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Susceptibility of *Enterococcus faecalis* to a Combination of Antibacterial Drugs（3Mix）*in vitro*

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Abstract：It has been reported that enterococci cause significantly persistent root canal infection, especially after $Ca(OH)_2$ intracanal dressing, and that they often show tolerance to certain antibacterial drugs. We aimed to evaluate the susceptibility of enterococci to a combination of antibacterial drugs, *i.e.* ciprofloxacin, metronidazole and minocycline（3Mix）, which is used for Lesion Sterilization and Tissue Repair（LSTR）therapy. The minimum inhibitory concentrations（MICs）of ciprofloxacin and minocycline on *E. faecalis*（6 strains）and *E. faecium*（1 strain）were 5—20 μg/mL, respectively, and no inhibitory effect was observed with metronidazole. However, 3Mix（100 μg each/mL）, as a mixture, inhibited the growth of every strain completely. In addition, 3Mix also inhibited all bacterial growth in faeces（16 samples）. The present result strongly indicates that 3Mix is sufficiently able to inhibit enterococcal growth, and may be useful for endodontic treatment, even in cases where enterococci are suspected to cause endodontic disorders.

Introduction

Many studies have indicated that the outcome of endodontic treatment is sometimes unsuccessful, especially in cases where bacteria remain in the root canal systems and cause inflammation of pulpal or periradicular tissues[1—6]. If so, the elimination of such microorganisms should be important in order to achieve successful outcomes[7—9].

Calcium hydroxide, $Ca(OH)_2$, is widely used as root canal dressing in endodontic treatment, obtaining excellent clinical outcomes[10,11]. However, after treatment with $Ca(OH)_2$, alkali-resistant enterococci[12—17], especially *Enterococcus faecalis*[13—15], have been isolated from some lesions and led to persistent root canal disorders.

To eliminate bacteria remaining in endodontic lesions, the application of antibacterial drugs may be useful. It has been found that a mixture of three antibacterial drugs（3Mix）, *i.e.* ciprofloxacin, metronidazole and minocycline, used under the concept of Lesion Sterilization and Tissue Repair（LSTR）therapy, could kill all bacteria taken from carious lesions, necrotic pulps, infected root dentine and endodontic lesions of permanent teeth[18—20] and deciduous teeth[21—23], and, in fact, treatment using 3Mix-MP（M：macrogol, P：propylene glycol）gave excellent outcomes[20,23]. 3Mix has not caused any observed pathological changes when placed onto human pulp tissue[24].

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3Mix was also effective against all isolates taken from various oral sites[18—23]. Since enterococci may also reside in oral cavities, 3Mix seemed to be effective even against enterococci. However, confirmation of its effectiveness against enterococci is needed, because enterococcal strains are resistant to various antibacterial drugs[14].

In the present study, we aimed to evaluate the antibacterial efficacy of 3Mix against enterococci, especially *E. faecalis*. Moreover, we also aimed to evaluate the antibacterial potential of 3Mix against human faecal bacteria, because intestinal bacterial florae may be a possible source of enterococci invading endodontic lesions, and may contain various species of enterococci which are alkali-resistant and tolerant against antibacterial drugs.

Materials and Methods

1. Bacterial samples

From the American Type Culture Collections (ATCC), *E. faecalis* ATCC 19433^T and *E. faecium* ATCC 19434^T were purchased as reference strains.

Eight clinical isolates, used in the present study, were obtained from the Department of Medical Examination, Niigata University Hospital. All isolates were identified using PCR. In brief：Microbial organisms were suspended and centrifuged in sterilized saline in sterilized microcentrifuge tubes. After treatment with 200 μL of InstaGene Matrix (Bio-Rad, Hercules, CA, USA), genomic DNAs were purified according to the manufacturer's protocol. Specific PCR amplifications were carried out in 50 μL of reaction mixture containing 25 μL of TaKaRa Premix Taq solution (Takara, Tokyo, Japan), 4 pmol of a specific primer set and template DNA extracted from the microbial cells. Amplifications were performed on Programmable Thermal Controller PTC-100 (MJ Research, Watertown, MA, USA) with 35 cycles of 94℃ for 1 min, 54℃ for 1 min, and 72℃ for 2 min. The specific primer set of 5'-ATC AAG ACA GTT AGT CTT TAT TAG-3' and 5'-ACG ATT CAA AGC TAA CTG AAT CAG T-3'[25] was selected for the 16S rDNA 941 bp gene product of *E. faecalis*. Another specific primer set, 5'-TTG AGG CAG ACC AGT TGA CG-3' and 5'-TAT GAC AGC GAC TCC GAT TCC-3', was also used[25] for the 658 bp product of *E. faecium*. The specificity of these primer sets was confirmed using reference DNAs extracted from *E. faecalis* ATCC 19433^T and *E. faecium* ATCC 19434^T. Also, it was confirmed that these specific primer sets do not produce any amplicons with DNA templates from the other bacterial species.

Human faeces (ca. 2 mg) were collected from 16 healthy volunteers aged 19—34 (mean of 22) years old.

2. Experimental procedures

Each of ciprofloxacin, minocycline and metronidazole was powdered finely using a porcelain mortar and pestle after removing coatings, if any, and stocked in a dehumidified and tightly capped device, respectively.

Aliquots of 0.1 mL of each enterococcal sample (10^8—10^9 organisms, Table 1), suspended in sterile pre-reduced 40 mM potassium phosphate (pH 7.0) and diluted in a serial-10-fold mode, were inoculated onto surfaces of brain heart infusion-yeast extract-blood (sheep) agar plates (BHI-blood agar[26]) with or without a mixture of ciprofloxacin, minocycline and metronidazole (3Mix) at a drug concentration of 100 μg/mL each, and incubated in an anaerobic glove box (Model AZ-Hard, Hirasawa, Tokyo, Japan) containing 80% N_2, 10% H_2 and 10% CO_2 at 37℃ for 7 days. Colony-forming units (CFU) were counted.

Each of the faecal samples was suspended in sterile pre-reduced 40 mM potassium phosphate (pH 7.0) and dispersed well with a glass homogeniser. The same bacteriological procedures were taken as mentioned above.

In order to determine MIC, BHI broth media containing the respective drug were prepared with a serial 2-fold dilution method, and bacteria growth was determined by measuring the turbidity at 660 nm.

Plates, media and buffer solution were kept in the anaerobic glove box for at least 24 h prior to use. BHI-blood agar plates containing 3Mix were used within 3 days for bacterial inoculation.

Table 1 Bacterial recovery on BHI-blood agar plates with or without 3Mix

Bacteria	Without 3Mix	With 3Mix
E. faecalis		
strain 1	7.0×10^8	0
strain 2	8.4×10^8	0
strain 3	6.0×10^8	0
strain 4	8.9×10^8	0
strain 5	2.0×10^8	0
strain 7	4.7×10^8	0
strain 8	7.0×10^8	0
E. faecium		
strain 6	1.0×10^8	0
E. faecalis		
ATCC 19433^T	1.9×10^9	0

3Mix is a mixture of ciprofloxacin, minocycline and metronidazole at a drug concentration of 100 μg/mL each.

Table 2 Minimum inhibitory concentrations (MIC) of ciprofloxacin and minocycline against *E. faecalis* and *E. faecium* strains

	MIC (μg/mL)	
Bacteria	ciprofloxacim	minocycline
E. faecalis		
strain 1	5	20
strain 2	5	20
strain 3	5	20
strain 4	5	0.5
strain 5	5	2
strain 7	5	20
strain 8	5	20
E. faecium		
strain 6	20	20
E. faecalis		
ATCC 19433^T	5	0.25

The MIC of each strain was the same in duplicated determinations. Metronidazole did not inhibit the growth of *E. faecalis* and *E. faecium* strains at a drug concentration of less than 1,000 μg/mL.

Results

Out of a total of 8 clinical isolates of enterococci, 7 strains were identified as *E. faecalis* and one strain as *E. faecium* (strain 6) using PCR specific primers.

None were recovered in the presence of 3Mix at a concentration of 100 μg/mL each, while more than 10^8 CFU/mL (range 1.0×10^8 to 8.9×10^8, average 5.6×10^8) bacterial colonies occurred in the same samples of 8 respective enterococcal strains (Table 1), suggesting that these strains of *E. faecalis* and *E. faecium* were completely inhibited by 3Mix.

The MIC of ciprofloxacin against seven isolates of *E. faecalis* and one isolate of *E. faecium* was 5—20 μg/mL, respectively (Table 2). The MIC of minocycline against all the isolates of *E. faecalis* and *E. faecium* was 0.5—20 μg/mL (Table 2). Metronidazole did not have an inhibitory effect on any of the 8 enterococcal isolates tested.

About 10^8 CFU/mL (range 7.3×10^7 to 15.0×10^8, average 6.8×10^8) bacteria were recovered from human faecal samples. However, no bacteria were recovered from the same samples in the presence of 3Mix at a concentration of 100 μg/mL each, although some fungal colonies were recovered from five faecal samples (Table 3). These results show that 3Mix has a significant antibacterial effect on bacteria in human faeces.

Discussion

One of the aims of using medicaments in endodontic treatment is to disinfect microorganisms of endodontic lesions. The high alkalinity of $Ca(OH)_2$ contributes to its antimicrobial activity, and, thus, $Ca(OH)_2$ is widely used as root canal dressing in endodontic treatment and gives excellent clinical outcomes[10,11].

Enterococci have been sometimes observed in endodontic lesions even after treatment using $Ca(OH)_2$[27,28] because of their resistance against alkaline pH[17]. Even so, enterococci do not seem to be so common in oral sites[29—31], so disinfections of such remaining bacteria in endodontic lesions should be of benefit for successful clinical outcomes[32]. 3Mix, as a combination of 3 antibacterial drugs, may be useful for this purpose, because the present study clearly demonstrated its antibacterial effects against *E. fae-*

Table 3 Microbial recovery from faecal samples on BHI-blood agar plates with or without 3Mix

Faeces	Without 3Mix	With 3Mix
1	2.0×10^8	0
2	7.8×10^8	0
3	1.2×10^8	0
4	3.8×10^8	0
5	7.3×10^7	0
6	8.5×10^8	0
7	1.4×10^9	*1.9×10^1
8	1.5×10^9	0
9	6.5×10^8	0
10	3.2×10^8	0
11	1.3×10^9	0
12	3.4×10^8	0
13	6.4×10^8	*3.2×10^2
14	7.6×10^8	*7.4×10^1
15	2.5×10^8	*3.0×10^0
16	6.7×10^8	*3.0×10^0

3Mix is a mixture of ciprofloxacin, minocycline and metronidazole at a drug concentration of 100 μg/mL each.
*Only *Candida*-like fungus observed.

calis and *E. faecium*. In addition, 3mix inhibited all bacterial growth in faecal samples, which might contain various other kinds of enterococci.

Enterococci have often revealed both intrinsic and acquired types of antimicrobial resistance properties against various antibiotics[33,34]. It has also been reported that *E. faecalis* isolates from persistent endodontic lesions are multi-resistant to antibiotics[14]. The present study also showed that all enterococcal isolates used in this study were resistant against metronidazole, in agreement with earlier studies[35,36]. However, the other 2 antibacterial drugs were still effective against *E. faecalis*, *E. faecium* and other probable enterococcal species in faeces, in agreement with reports in which the MIC range values of ciprofloxacin against *E. faecalis* were 0.25—8 μg/mL[37] and 0.5—32 μg/mL[38], and those of minocycline were 0.25—32 μg/mL[37]. As a drug combination of different antibacterial modes[39], 3 Mix can be used as a medicament in cases even when enterococci are suspected to cause endodontic disorders, because it is effective against all types of oral bacteria[18,19,21,22] which can possibly invade endodontic lesions. In fact, 3Mix, in the form of 3Mix-MP in endodontic treatment, provided excellent clinical outcomes[40].

In the present study, however, some fungal strains were recovered from five human faecal samples in the presence of 3Mix. Fungus such as *Candida albicans* has been isolated as a minor constituent from human dental plaques and endodontic lesions[12,13,15,16,41]. *C. albicans* and some other *Candida* species have been shown to be resistant to $Ca(OH)_2$ *in vitro*[42]. If so, LSTR 3Mix-MP treatment may need to be considered in modified treatment procedures[40] in cases when *Candida* is associated with endodontic disorders.

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