An *in-vitro* investigation of the antibacterial effect of nisin in root canals and canal wall radicular dentine

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Abstract

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Aim To determine whether nisin, a bacteriocin, would be effective at killing *Enterococcus faecalis* and *Streptococcus gordonii* cells in solution and within the root canal system.

Methodology Bacterial isolates of *E. faecalis* and *S. gordonii* were grown from glycerol stocks in closed tubes containing BHY broth at 37 °C. The minimum bactericidal concentration (MBC) of nisin for both bacterial species was determined by a microdilution method. Extracted human teeth were decoronated to produce roots of equal length with a single canal and divided into six groups of 10 roots. The canals were prepared to a master apical size 30 file using 0.04 taper Ni-Ti rotary instruments. Bacterial samples of each species were inoculated into three groups of prepared roots and incubated in closed tubes at 37 °C for 21 days. The root canals in each group were then medicated with water (control), calcium hydroxide powder mixed with sterile water [$Ca(OH)_2$], or nisin and incubated for a further 7 days. Rotary Ni-Ti files were used to take radicular dentine samples from the walls of each canal which were then incubated in BHY broth for 24 h. Optical density (OD_{600}) readings were taken as a measure of bacterial growth.

Results The MBC of nisin for *E. faecalis* and *S. gordonii* was 70 and 20 mg mL^{-1} respectively. Calcium hydroxide and nisin medication eradicated infection within the root canal while cells remained viable in the control group. Mean optical density (OD_{600}) readings from canal wall dentine shavings infected with *E. faecalis* were 1.32 ± 0.98, 0.73 ± 0.27 and 0.69 ± 0.38 for the control, $Ca(OH)_2$ and nisin samples respectively. Corresponding mean readings for *S. gordonii* were 1.19 ± 0.18, 0.73 ± 0.15 and 0.60 ± 0.29. The $Ca(OH)_2$ and nisin group readings were significantly ($P < 0.01$) lower than the control for each species as tested by Student's *t*-test and Mann–Whitney *U* statistical analysis. Values for $Ca(OH)_2$ and nisin were not significantly ($P > 0.01$) different.

Conclusion Nisin was effective at eradicating *E. faecalis* and *S. gordonii* cells in pure culture and was comparable with $Ca(OH)_2$ in the elimination of these species from within the root canal system.

Keywords: calcium hydroxide, endodontic medicament, *Enterococcus faecalis*, nisin.

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Introduction

Bacteria are considered the primary cause of pulp and periapical disease (Kakehashi *et al.* 1965, Sundqvist 1976, Möller *et al.* 1981). Infection of the root canal system results in apical periodontitis, an inflammatory response in the periradicular tissues, causing pain and resorption of periradicular structures. Successful root canal treatment is based around a mechanical and chemical procedure of root canal disinfection (Byström & Sundqvist 1981, 1983, 1985). However, this chemomechanical method of instrumentation is not always effective at consistently disinfecting the root

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canal system and radicular dentine (Byström & Sundqvist 1985). As a consequence, in an attempt to eliminate residual bacteria, an intracanal dressing of calcium hydroxide [$Ca(OH)_2$] is commonly placed within the root canal for a minimum of 1 week (Sjögren *et al.* 1991). This treatment triad of mechanical cleaning, chemical disinfection and antibacterial dressing is aimed at producing canals that are clean and free from infection.

Calcium hydroxide has a wide spectrum of action against many endodontic pathogens and as an intracanal medicament commonly results in the elimination of the majority of microorganisms from the root canal system (Sjögren *et al.* 1991). The main mechanism of action of calcium hydroxide, while not completely understood, is thought to be due to its high alkalinity (Tronstad *et al.* 1980). Substances with high alkalinity produce destruction of cell membranes (Gordon & Alexander 1986) and the majority of microorganisms within root canals are killed at pH 9.5 (Fisher & Huerta 1984).

However certain bacteria, commonly *Enterococcus faecalis*, are able to survive in a highly alkaline environment (Evans *et al.* 2002) and are resistant to its effects. Further, *E. faecalis* is commonly recovered from the root canals of failed endodontic cases (Molander *et al.* 1998, Sundqvist *et al.* 1998). The ability of enterococci to invade dentinal tubules, even under stressed environmental conditions, is thought to play a role in its ability to resist chemomechanical instrumentation and reinfect the root canal system (Love 2001). Similarly, some cells present in dentine infected with *E. faecalis* may survive following exposure to sodium hypochlorite *in vitro* (Ørstavik & Haapasalo 1990). The presence of a proton pump has been illustrated as a primary resistance mechanism of *E. faecalis* to resist the alkaline effects of calcium hydroxide *in vitro* even in an alkaline environment up to pH 11.1 (Evans *et al.* 2002). The buffering capacity of dentine provides a decreasing pH gradient from inner to peripheral root dentine (Wang & Hume 1988, Nerwich *et al.* 1993, Haapasalo *et al.* 2000). A pH of 11.5 is required to effectively kill *E. faecalis* (Evans *et al.* 2002) and this is not commonly achieved within radicular dentine by a $Ca(OH)_2$ medicament, with an alkalinity of only pH 10.3 being reported *in vitro* (Nerwich *et al.* 1993, Miñana *et al.* 2001).

Enterococcus faecalis has also been frequently isolated as the sole species from the canals of root filled teeth with persistent apical pathology that were treated a number of years before (Sundqvist *et al.* 1998, Peciuliene *et al.* 2001). Initially, Love (2001) demonstrated the ability of *E. faecalis* to survive in serum for several days (56 days); serum could be thought of as being similar to the fluid within dentinal tubules in radicular dentine. Additionally, the ability to survive alone, in isolation, in a nutrient-poor environment for extended periods has recently been confirmed *in vitro* (Figdor *et al.* 2003). In that study *E. faecalis* cells survived starvation in water for a period of 4 months and were able to resume normal growth following the addition of human serum.

These findings indicate that an endodontic medicament, able to effectively kill therapy resistant bacteria within root canals and radicular dentine with minimal or no host toxicity would be a valuable addition to endodontic treatment.

Nisin is a naturally occurring antimicrobial peptide and was discovered in 1928 (Hurst 1967, Montville & Chen 1998). Produced by strains of *Lactococcus lactis*, nisin is an antibiotic peptide, and is a class I bacteriocin (Klaenhammer 1993). Nisin is safe to humans and is used extensively as a food preservative in over 40 countries (Delves-Broughton 1990), mainly in preservation of meat and dairy products (Hurst 1981), and is approved for use in unlimited concentrations in Australia, UK and France (Cleveland *et al.* 2001). The mode of action of nisin, while not completely understood, is due to interaction with the phospholipid membrane of the target bacterial cell (Driessen *et al.* 1995). Nisin disrupts the cellular membrane inducing leakage of small intracellular contents from the cell (Montville & Chen 1998).

The use of nisin in dentistry has been limited. Investigation into the effect of a nisin containing diet on dental plaque in laboratory monkeys was assessed and found not to significantly alter the oral microflora (Johnson *et al.* 1978), while a nisin-based mouthwash, tested on beagle dogs, was found to significantly decrease the number of bleeding sites compared with a placebo (Howell *et al.* 1993). Presently, the use of nisin in endodontics appears to be unreported. The aim of this study was to provide a preliminary assessment of the effects of nisin, a naturally occurring antimicrobial agent, against endodontic pathogens in solution and within the root canal system.

Materials and methods

Bacterial isolates of *Streptococcus gordonii* DLI (Challis) and *E. faecalis* (JH2-2) were used in this experiment. Cells were grown as colonies on TSBY agar plates

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[tripticase soy broth (30 g L^{-1}; Becton Dickinson Microbiology Systems, Cockeysville, MD, USA), yeast extract (5 g L^{-1}; Difco Laboratories, Detroit, MI, USA), agar 15 g L^{-1} containing bactopeptone (5 g L^{-1}; Difco)] at 37 °C in a reduced atmosphere. Glycerol stocks were prepared by growing cells for 16 h in brain–heart infusion broth (37 g L^{-1}; Difco) containing yeast extract (5 g L^{-1}; Difco) (BHY) at 37 °C in closed tubes without shaking. The cells were harvested by centrifugation (6000 ***g***, 10 min, 4 °C) and resuspended in BHY broth containing 15% (v/v) glycerol and stored as a stock suspension at −80 °C until required.

Cultures for experimental procedures were inoculated from glycerol stock suspension (50 μL) into BHY (1.5 mL) and incubated at 37 °C in reduced O_2 atmosphere for 24 h and adjusted to an optical density (OD_{600}) of 1.

Minimum bactericidal and inhibitory concentration

The minimum bactericidal concentration (MBC) and minimum inhibitory concentration (MIC) of nisin (2.5%; Sigma-Aldrich Co., St Louis, MO, USA) were assessed by adapting the methodology described by Muroi & Kubo (1996) and Steinberg *et al.* (1999) using a microdilution method.

Minimum bactericidal concentration

Briefly, bacterial cells were cultured, as described previously, and 15 portions (50 μL each) were inoculated into sterile microtitre plastic wells. Nisin was prepared by dissolving in sterile distilled water to a concentration of 100 mg mL^{-1} and various concentrations were placed into the corresponding microtitre wells. The plates were covered with sterile plastic film and incubated for 24 h at 37 °C. Bacterial samples then were taken from each well and inoculated onto TSBY agar plates and incubated for 24 h at 37 °C. The MBC was calculated as the lowest concentration of nisin resulting in no bacterial growth following 24 h incubation. The experiments were performed in triplicate.

Minimum inhibitory concentration

To determine the MIC of nisin, overnight cultures of cells were prepared as above and 1 mL was dispensed into Eppindorf tubes (Paylab, Auckland, New Zealand). The tubes were arranged into groups of five and each group had nisin incorporated at various concentrations. The optical density (OD_{600}) of each sample was measured using a spectrophotometer (Shimadzu, Kyoto, Japan) and the cultures were then incubated for 24 h at 37 °C. The OD_{600} was then remeasured following 24 h incubation. The MIC was determined as the lowest concentration of nisin yielding no growth after 24 h.

Preparation of teeth

Freshly extracted human teeth were collected and stored in water until required. The teeth were decoronated using a rotary diamond saw with water irrigation (Gillings-Hamco, Rochester, NY, USA) and only roots with a Type-I root canal configuration (Vertucci 1984) and a round root canal at the cut surface were chosen. The pool of roots was composed mainly of palatal roots of maxillary molars, mandibular premolars, and distal roots of mandibular molars. The roots were divided into six groups of 10 roots. The root lengths were measured and the working length was determined as 1 mm less than the file length when the tip was visible at the apical foramen. Coronal root structure was removed perpendicular to the long axis of the root to produce roots with a working length of 10 mm.

The canals were instrumented to a standardized apical size (0.3 mm) and taper (0.04 mm mm^{-1}) with engine driven nickel-titanium rotary files (Dentsply Maillefer ProFile .04 taper; Maillefer Instruments, Ballaigues, Switzerland) used in a sequential crown-down technique with sodium hypochlorite (10 mL, 2.5% v/v) irrigation using a sterile endodontic needle (Monoject; Sherwood Medical, St Louis, MO, USA) and 5 mL luer lock syringe (Monoject). The roots were then rinsed in water for 30 min, then rinsed in ethylenediaminetetraacetic acid (17% w/v) for 5 min in an ultrasonic bath to remove the smear layer and rinsed in water for a further 30 min. The teeth were stored in sterile water until used.

Each root was dried and three layers of clear nail varnish were placed over all external root surfaces with care not to occlude the root canal entrance. The roots were then mounted in groups in resin dye stone (Exacto-Form, bredent; Weissenhorner, Senden, Germany) with approximately 3 mm of coronal root structure above the stone. The mounted roots were placed in a sterilizing bag and sterilized in an autoclave (Mocom B, Buccinasco, Italy; 20-min cycle).

Root canal infection

Bacterial samples of *S. gordonii* and *E. faecalis* were cultured as described above. Each root canal was

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inoculated with a bacterial solution up to the canal entrance using a sterile endodontic needle (Monoject) and 5 mL luer lock syringe (Monoject). Each canal was sealed with dental wax and all samples were incubated in a closed container at 37 °C for a period of 21 days. The canals were reinoculated with fresh bacterial samples every 3 days. To check for cell viability and purity of culture, samples were taken from each canal with a sterile paper point and inoculated onto TSBY agar and incubated aerobically for 24 h at 37 °C.

Root canal medication

After 21 days the canal contents were aspirated and each canal was rinsed with 5 mL saline using a sterile endodontic needle (Monoject) and 5 mL luer lock syringe (Monoject), then dried with sterile paper points. The test medicament was applied to the corresponding group, sterile water (control) and nisin (100 mg mL^{-1}; determined from the MBC data) was placed using a sterile endodontic needle and syringe as above, and calcium hydroxide paste (Dental Therapeutics AB, Saltsjö-Boo, Sweden) was applied using a sterile Past-inject (Micro Mega S.A., Geneve, Switzerland) spiral filler. The canals were sealed with dental wax and all samples were incubated for 7 days at 37 °C. The wax seal was then removed from each of the canals, and bacterial samples of each canal were obtained with sterile paper points and inoculated onto BHY agar and incubated for 24 h at 37 °C. Each canal was then irrigated with 5 mL of saline using a sterile endodontic needle and syringe and dried with paper points.

To investigate the degree of infection of the canal wall and associated radicular dentine, specimens of dentine chips from the full length of the root canal were obtained from each canal using a sterile number 40 size 0.04 taper engine driven nickel-titanium rotary file (Dentsply Maillefer ProFile). The dentine samples were removed by placing the files into a sterile Eppindorf tube containing 1.5 mL of BHY broth. The Eppindorf was placed in a vortex mixer (Scientific Industries Inc., New York, NY, USA) for 30 s. The files were removed and inspected for dentinal debris remaining in the flutes. If debris remained the file and Eppindorf were mixed for a further 30 s. This was repeated until the files were free from dentinal debris. The samples were incubated for 24 h at 37 °C.

Following incubation each sample was mixed in a vortex mixer (Scientific Industries Inc.) for 15 s and 1 mL of solution pipetted into a cuvette (LP; Italiana Spa, Milano, Italy). The OD_{600} of each sample was measured and any differences between the groups was determined using Student's *t*-test and Mann–Whitney *U* statistical analysis.

Results

The MBC of nisin for *S. gordonii* was 20 mg mL^{-1} and the MBC of nisin for *E. faecalis* was 70 mg mL^{-1} (Table 1). The MIC of nisin for *S. gordonii* was 10 mg mL^{-1} (Table 2) and the MIC of nisin for *E. faecalis* was 60 mg mL^{-1} (Table 3).

All bacterial samples in the root canals remained viable and as a pure culture during the 21-day infection phase. At the end of the root canal medication phase all control (sterile water) intracanal samples showed bacterial growth of both species while no intracanal samples from either the calcium hydroxide or nisin groups showed any growth of either species. For both bacteria optical density readings from dentine

Table 1 MBC of nisin

	Nisin concentration (mg mL^{-1})											
Bacteria	90	75	70	60	50	30	20	10	5	2	1	0.5
Streptococcus gordonii	–	–	–	–	–	–	–	+	+	+	+	+
Enterococcus faecalis	–	–	–	+	+	+	+	+	+	+	+	+

–, No bacterial growth; +, bacterial growth, $n = 3$.

Table 2 MIC of nisin for *Streptococcus gordonii*

Nisin (mg mL^{-1})	20	10	5	2	1	0.5
Initial OD_{600} (mean ± SD)	0.09 ± 0.003	0.09 ± 0.003	0.03 ± 0.002*	0.02 ± 0.005*	0.03 ± 0.006*	0.01 ± 0.003*
24-h incubation OD_{600} (mean ± SD)	0.10 ± 0.004	0.08 ± 0.003	0.06 ± 0.002	0.05 ± 0.002	0.05 ± 0.001	0.04 ± 0.001

*Significant difference ($P < 0.05$) between initial and 24-h incubation OD_{600} readings, $n = 5$.

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Table 3 MIC of nisin for *Enterococcus faecalis*

Nisin (mg mL^{-1})	70	60	50	30	20	10
Initial OD_{600} (mean ± SD)	0.37 ± 0.05	0.41 ± 0.09	0.33 ± 0.03*	0.31 ± 0.03*	0.33 ± 0.04*	0.25 ± 0.01*
24-h incubation OD_{600} (mean ± SD)	0.42 ± 0.09	0.47 ± 0.09	0.48 ± 0.05	0.41 ± 0.05	0.41 ± 0.05	0.34 ± 0.05

*Significant difference ($P < 0.05$) between initial and 24-h incubation OD_{600} readings, $n = 5$.

Table 4 Bacterial growth from dentine shavings

	Bacteria					
	Streptococcus gordonii			*Enterococcus faecalis*		
Group	Control	$Ca(OH)_2$	Nisin	Control	$Ca(OH)_2$	Nisin
OD_{600} (mean ± SD)	1.19 ± 0.18	0.73 ± 0.15*	0.60 ± 0.29*	1.32 ± 0.98	0.73 ± 0.27*	0.69 ± 0.38*

*Significantly different from control, $n = 10$.

shavings following 24 h incubation showed significantly ($P < 0.01$) lower values in the calcium hydroxide and nisin groups compared with control groups (Table 4).

There was no significant difference in the OD_{600} readings ($P > 0.01$) between the calcium hydroxide and nisin groups for both bacterial species tested. Compared with control values the number of viable bacterial cells associated with canal wall radicular dentine was reduced by the medicaments. Canals medicated with calcium hydroxide reduced *S. gordonii* by 39% and reduced *E. faecalis* by 45%, while nisin medication reduced *S. gordonii* by 49% and *E. faecalis* by 48%.

Discussion

The implication of *E. faecalis* in endodontic treatment failure due to its resistance to the highly alkaline environment produced by calcium hydroxide (Evans *et al.* 2002), the ability to invade dentinal tubules under stressed conditions (Love 2001) and its common recovery from root canals of previously root filled teeth with persistent periapical pathology (Molander *et al.* 1998, Sundqvist *et al.* 1998) are indicative that an alternative method of killing this species would be beneficial to endodontic treatment. Recent investigations involving the use of a tetracycline, acid and detergent mixture (MTAD) (Torabinejad *et al.* 2003), slow release chlorhexidine (Lin *et al.* 2003) and calcium hydroxide mixed with iodine and electrophoretically activated copper (Fuss *et al.* 2002) have all shown promising results in eradicating *E. faecalis* within the root canal and infected dentinal tubules.

To date there appears to be no other reports investigating the effects of nisin with regard to endodontic infections. Initial information from this study indicates that nisin is able to effectively kill *S. gordonii* and *E. faecalis* both in solution and in an *in vitro* intracanal environment. Determination of the MBC and MIC concentrations for nisin against both *S. gordonii* and *E. faecalis* revealed that the MBC for *E. faecalis* was approximately 3.5 times that required for *S. gordonii*. This may be due to the resistance and survival techniques of *E. faecalis* previously mentioned. However, as the mode of action of nisin differs to that of calcium hydroxide, and is not reliant on a highly alkaline environment for effective killing, the reason for this difference is unclear. The ability of *E. faecalis* to survive in a stressed environment has been highlighted (Evans *et al.* 2002) and the increased concentration of nisin required to kill *E. faecalis* may be due to an undiscovered survival mechanism. Despite this, the MBC of nisin for *E. faecalis* is several orders of magnitude lower than the maximum safe dose (Cleveland *et al.* 2001).

Assessment of the antibacterial effects of intracanal medication quantified indirectly using optical density readings as a measure of cell number has been described previously (Han *et al.* 2001). Whilst removal of bovine dentine using round burs has previously been undertaken problems in using this technique when using human teeth have been highlighted (Han *et al.* 2001). Similar to the study by Han *et al.* (2001), the present study used engine driven rotary nickel-titanium files to prepare the canals and collect dentine from the infected canal wall. This preparation technique allows a precise canal shape with a constant apical diameter and canal taper to be readily achieved. The volume of

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dentine chips removed for bacterial sampling is constant between root samples due to the consistent increase in canal dimensions produced by the file and standardized root canal length. Theoretically, 100 μm of dentine is removed from all portions of the canal wall in stepping from a number 30 0.04 taper ProFile to a number 40 0.04 taper ProFile. *Streptococcus gordonii* cells have been shown to penetrate into dentinal tubules up to 200 μm in the coronal area and 60 μm in the apical region of human teeth (Love 1996). Invasion of dentinal tubules by *E. faecalis* usually occurs to a depth of 50–100 μm (Safavi *et al.* 1990). Therefore an incremental cut of 100 μm should have incorporated the majority of infected dentine for sampling. However the dentine sampling technique may result in the inclusion of the test medicaments into the growth media. It is possible that optical density readings were affected by this carry-over of medicament, though the canals were thoroughly washed and dried prior to dentine sampling to minimize this possibility.

The optical density readings in this study were considerably higher than those previously reported (Han *et al.* 2001). This can be explained by differences in experimental technique. In the study by Han *et al.* (2001), following 21 days incubation the infected roots were immersed in and thoroughly washed in a tube containing 30 mL of sterile saline. In the present study the canals were irrigated with 5 mL sterile saline to simulate the clinical environment. The optical density values measures cell growth and the relative values between samples in the two studies reflect the number of bacteria that were initially present. Consequently, the canals in the present study were likely to be more heavily infected (higher number of cells present initially) and as a result the optical density readings higher due to more bacterial growth and an overall lower kill rate of bacteria within the radicular dentine. This methodology was chosen to provide a worst-case scenario clinical situation, that is, the medication of a canal known to be contaminated. Notwithstanding, kill rates of bacteria associated with canal wall radicular dentine exceeded 50% for both nisin and calcium hydroxide in both the *S. gordonii* and *E. faecalis* groups and were significantly higher than for the corresponding un-medicated control groups.

Nisin was able to effectively kill all bacteria within the intraradicular canal space and provided comparable results to calcium hydroxide in its ability to kill these two bacterial species. The delivery mechanism of the nisin, dissolved in sterile water, resulted in a kill rate in canal wall dentine comparable with calcium hydroxide paste. Interestingly, both bacterial species survived in the control samples containing sterile water. *E. faecalis* has been shown to survive in water for extended periods of up to 4 months (Hartke *et al.* 1998, Figdor *et al.* 2003). The MBC required to kill *S. gordonii* and *E. faecalis* was vastly lower than the minimal safe permitted dose for humans and the incorporation of nisin into a paste or gel emulsion at a higher concentration may result in an increased quantity of nisin available in the canal for dentinal tubule disinfection and may increase its antibacterial effectiveness.

The present study, investigating nisin as an endodontic medicament has provided some encouraging results. Nisin is comparable with calcium hydroxide in its ability to eliminate the species tested, both within the root canal and associated canal wall radicular dentine *in vitro*. The ability of nisin to effectively kill *E. faecalis* by a mechanism that is in not reliant on achieving a high pH may provide a means to eliminate this species by a method to which it has no defence mechanism. Nisin is reported to be active against a broad range of Gram-positive bacteria (Kuipers *et al.* 1992). In the early stages endodontic infections are a mixed flora with a predominance of obligate anaerobes with increasing time (Sundqvist 1976). It is uncertain as to the effectiveness of nisin against a mixed endodontic infection consisting of predominantly Gram-negative species. However, infection of radicular dentinal tubules is dominated by Gram-positive species namely, Streptococci and Enterococci (Love & Jenkinson 2002). Given the association of *E. faecalis* in cases of chronic failure in endodontically treated teeth a medication aimed specifically at this species may be of value. This may be especially pertinent in cases of conventional endodontic retreatment where *E. faecalis* is the most commonly recovered bacterial species (Sundqvist *et al.* 1998).

Conclusion

Within the limits of the study nisin was effective at eradicating *E. faecalis* and *S. gordonii* cells in pure culture and was comparable with $Ca(OH)_2$ in the elimination of these species from within the root canal system.

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