeso reamer, the canal walls of each specimen were ground so that the dentin shavings would fill an 1 ml Eppendorf tube approximately half-way. Eppendorf tubes were weighed separately and then together with specimens to determine the exact weight of the dentin shavings. Seven hundred microliters of sterile saline was added to each tube and specimens were agitated for five hours on a rotary agitator (Roto-torque, Cole Parmer, Vernon Hills, Il, USA). Immediately after agitation, specimens were centrifuged for 20 minutes at 5000rpm in an Eppendorf centrifuge (Brinkman Instruments, Hamburg, Germany). Supernatants were aspirated and these dentin extracts were submitted for spectrophotometric analysis to determine quantity of retained CHX.
The quantitation of CHX is facilitated by the fact that it absorbs ultraviolet light and displays distinct maxima around 230nm and 253nm\(^8\). A Spectrophotometer (Ultraspec 4050, LKB Biochem, Cambridge, England) was, therefore, set to read absorbency at 253nm. Absorbency was referenced to zero using the same sterile saline used to extract CHX from dentin shavings by placing 500µl of saline into a 500µl quartz cuvette. Five hundred microliters of dentin extract was placed into a 500µl quartz cuvette and ultraviolet absorbence was recorded as optical density at 253nm (OD\(_{253}\)). Cuvettes were washed thoroughly with sterile saline between samples. Samples were recovered and stored in a freezer at -10\(^\circ\) C.

C. Processing of Specimens: Qualification of CHX

To determine whether the residual CHX detected from dentin samples remained antimicrobial, the extracts were mixed with cultures of *E. hirae*. Eight milliliters of Brain Heart Infusion (BHI) broth (Sigma, St Louis, MO, USA) was inoculated with *E. hirae*. After 24 hours of incubation at 37\(^\circ\)C, the culture tube was centrifuged and at 1500 rpm for 10 minutes. The supernatant was poured off and replaced with 8ml sterile saline, the bacteria were re-suspended, and re-centrifuged. This was repeated a total of three times. After pouring off the final supernatant, only 4ml of sterile saline was added. Using a spectrophotometer, 3ml of #1 MacFarland standard (3x10\(^8\))
CFU/ml) was placed in a cuvette and absorption was measured at 580 nm to be approximately 0.25. Sterile saline was used as a zero reference. The culture was diluted with sterile saline to have the same absorbance as the #1 MacFarland standard, resulting in a final concentration of approximately $3 \times 10^8$ colony forming units per ml. The standardized culture was diluted with sterile saline to $3 \times 10^4$ CFU. An additional control dilution of 300 CFU was made to analyze and ensure precision of procedures. One milliliter of this dilution was plated on sheeps blood agar.

In order to determine sensitivity of this method serial dilutions were prepared from a stock solution of 20% CHX (Sigma, St Louis, MO). One ml of 20% CHX was added to 9 ml of sterile de-ionized water to make a 2% solution. One ml of the 2% solution was added to 9ml of sterile de-ionized water. This was continued until a concentration of 0.000002% CHX was reached. One hundred microliters of the $3 \times 10^4$ CFU culture was added to 900μl of each serial dilution of 20% CHX in the following groups:

- Serial Dilution (SD) Groups: **SD1** (n=6) 2% CHX; **SD2** (n=6) 0.002% CHX; **SD3** (n=6) 0.00002% CHX; **SD4** (n=6) 0.000002% CHX; **SD5** (n=6) 0.0000002% CHX; **SAL** (n=6) saline control

In order to determine the antimicrobial effectiveness of CHX remaining in the dentin extracts, $3 \times 10^4$ CFU culture was added. One hundred microliters
of culture was added to 900μl of experimental and control dentin extracts in the following groups:

- **Experimental (E) Groups:** E1(n=3) 1 day storage; E2(n=3) 3 week storage; E3(n=3) 6 week storage; E4(n=3) 12 week storage
- **Control (C) Groups:** C1(n=3) 1 day storage; C2(n=3) 3 week storage; C3(n=3) 6 week storage; C4(n=3) 12 week storage

After 30 minutes 400μl of this mixture was added to 400μl of an aqueous CHX inactivation solution consisting of 3% Tween 80® (Sigma, St Louis, MO, USA), 0.3% lecithin, and 0.5% sodium thiosulfate (Sheikh 1981, Fitzgerald et al 1989). The mixture was allowed to stand for 10 minutes in order to neutralize any remaining CHX. Six hundred microliters of this mixture was plated on sheeps’ blood agar (BBE) and incubated at 37°C for 24 hours. After incubation the number of colonies were counted on all plates and recorded.

**Results**

**A. Quantification of CHX**

Results are summarized in Table I. After storage of CHX treated and obturated root segments in saline for varying time periods, CHX was recovered from root canal dentin in all specimens. Based on a “standard
curve” developed from pilot experiments (see Figure I and Appendix), the approximate concentration of CHX was calculated. It was also determined in pilot experiments that the AH26 sealer used in the obturated root segments released a soluble constituent that weakly absorbed UV light (Appendix). Therefore, OD$_{253}$ readings from controls were subtracted from OD$_{253}$ readings from experimental groups to give a resultant concentration of CHX.

As indicated in Table I, CHX was retained in relatively high amounts in root canal dentin even after 12 weeks. After storage of CHX treated and obturated root segments (Group E4) in saline for 12 weeks, the mean OD$_{253}$ of dentin extracts was 0.64±0.08. Saline treated controls (Group C4) displayed a mean OD$_{253}$ of 0.29±0.07. This gave a resultant OD$_{253}$ of 0.35 and an approximate CHX concentration of 0.0010%. After storage of CHX treated and obturated root segments (Group E3) in saline for 6 weeks, the mean OD$_{253}$ of dentin extracts was 0.90±0.08. Saline treated controls (Group C3) displayed a mean OD$_{253}$ of 0.36±0.05. This gave a resultant OD$_{253}$ of 0.53 and an approximate CHX concentration of 0.0016%. After storage of CHX treated and obturated root segments (Group E2) in saline for 3 weeks, the mean OD$_{253}$ of dentin extracts was 1.13±0.2. Saline treated controls (Group C2) displayed a mean OD$_{253}$ of 0.33±0.05. This gave a resultant
OD$_{253}$ of 0.80 and an approximate CHX concentration of 0.0023%. After storage of CHX treated and obturated root segments (Group E1) in saline for one day, the mean OD$_{253}$ of dentin extracts was 1.87±0.33. Saline treated controls (Group C1) displayed a mean OD$_{253}$ of 0.26±0.08. This gave a resultant OD$_{253}$ of 1.61 and an approximate CHX concentration of 0.0048%.

B. Qualification of CHX

Results are summarized in Table II. Three samples from each experimental (E1, E2, E3, and E4) and control (C1, C2, C3, and C4) were mixed with cultures of *E. hirae*. In addition, serial dilutions of CHX and saline controls were also mixed with culture as summarized in Table III.

Extracts from the one-day storage group (E1) were found to be highly antimicrobial with only 1 CFU being found among the three samples. After 3 weeks, group E2 remained antimicrobial with a mean CFU of 41.33±18.23. Groups E3 and E4 displayed progressively less antimicrobial activity with mean CFU’s of 169±79.64 and 577.33±177.78 respectively. Control groups C1, C2, C3, and C4 displayed no antimicrobial activity as their mean CFU’s were, respectively, 1026.33±73.58, 925.67±26.95, 1026±54.37, 955.33±73.87.
As displayed in Table III, serial dilutions of CHX were highly antimicrobial until groups SD4 (0.00002% CHX) and SD5 (0.000002% CHX) which had mean CFU’s of 992.83±104.6 and 1040.33±136.4. The saline control group, SAL, displayed a mean CFU count of 1000.83±87.58.

The “300 CFU control plate”, as described in materials and methods had a mean CFU count of 300.33±89.13 which assured a reasonably accurate laboratory methods.

**Discussion**

The results of this study indicate that CHX, when used as an endodontic irrigant, is retained in root canal dentin in antimicrobial amounts for at least 12 weeks. Previous studies that have investigated the substantive properties of CHX in the root canal have only tested for its presence for up to 3 weeks (White et al 1997, Komorowski 2000, Leonardo 1999). In addition, these studies only analyzed the antimicrobial activity. In the current study, UV spectrophotometry was utilized as an adjunct to estimate the amount of CHX that is retained in canal wall dentin. This method was used by Rölla et al (1970), to analyze the affinity of CHX for hydroxyapatite. This study was pivotal in that it was among the first to show definitively that CHX does indeed bind to hydroxyapatite and that the absorbed CHX is released when the environmental concentration is low
(Rölla et al 1970). In a study *in vitro* by White et al (1997), the substantivity of CHX in the root canal was analyzed by measuring the zones of inhibition around paper point samples from treated canals. A study *in vivo* by Leonardo et al (1999) also evaluated the retained amounts of CHX, in part, by a similar method. In the current study, a similar pilot experiment was devised. Filter disks were soaked in serial dilutions of CHX and placed on agar plates with cultures of *E. hirae* (see Appendix 5). This method was found to be particularly insensitive not only to different concentrations of CHX but also to the small concentrations expected during the study. The current study was successful in demonstrating that UV spectrophotometry is an accurate, sensitive, and easy method for measuring small quantities of CHX.

The CHX concentration of dentin extracts was estimated for experimental groups E1, E2, E3, and E4 (Table I). The dentin from which these were extracted, presumably, still contained high concentrations of CHX after the extraction process. This is due to the fact that dentin shavings were placed in 700µl of sterile saline, which diluted and, most likely, resulted in an under-estimation of CHX concentration within the dentin. However, assuming that an equilibrium was reached between the dentin and the saline, CHX in the dentin was estimated based on the weight of the dentin samples. These estimations were E1-0.0096% CHX in dentin, E2-
0.0046%, E3-0.0032 %, and E4-0.002 %. Given that these higher concentrations are within the dentin, a greater antimicrobial effect would be expected against bacteria in the dentin.

When dentin extracts were mixed with cultures of *E. hirae*, the effective concentration was reduced by 9/10. This is due to the mixture of 900 µl of dentin extract and 100 µl of culture. Therefore, the effective concentrations in this experiment were E1- 0.0043 % CHX, E2- 0.0021 % CHX, E3- 0.0014 % CHX, and E4- 0.0009 % CHX.

Infected root canals and endodontically treated teeth with persistent apical periodontitis are frequently found to be infected with *Enterococcus faecalis* (Sundqvist et al 1998, Molander et al 1998, Winkler et al 1959). In addition, *E. faecalis* has been found to adapt well to the substrate-scant environment of the filled root canal system, where it can sustain mono-infections (Molander et al 1998). Because of its characteristics, *E. faecalis* is difficult to eradicate during re-treatment of teeth with persistent apical periodontitis and is also resistant to commonly used antimicrobial dressings such as calcium hydroxide (Sundqvist et al 1998, Ørstavik and Haapasalo 1990). However it is known to be particularly susceptible to the antimicrobial effects of CHX (Emilson 1977). For these reasons, *E. hirae* (ATCC 9790, formerly *Enterococcus faecalis*) was used in the current study.
along with the fact that it is readily available, easy to culture, and grows rapidly.

A recent study by Haapasalo et al (2000) demonstrated that the presence of dentin powder displayed an inhibitory effect on calcium hydroxide, sodium hypochlorite, chlorhexidine acetate, and iodine potassium iodide. The effect of 0.05% chlorhexidine on E. faecalis was reduced but not totally eliminated by the presence of dentine (Haapasalo et al 2000). This observation is important to the results of the current study. Although the approximate concentration of CHX from the 12 week dentin sample was 0.001%, the antimicrobial activity was clearly less than expected based on serial dilution samples (Table III).

Chlorhexidine has been suggested as an endodontic intracanal irrigant by a number of authors (Vahdaty et al 1993, Ohara et al 1993, White et al 1997, Ringel et al 1982, Delaney et al 1982, Jeansonne and White 1994, Komorowski et al 2000, Leonardo et al 1999). CHX is known to be particularly effective against many strains of bacteria found in infected root canals (Ohara et al 1993). In a study comparing common endodontic disinfectants, 0.5% CHX was significantly more effective at killing Candida albicans than Ca(OH)₂, 5% and 0.5% NaOCl, and 2% IKI (Waltimo et al 1999). While these substantive and antimicrobial properties of CHX are
promising, it does not possess the tissue dissolving properties of sodium hypochlorite (Gordon et al 1981). Although sodium hypochlorite is still considered the irrigant of choice, the use of CHX may be advantageous as a “final rinse” prior obturation, an alternate irrigant during re-treatments, or even incorporated into antimicrobial dressings.
### Table I

**Optical Density (OD) of Chlorhexidine Treated Dentin and Control Extracts**

<table>
<thead>
<tr>
<th>Experimental (E) and Control (C) Groups</th>
<th>Mean Weight (gr)</th>
<th>Standard Deviation</th>
<th>Mean OD$_{253}$</th>
<th>Standard Deviation</th>
<th>Resultant OD$_{253}$</th>
<th>Approx. CHX conc. *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Day</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>0.07</td>
<td>0.01</td>
<td>1.87</td>
<td>0.34</td>
<td></td>
<td>1.61</td>
</tr>
<tr>
<td>C1</td>
<td>0.07</td>
<td>0.02</td>
<td>0.26</td>
<td>0.08</td>
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<td></td>
</tr>
<tr>
<td>E2</td>
<td>0.07</td>
<td>0.01</td>
<td>1.13</td>
<td>0.2</td>
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<td></td>
</tr>
<tr>
<td>3 Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>0.07</td>
<td>0.001</td>
<td>.9</td>
<td>.08</td>
<td></td>
<td>0.53</td>
</tr>
<tr>
<td>C2</td>
<td>0.07</td>
<td>0.01</td>
<td>.36</td>
<td>.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>0.07</td>
<td>0.01</td>
<td>.64</td>
<td>.08</td>
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</tr>
<tr>
<td>12 Week</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4</td>
<td>0.07</td>
<td>0.01</td>
<td>.29</td>
<td>.07</td>
<td></td>
<td>0.35</td>
</tr>
<tr>
<td>C4</td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

*Calculated by subtracting Control mean OD$_{253}$ from Experimental mean OD$_{253}$

*Estimation based on standard curve (Figure I)
Table II

CHX (E) and Control (C) Extracts mixed with cultures of *Enterococcus hirae*

<table>
<thead>
<tr>
<th>Experimental and Control Groups</th>
<th>Sample 1†</th>
<th>Sample 2†</th>
<th>Sample 3†</th>
<th>Mean</th>
<th>SD*</th>
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</thead>
<tbody>
<tr>
<td>1 Day</td>
<td>E1</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>C1</td>
<td>1002</td>
<td>968</td>
<td>1109</td>
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<tr>
<td>3 Week</td>
<td>E2</td>
<td>25</td>
<td>61</td>
<td>38</td>
<td>41.33</td>
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<tr>
<td></td>
<td>C2</td>
<td>902</td>
<td>955</td>
<td>920</td>
<td>925.67</td>
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<tr>
<td>6 Week</td>
<td>E3</td>
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<td>260</td>
<td>112</td>
<td>169</td>
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<tr>
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<td>C3</td>
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<td>12 Week</td>
<td>E4</td>
<td>490</td>
<td>460</td>
<td>782</td>
<td>577.33</td>
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<tr>
<td></td>
<td>C4</td>
<td>1040</td>
<td>922</td>
<td>904</td>
<td>955.33</td>
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</table>

†Number of Colony-Forming Units (CFU’s)

*Standard Deviation
### Table III

Serial Dilutions of CHX Mixed With Cultures of *Enterococcus hirae*

<table>
<thead>
<tr>
<th>CHX conc.</th>
<th>Sample 1†</th>
<th>Sample 2†</th>
<th>Sample 3†</th>
<th>Sample 4†</th>
<th>Sample 5†</th>
<th>Sample 6†</th>
<th>Mean</th>
<th>SD*</th>
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<tr>
<td>2%</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.002%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.0002%</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>0.67</td>
<td>0.62</td>
</tr>
<tr>
<td>0.00002%</td>
<td>978</td>
<td>1082</td>
<td>922</td>
<td>850</td>
<td>987</td>
<td>1138</td>
<td>922.83</td>
<td>104.6</td>
</tr>
<tr>
<td>0.000002%</td>
<td>1007</td>
<td>875</td>
<td>968</td>
<td>1006</td>
<td>1269</td>
<td>1117</td>
<td>1040.33</td>
<td>136.4</td>
</tr>
<tr>
<td>Saline</td>
<td>1102</td>
<td>906</td>
<td>1097</td>
<td>902</td>
<td>992</td>
<td>1006</td>
<td>1000.83</td>
<td>87.58</td>
</tr>
</tbody>
</table>

†Sample size denoted in colony-forming units (CFU’s)
*Standard Deviation
Figure I

Serial dilutions of CHX plotted against optical density (OD) at 253nm
Chlorhexidine is a symmetric cationic molecule consisting of two 4-chlorophenyl rings and two biguanide groups connected by a central hexamethylene chain.
Appendix

Pilot Studies

Having established the aims of the project, it was necessary to determine in pilot studies if the project was feasible. Therefore the following pilot studies were conducted to determine if:

1. UV spectrophotometry is a suitable method for quantifying small amounts of CHX.

2. CHX can be recovered from CHX treated bovine root segments.

3. the sealer used to obturate canals releases constituents that will be detected at the same wavelength as CHX.

4. sufficient quantities of CHX can be recovered from root segments that have been obturated with gutta percha and AH26 then stored in Saline for 1 to 2 weeks.

5. there is a suitable method to measure the antimicrobial effects of small concentrations of CHX.

(1) Detection Levels  As displayed in Figure I, 10 serial dilutions were made starting with 2% CHX and sterile, de-ionized water. These concentrations were then plotted against their respective optical densities at 253 nm (OD_{253}) obtained from the spectrophotometer (Ultraspec 4050, LKB...
Biochem, Cambridge, England). These results show that concentrations of CHX as small as 0.00078% may be measured with accuracy.

(2) **Retrieval of CHX** Twelve bovine root segments were treated with 1M EDTA for 5 minutes and 2% CHX for 10 minutes then blotted dry. Six segments treated with EDTA then followed by saline served as controls. The root canals were ground with Peeso reamers to obtain dentin shavings. The dentin shavings were placed in Eppendorf tubes and 700 μl of sterile saline was added. These were placed on a rotary agitator (Roto-torque, Cole Parmer, Vernon Hills, IL, USA) for two hours to extract the CHX. Specimens were centrifuged for 10 minutes and supernatants were obtained. Optical density at 253 nm was measured by means of a spectrophotometer (Ultraspec 4050, LKB Biochem, Cambridge, England). The dentin extracts obtained from CHX treated roots resulted in an average OD$_{253}$ of 0.980 while controls showed an average of 0.048. This data represents three identically repeated trials. Results indicate that the spectrophotometer is useful in determining the presence and estimating the amount of CHX in dentin.

(3) **Effect of AH26** Freshly mixed AH$_{26}^\text{®}$ (DeTrey, Dentsply, Konstanz, Germany) cement was placed on two gutta-percha points and placed in two eppendorf tubes containing 1ml of sterile de-ionized water for
1 minute. After centrifugation for ten minutes, the OD$_{253}$ of the supernatants were measured. Results show an average OD$_{253}$ of 0.245.

Freshly mixed AH26 was placed into a multi-well culture dish. Four wells contained 0.05 ml of AH26, another four wells contained 0.1 ml AH26 of and four additional wells with sterile de-ionized water served as controls. One milliliter of sterile de-ionized water was placed into each well and the cement was allowed to set for 1 week. The water was then aspirated and centrifuged for 10 minutes. After measuring the OD$_{253}$ of the supernatants, the following results were obtained: wells containing 0.05 ml AH26 had an average OD$_{253}$ of 0.135, 0.01 ml of AH26 averaged 0.177, while controls averaged 0.01.

Freshly mixed AH26 cement was aspirated into six eight-gauge 15mm needles. These were placed into 2ml of saline and allowed to set for 1 week. Two needles without AH26, otherwise treated identically, served as controls. After centrifugation for ten minutes, the OD$_{253}$ of the supernatants were measured. Needles with AH26 showed an average OD$_{253}$ of 0.076 while controls showed an average of 0.01.

These results indicate that AH26 cement releases a soluble constituent that absorbs UV light at 253 nm. This will be taken into account during future experiments.
(4) **Effect of Storage Periods**  Two separate trials were performed utilizing CHX treated and obturated root segments. In the first trial two segments were treated with 1M EDTA for 5 minutes then 2% CHX for 10 minutes. These segments were then obturated with warm gutta percha (Obtura®, Spartan USA, Fenton, MO) and AH26® (DeTrey, Dentsply, Konstanz, Germany) cement. For controls, two more segments were treated identically but with saline instead of CHX. All four segments were then placed in separate culture tubes containing saline and stored for 1 week. After storage segments were split in half, and the gutta percha was removed. Canal wall dentin was then removed using a slow-speed hand piece and #8 round bur and dentin shavings were collected in pre-weighed eppendorf tubes. After weight of dentin shavings was determined, 700 μl of saline was added and tubes were agitated for 3 hours. These samples were then centrifuged for 10 minutes after which the OD$_{253}$ of the supernatants was measured. The following results were obtained: experimental sample 1 weighed 0.0508 grams with an OD$_{253}$ of 0.797 or 0.0508g (0.797), sample 2, 0.0203g (0.237); control sample 1, 0.0557g (0.180), and control sample 2, 0.0392g (0.120).

The second trial utilized four experimental segments and 2 control segments. Treatment was identical as in the first trial except storage was for
two weeks. The following results were obtained: experimental sample 1 weighed 0.0654 grams with an OD\textsubscript{253} of 0.823 or 0.0654g (0.823), sample 2, 0.0623g (0.764), sample 3, 0.0548g (0.772), sample 4, 0.0510g (0.527); control sample 1, 0.0491g (0.091), sample 2, 0.0587g (0.102).

Results from this pilot study indicate that CHX can be retrieved and from dentin and detected after treated root segments have been stored for up to two weeks.

(5) Antimicrobial dose-response information for CHX A test was devised to determine the antimicrobial activity CHX. Five hundred microliters of a 24 hour suspension of \textit{Enterococcus hirae} (ATCC 9790, formerly \textit{Enterococcus faecalis}) in brain heart infusion (BHI) broth (Sigma Scientific, St. Louis, MO, USA) was spread on a plate of sheeps’ blood agar (Sigma Scientific, St. Louis MO, USA). Seven millimeter filter disks were then prepared along with serial dilutions of 20% stock solution of CHX (Sigma Scientific, St. Louis, MO, USA) consisting of 10\%, 5\%, 2.5\%, 1.25\%, 0.2\%, 0.1\%, 0.05\%, 0.025\%, 0.0125\%, and 0.00625\%. Immediately after the bacterial suspension was spread on the plate, filter disks were soaked with respective solutions of CHX and divided among 4 plates each with a single saline control. After 24 hours of incubation at 37°C, zones of inhibition around filter disks were measured in diameter. Results were as
follows: 20% CHX (22mm), 10% (20mm), 5% (16mm), 2.5% (16mm), 
1.25% (14mm), 0.2% (12mm), 0.1% (12mm), 0.05% (9mm), 0.025% 
(9mm), 0.0125% (8mm), 0.00625% (8mm), saline control (7mm).
Experiment was repeated with identical results. While some amount of 
inhibition was present around low concentrations of CHX, it was concluded 
that this method was not sensitive enough to distinguish among the small 
concentrations of CHX expected from experiments.

In order to find a more sensitive method to determine the 
antimicrobial activity of CHX another experiment was devised. First, a 
standardized culture of *E. hirae* was prepared. Eight milliliters of Brain 
Heart Infusion (BHI) broth (Sigma, St Louis, MO, USA) was inoculated 
with *E. hirae*. After 24 hours of incubation at 37°C, the culture tube was 
centrifuged and at 1500 rpm for 10 minutes. The supernatant was poured off 
and replaced with 8 ml sterile saline, the bacteria were re-suspended, and re-
centrifuged. This was repeated a total of three times. After pouring off the 
final supernatant, only 4 ml of sterile saline was added. Using a 
spectrophotometer, 3 ml of #1 MacFarland standard (3x10^8 CFU/ml) was 
placed in a cuvette and absorption was measured at 580 nm to be 
approximately 0.25. Sterile saline was used as a zero reference. The culture 
was diluted with sterile saline to have the same absorbance as the #1
MacFarland standard, resulting in a final concentration of approximately $3 \times 10^8$ CFU. The culture was diluted with sterile saline to $3 \times 10^4$ CFU. This standardized culture was used for the following experiments.

**Experiment 1:** A 20% stock solution of CHX (Sigma, St Louis, MO, USA) was serial diluted to 0.002%, 0.02%, and 0.2% while sterile saline was used as a control. These dilutions were divided into groups A, B, C, and D respectively. Group A consisted of 6 test tubes containing 900 μl of 0.002% CHX and 100 μl of $3 \times 10^4$ CFU *E. hirae* culture. In 3 of these tubes, CHX was allowed to remain in contact with the culture for 30 minutes and in the other 3 tubes for 60 minutes. After these time periods, 400 μl from each tube was mixed with 400μl of an aqueous solution of 3% Tween 80® (Sigma, St Louis, MO, USA), 0.3% lecithin, and 0.5% sodium thiosulfate for 10 minutes in order to neutralize any remaining CHX. After this time period, 600 μl from each of the six tubes for the 30 and 60 minute groups were placed on sheeps’ blood agar plates, while the remaining 200 μl was added to 400 μl of sterile saline then plated. All groups were treated identically. After 24 hours of incubation at 37°C colonies were counted.

In Group A (0.002%), 3 of 12 plates had a single colony. In Group B (0.02%) 1 of 12 had a single colony. In Group C (0.2%), 0 of 12 had colonies. In Group D (saline control), the 30 min group with 600 μl had an
average of 285 colonies, while 200 μl showed an average of 175.3 colonies. The 60-minute group with 600 μl had an average of 240.3 colonies, while 200μl showed an average of 194.6 colonies.

An additional control consisted of a single plate inoculated with 100μl of 3x10^4 CFU suspension of *E. hirae*. This plate contained 1628 colonies although it should have had 3000 colonies based on the dilution. The following conclusions can be drawn from this pilot experiment: (1) Allowing CHX to remain in contact with culture for 30 or 60 minutes did not appear to have any effect. (2) Dividing plates into 600 μl and 200 μl to facilitate colony counting was determined to be unnecessary. (3) Solutions of CHX down to 0.002% CHX were highly bacteriocidal. (4) Based on controls it is evident that great care must be taken to ensure accuracy of measurement of bacterial CFU’s and dilution methods.

**Experiment 2:** The standardized bacterial suspension of *E. hirae* was prepared as described. Serial dilutions of CHX were prepared for the following groups: **Group A** (n=2) 0.0002% CHX, **Group B** (n=2) 0.00002% CHX, **Group C** (n=2) 0.000002% CHX, **Group D** (n=2) saline control. One hundred microliters of standardized culture (3x10^4 CFU) was added to 900μl of each dilution of CHX. Four hundred microliters of this mixture was added to 400μl of inactivating solution as described. After 10 minutes, 600
μl of this mixture was plated on sheep's blood agar. An additional control consisted of a dilution of standard culture to 300 CFU that was also plated. After 24 hours of incubation at 37°C the colonies were counted.

In **Group A**, only 1 colony was found among the two plates. In **Group B**, one plate had 722 colonies and the other had 798 colonies. **Group C** plates showed 804 and 852 colonies respectively. **Group D** showed 920 and 802 colonies respectively. The 300 CFU control plate had 320 colonies. Based on dilutions of the $3 \times 10^4$ CFU culture, **Group D** controls should contain 900 colonies. The following conclusions can be drawn from this pilot experiment: (1) Concentrations of CHX below 0.0002% do not appear to have an antibacterial qualities. (2) Through careful laboratory methods good accuracy can be obtained. This is evident from the number of colonies counted in controls being close to what was predicted by calculations.
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