Sterilization of infected root-canal dentine by topical application of a mixture of ciprofloxacin, metronidazole and minocycline *in situ*

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Summary

The aim of this study was to observe the potential of a mixture of ciprofloxacin, metronidazole and minocycline to kill bacteria in the deep layers of root canal dentine *in situ*. After the crowns of extracted teeth had been removed, the drug combination (0.5 mg of each drug), or sterile saline, as the control, was placed in the root canals which had been previously irrigated ultrasonically with 0.4 M EDTA. The penetration and bactericidal efficacy were estimated by various procedures as follows. (1) A cell suspension of *E. coli* was placed into small cavities prepared parallel to the root canals on the cut planes of nine single-rooted teeth. The teeth were then entirely covered with blue inlay wax. At time 0, and at 5h, 24h and 48h after the drug combination had been applied, cells of *E. coli* were recovered from the cavities by washing the cavities several times with sterile saline solution, and were cultured on the surfaces of heart-infusion (HI) agar plates. Total colony-forming units were tuen counted. Bacterial recoveries decreased with time, and no bacteria were recovered 48 h after application of the drug combination, while bacteria survived in all cases with the controls. (2) After the drug combination or sterile saline had been placed into and sealed in the root canal with blue inlay wax, the teeth were placed into HI agar plates where cells of *E. coli* had been inoculated. After culturing, a clear zone caused by the inhibition of bacterial growth was observed around the teeth, but not in the control experiment. (3) After sampling infected root dentine of 12 freshly extracted teeth as positive controls, the drug combination (0.5 mg each) was placed in the root canals. No bacteria were recovered from the infected dentine of the root canal wall 24 h after application of the drug combination, except in one case in which a few bacteria were recovered. On the basis of these results, penetration through dentine and antibacterial efficacy of the drug combination can be expected against bacteria infecting the dentine of the root canal wall *in situ* when the drugs were placed in root canals which had been irrigated ultrasonically.

Keywords: ciprofloxacin, disinfection, infected root dentine, metronidazole, minocycline

Introduction

Bacteria present in root canals may be removed by filing or by chemical irrigation during conventional root canal treatment. However, bacteria in the deeper layers of infected root dentine may sometimes remain even after conventional root canal treatment (Ando & Hoshino 1990), and may occasionally cause periapical complications (Yamata 1973, Byström *et al.* 1987). Such bacteria should be eliminated to ensure a successful outcome. Various medicaments, including non-specific antiseptics and antibiotics, have been used in root canal treatment, and each of them has both advantages and disadvantages (Grossman 1965, 1972, Haapasalo & Orstavik 1987, Morse 1987, Seltzer 1988, Abbott *et al.* 1990, Safavi *et al.* 1990, Spångberg 1994). Besides the use of non-specific antiseptics (Haapasalo & Orstavik 1987, Safavi *et al.* 1990), the application of antibacterial drugs may represent a way to eradicate bacteria during root canal treatment. The selection of these antibacterial drugs should be based on the most up-to-date microbiological knowledge.

With the technology for culturing obligate anaerobes, including the use of an anaerobic glove box, bacteria invading deep layers of root canal walls have been

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detected and reported to be predominantly obligate anaerobes (Ando & Hoshino 1990). Metronidazole has a wide spectrum of bactericidal action against oral obligate anaerobes (Ingham *et al.* 1975), even against isolates from infected necrotic pulps (Sundquvist 1976) and, in fact, more than 99% of the bacteria found in carious lesions (Hoshino *et al.* 1988) and infected root dentine (Hoshino *et al.* 199?) were not recovered in the presence of 10 µg ml^{-1} metronidazole in *in-vitro* experiments. However, metronidazole, even at a concentration of 100 µg ml^{-1}, could not kill all the bacteria (Hoshino *et al.* 199?), indicating that other drugs may be needed to sterilize infected root dentine. It has been reported that a mixture of antibacterial drugs, i.e. ciprofloxacin, metronidazole and minocycline, can sterilize root dentine (Sato *et al.* 1992, Hoshino *et al.* 199?).

The aim of the present study was to observe the capacity of a mixture of ciprofloxacin, metronidazole and minocycline to kill bacteria localized in the deep layers of dentine of infected necrotic pulps *in situ*.

Materials and methods

Test organisms

Escherichia coli strain NIHJ-JC-2 was obtained from the Japan Collection of Microorganisms (RIKEN, Saitama, Japan). The bacterial cells were cultured in heart infusion (HI) broth, harvested, washed three times with sterile saline and resuspended in sterile saline (to give a final concentration of 10^8 cells ml^{-1}). The cells of *E. coli* used in this experiment were very sensitive to the drug combination, and did not grow in the presence of the mixture (1 µg ml^{-1} each).

Experimental procedure 1

Experimental model. The crowns of 14 single-rooted teeth, which were free of dental caries, restorations and cracks, were cut off at the level of the cemento-enamel junction. The opening of each root canal was enlarged with an engine-driven enlarger (type F, ISO size 120) to make it easier to place the medicament. The remaining root canal was not instrumented. Small cavities, 3 mm in depth and 1 mm in diameter were prepared on the cut planes at sites 1 mm apart from and parallel to the root canal (Fig. 1). Some of the teeth were further treated ultrasonically under the following conditions: apparatus, ENAC (Osada Electric Co., Ltd, Tokyo, Japan), 33 kHz at level 3 using an irrigating solution consisting of 0.4 M EDTA solution (pH 7.6).
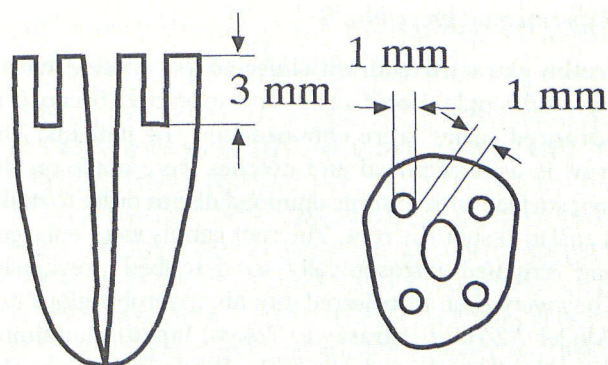

Fig. 1 Preparation of artificial cavities. The crown of the tooth was removed. Four cavities of depth 3 mm and diameter 1 mm were prepared at sites 1 mm apart from and parallel to the root canal on the cut surface.

Survival of test organisms. The suspensions of *E. coli* (1 µl) were placed into the cavities, and a mixture of ciprofloxacin, metronidazole and minocycline (0.5 mg each), or sterile saline as the control, was placed into the root canals. The teeth were then completely covered with blue inlay wax. At time 0, and at 5h, 24h and 48h after the drug combination had been applied, cells of *E. coli* were recovered from the cavities by washing the cavities several times with sterile saline. Organisms were cultured on the surfaces of HI agar plates for 1 week. The total number of colony-forming units was determined and monitored after incubation for 3 weeks.

Experimental procedure 2

Penetration of the drug mixture from the root canal to the dentine-cementum junction through the dentinal tubules was also determined. For this experiment, the root surfaces were carefully checked for cracks and collateral branches of root canals. Following removal of the crown and cementum, the root canals were carefully prepared as described previously. A mixture of ciprofloxacin, metronidazole and minocycline (0.5 mg each) was placed in the root canals after the ultrasonic treatment and the openings of the root canals were closed with blue inlay wax. To avoid leakage of the drugs through apical ramifications, the apical one-fifth of the root surface was also covered with blue inlay wax. To allow the drug combination to penetrate into the dentine, the teeth were allowed to stand for 6h. They were then placed on HI agar plates on which *E. coli* was inoculated. The plates were incubated for 24h at 37°C. Preliminary experiments had shown that *E. coli* grew well on HI agar plates. As a control, the drug combination was replaced by sterile saline.

Experimental procedure 3

Freshly extracted teeth with infected root dentine, which were all single-rooted teeth and extracted because of advanced caries, were obtained from 14 patients. The crowns were removed and notches were made on the root surface with a sterile diamond disc in order to make it easier to split the root. The root canals were enlarged and irrigated ultrasonically as described previously. They were then transferred into an anaerobic glove box (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2. While in the box, the root was split in half with forceps. Infected root dentine was taken to represent time 0 from the split surface of each tooth (Fig. 2a), using a sterile excavator or a dental bur at low speed (<120 rpm; Edwardsson 1974, Hoshino 1985, Ando & Hoshino 1990). A mixture of ciprofloxacin, metronidazole and minocycline (0.5 mg each) or sterile saline as the control was placed in the root canal, and the root was completely covered with blue inlay wax.

After 24h, the root was split again and, from the new split surfaces of the same lesion, the second sample was obtained (Fig. 2b). The samples were dispersed in sterile 40 mM potassium phosphate (pH 7.0) with a motor-driven homogenizer (Tissue-Tearor, Biospec Products, Bartlesville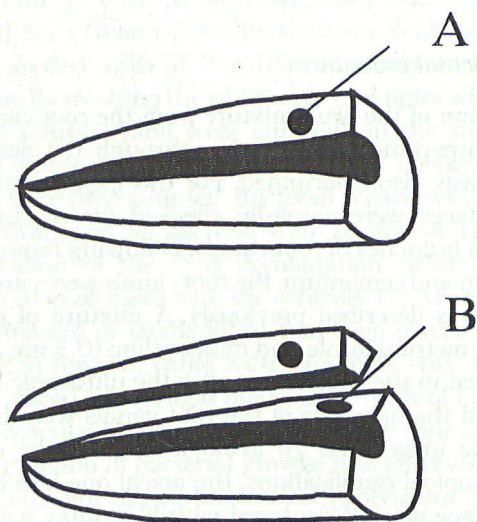, OK, USA) and a glass homogenizer. The suspension was inoculated with an automatic spiral plater (Model D, Spiral System Instruments, Inc., Bethesda, MD, USA) on to the surfaces of BHI-blood (sheep) agar plates (Holdeman *et al.* 1977) and cultured in an anaerobic glove box at 37°C for 7 days. Colony-forming units when then counted. Bacteria in the second samples, if any grew, were further inoculated on BHI-blood agar plates containing a mixture of ciprofloxacin, metronidazole and minocycline (25 μg ml^{-1} each) to confirm the sensitivity to the drug combination. All plates, media, buffer solution and experimental instruments were kept in the anaerobic glove box for at least 24h before use. To ensure aseptic procedures, intact dentine from the split surfaces was also taken, and treated in the homogenizer, diluted, and spread over the blood agar plates and cultured. It was confirmed that there was no bacterial contamination.

Results

Recovery of E. coli *placed in the artificially prepared cavities*

Cells of *E. coli* (10^5) could survive for at least 48h after the cells were placed in the prepared cavities in dentine (Table 1; samples 12–14). The bacterial recovery decreased, however, according to the time of the application of the drug mixture. Thus no bacteria were recovered from four samples after 24h (Table 1; samples 5, 6, 8 and 9) and from any of the samples after 48h (Table 1; samples 1–9). This incidates that the mixture of ciprofloxacin, metronidazole and minocycline penetrated 1-mm-thick dentine to the cavities after 24 or 48 h. In addition to these data for the ultrasonically irrigated teeth, *E. coli* was recovered after 48h when the drug combination was applied to root canals that had not been irrigated ultrasonically (Table 1; samples 10 and 11). This suggests that ultrasonic treatment may make the drug combination penetrate through dentine more easily.

Penetration of the drug combination through entire dentine

A wide inhibitory zone of *E. coli* was observed along the root placed on HI agar plates when the drugs were placed in the root canals and allowed to penetrate through dentine (Fig. 3.) No inhibitory zone was detected when the drug combination was replaced by sterile saline. This indicates that the bacterial growth was inhibited by the drugs which passed through the dentine from the root canals.

Fig. 2 Sampling areas in experimental procedure 2. The tooth root was split in half in the anaerobic glove box. Infected root dentine was taken as time-0 samples from the split surface (a). A mixture of ciprofloxacin, metronidazole and minocycline was placed in the root canal chamber, and the root was then completely covered with blue inlay wax. After 24h of application of the drug combination, the root was split again and, from the new split surfaces of the same lesion (b), the second sample was obtained.

Table 1 Bacterial recovery by experimental procedure 1*

Sample no	Time-0	After 5 h	24 h	48 h
		log (colony-forming units mg^{-1})		
1	5.43	5.00	2.30	0**
2	5.20	5.30	2.00	0
3	5.93	4.86	2.00	0
4	5.62	4.93	2.00	0
5	5.46	4.08	0	0
6	5.49	5.11	0	0
7	5.11	4.58	2.30	0
8	5.86	4.30	0	0
9	5.79	4.66	0	0
10[c]	5.59	5.62	4.78	2.30
11[c]	5.93	5.89	4.93	2.45
12[d]	5.00	5.26	5.66	5.64
13[d]	5.11	5.68	4.34	4.75
14[d]	5.72	5.36	4.70	5.18

*Recovery of *E. coli* from cavities prepared artificially 1 mm from the root canals where a mixture of ciprofloxacin, metronidazole and minocycline (0.5 mg each, sample 1–11) or sterile saline (samples 12–13) was placed. The root canals were treated (samples 1–9) or not treated (samples 10–11) ultrasonically.

**No bacteria were recovered.

Bacterial recoveries after 24 and 48 h (samples 1–9) were significantly different from those of time-0 samples (paired *t*-test; $P<0.0005$, while those of the control samples (samples 12–14) were not significantly different.

Antibacterial efficacy of the drug combination in situ against bacteria in infected root dentine

Substantial numbers of bacteria (10^1–10^5) occurred in the time-0 samples taken from the infected root canal dentine (Table 2; samples 1 to 12). However, none was recovered after a 24-h application of a mixture (0.5 mg each) of ciprofloxacin, metronidazole and minocycline (Table 2; samples 1 to 3 and 5 to 12), except for one case (Table 2; sample 4), from which 21 bacteria were recovered even after a 24-h application of the drug combination. However, all the isolates from the sample were sensitive to the drug combination, because they did not grow on BHI-blood agar plates in the presence of the drug mixture (25 μg ml^{-1} each). This may indicate that the bactericidal efficacy of the topically applied drug mixture is potent enough to sterilize root dentine within 48h.

Discussion

Bacteria in the main root canals and superficial layers of infected root canal walls may be easily removed by conventional root canal treatment. Bacteria, which remain in the deep layers of root canal dentine, may leak out to periapical regions and cause complications (Yawata 1973, Byström *et al.* 1987). It has been
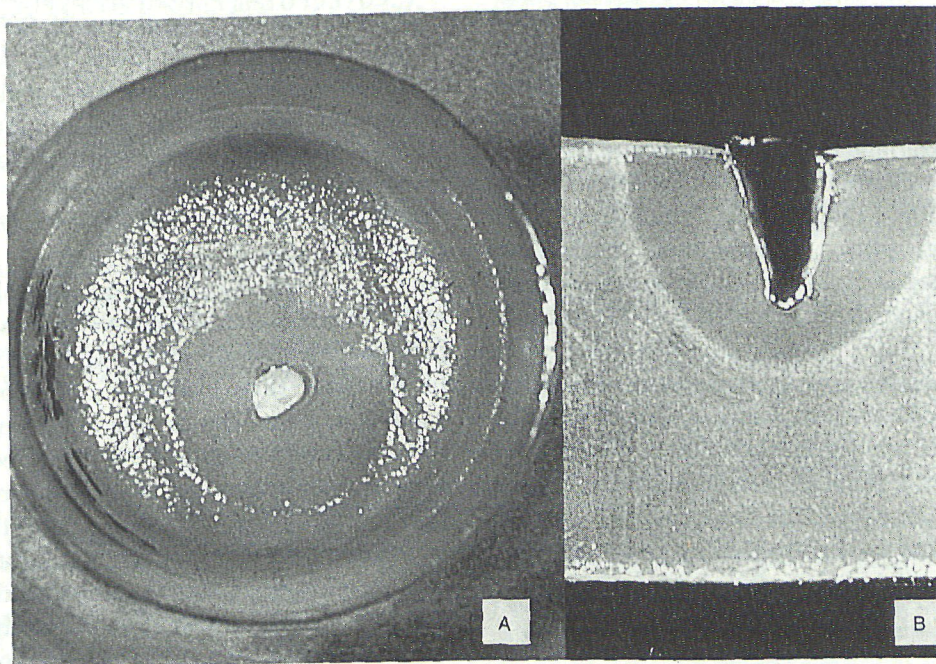

Fig. 3 The inhibitory zone of bacterial growth. (a) surface view of the inhibition. (b) The transverse section.

Table 2 Bactericidal efficacy of a mixture of ciprofloxacin, metronidazole and minocycline *in situ* against bacteria of infected root dentine

Sample No.	After	
	Time-0	24 h
	log (colony-forming units mg^{-1})	
1	3.76	0*
2	1.51	0
3	3.71	0
4	3.38	1 (21 colonies)
5	3.56	0
6	3.20	0
7	4.76	0
8	2.30	0
9	2.23	0
10	5.98	0
11	3.57	0
12	5.76	0
13**	2.38	2.77
14**	4.15	1.15

*No bacteria were recovered.
**Sterile saline was applied to the root canal canals.
Bacterial recoveries after 24 h (samples 1–12) were significantly different from thsoe of time-0 samples (paired *t*-test; $P<0.0005$), while those of the control samples (samples 12–14) were not significantly different.

reported that sterilization of the root canal and periradicular region results in good healing of periapical diseases in adults (Byström *et al.* 1987). In order to sterilize infected root dentine, especially the deep layers, antibacterial medicaments are useful. These compounds should reach the deeper layers of infected dentine. The experimental models in the present study were designed to test the potential of root medicaments to penetrate dentine and eradicate bacteria in deeper layers of dentine. Other *in vitro* models for examining infection and disinfection of root canal dentinal tubules have demonstrated that non-specific antiseptics (camphorated paramonochlorophenol, Haapasalo & Orstavik 1987; iodine potassium-iodide, Safavi *et al.* 1990) disinfected the dentinal tubules when these were contaminated with *Enterococcus faecalis*. It is also clearly demonstrated in the present *in situ* study that the mixture of ciprofloxacin, metronidazole and minocycline penetrated through the dentine from the root canal and eradicated bacteria from the infected root dentine. This strongly suggests that infected root dentine can be sterilized by topical application of the drug combination to root canals in root canal treatment.

Since the overwhelming majority of bacteria in the deep layers of infected dentine of the root canal wall consist of obligate anaerobes (Ando & Hoshino 1990),

metronidazole was selected as the first choice among antibacterial drugs. It is reported that metronidazole can penetrate the deep layers of carious lesions and disinfect the lesions *in vivo* (Hoshino *et al.* 1989a) and, moreover, diffuse throughout the dentine (Csukás *et al.* 1987). Since various types of bacteria have been isolated from periradicular lesions and root canals in previous studies by the present authors (Hoshino 1985, Hoshino & Sato 1988, Hoshino *et al.* 1989b, Ando & Hoshino 1990, Uematsu & Hoshino 1992, Hoshino *et al.* 1992, Sato *et al.* 1993, Sato *et al.* 1993a, Kiryu *et al.* 1994) or by others (Bergenholtz 1974, Edwardsson 1974, Wittgow & Sabiston 1975, Sundquvist 1976, Zavistoski *et al.* 1980, Yoshida *et al.* 1987, Sugita 1994), metronidazole cannot kill all bacteria (Hoshino *et al.* 199?) indicating that other drugs may be necessary to sterilize infected root dentine. Thus, ciprofloxacin and minocycline, in addition to metronidazole, were required to sterilize infected root dentine.

The permeability of root canal disinfectants is thought to be affected by the conditions prevailing in the dentinal tubules, for example, the presence of a smear layer. One of the ways to achieve efficient penetration of the drug mixture through dentine is by ultrasonic cleaning and chemical irrigation of the root canal. Smear layer and debris may be removed by ultrasonic irrigation and, as a result, the dentinal tubules may become open (Cameron 1983, Imamura *et al.* 1989), and they may also become enlarged. Consequently, the drugs diffuse more easily. In addition, chemical irrigation with EDTA may help to remove calcified debris from the dentinal tubules, although further ultrasonic treatment appears to be necessary for efficient drug delivery (Table 2). Another study to establish efficient penetration of the drugs through dentine is now in progress.

In endodontic diseases, bacteria may invade not only dentine but also cementum (Kiryu *et al.* 1994). Such bacteria are reported to be mainly obligate anaerobes and are sensitive to the drug combination used in the present study (Kiryu *et al.* 1994). It appears to be difficult to eliminate these bacteria using conventional root canal treatment. The present study demonstrates that the drug combination could be delivered to the dentine-cementum junction and, if so, it is probable that such bacteria are killed with local application of the drug combination to root canals. Mixed antibacterial drugs are also effective against bacteria in periodontal pockets and dental plaque (Hoshino 1990, Hoshino *et al.* 1990), suggesting that this drug combination may also be useful in the treatment of endodontic-periodontal diseases.

This research demonstrates that a mixture of ciprofloxacin, metronidazole and minocycline is useful for sterilization of infected root dentine, and that the drug mixture can be applied to root canals. The topical application needs only a low dose of the mixture, and is delivered for only a short period, e.g. 2 days (Tables 1 & 2). Thus any adverse systemic side-effects could be minimized although, as a matter of principle, the application of antibiotics should be limited if possible.

Minocycline sometimes causes pigmentation, especially in calcifying teeth, so the bactericidal efficacy of the mixture of ciprofloxacin and metronidazole plus amoxicillin (Mixed-drug I; Sato T *et al.* 1993b), cefaclor (Mixed-drug II), cefroxadine (Mixed-drug III), fosfomycin (Mixed-drug IV) or rokitamycin (Mixed-drug V) have been compared and it has been found that these new drug combinations ($100 \ \mu g \ ml^{-1}$ each) were able to sterilize carious and endodontic lesions (Sato *et al.* 1993b, Hoshino *et al.* 199?).

Acknowledgements

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