Cytotoxicity Versus Antibacterial Activity of Some Antiseptics *In Vitro*

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Abstract

Through the development of new techniques for bacterial growth and isolation, obligate anaerobes have been shown to be more prevalent than previously thought in the pathogenesis of periapical and pulpal diseases.

A follow-up study was conducted to examine the germicidal effect of 0.05% NaOCl, 10 mg/ml metronidazole and supernatant of Ca(OH)₂ (0.025 ml) on four anaerobic microorganisms commonly found in root canals, and to compare the toxicity of these substances on cell cultures. *In vitro* testing revealed that 0.05% NaOCl and Ca(OH)₂ were both equally effective on these anaerobes. Also, metronidazole was found to be germicidally effective against *Bacteroides melaninogenicus*, *Bacteroides oralis* and *Peptostreptococcus anaerobius*, but ineffective against *Veillonella alcalescens*.

Furthermore, it was found that NaOCl and Ca(OH)₂ had a very destructive effect on cell cultures compared with their antimicrobial effect, whereas metronidazole was less toxic among the agents tested.

Introduction

Modern endodontic practice is related more to mechanical disinfection of the root canal system, rather than making root canal residues inert or killing microorganisms by chemical means. However, proper debridement of the canal system cannot be achieved because of the complexity of the pulp cavity. Thus, control of infection may be problematic in some cases, and the potential value of an antimicrobial agent between dental appointments cannot be disregarded.

Unfortunately, many commonly used medications are believed to possess a high potential for toxicity. Therefore, assessment of the biological balance between germicidal activity and cytotoxicity should be the overall aim and philosophy of root canal treatment.

New techniques for the growth and regulation of obligate anaerobes have shown that anaerobic microorganisms are much more prevalent in the root canal spaces than previously thought.

The purpose of the present study was to examine the germicidal effects of Ca(OH)₂, metranidazole and NaOCl on certain anaerobic bacteria and to compare their toxicity on cell cultures.

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Materials and Methods

The medicaments used were calcium hydroxide (Sigma), NaOCl (0.05%), and metronidazole (10 mg/ml). The obligate anaerobes investigated were *Bacteroides melaninogenicus*, *Bacteroides oralis*, *Veillonella alcalescens* and *Peptostreptococcus anaerobius*. In preliminary experiments, the antibacterial activity of the disinfectants had been estimated by superior coefficient test. For this purpose, dilutions of disinfectants were prepared. One fifth of a milliliter of 1:1000-diluted bouillon culture of *Bacillus subtilis* was added to these solutions in test tubes, and incubated at 37°C. The lowest concentration at which no growth was seen was recorded as the superior lethal coefficient of the respective disinfectant. The effects of these concentrations were then tested on the chosen microorganisms.

A 24-28 h turbid culture of *B. melaninogenicus* (10^6 organisms per ml) in 50 ml of trypticase soy broth was used in this experiment. The culture was poured into a sterile beaker. Twenty-four absorbent paper points were sterilized, and twenty of them were submerged in the beaker containing the inoculum for at least 3 min. The remaining 4 paper points were used as controls.

Five milliliters of each sterile test solution was placed in each of 20 sterile tubes and the 20 paper points that had been submerged in the inoculum were then placed in the beakers. Ten of the points were removed from the test solution beakers after 5 min, and 10 were removed after 15 min. Upon removal, the points were placed in a test tube with 10 ml of thioglycolate medium in which anaerobic bacteria could be grown.

After a 72-h incubation at 37°C, the presence of turbidity in each tube was recorded. Each tube, including those that had a negative growth response in the thioglycolate, was then subcultured on blood agar plates. The presence of colonies of the test organisms on the plates was confirmed by gram staining.

Two of the four remaining sterilized paper points, which were used as controls, were placed in the beaker containing the bacterial culture for 3 min and then transferred to separate tubes containing 10 ml of the thioglycolate solution. The two remaining sterilized points were placed in two thioglycolate tubes. These four tubes were then incubated and subcultured.

The entire procedure was repeated with cultures of the other three microorganisms (*B. oralis*, *V. alcalescens* and *P. anaerobius*).

For the second stage of our investigation, the cytopathologic effects (CPE) of the germicidally effective dilutions of Ca (OH)₂, metronidazole, and NaOCl were tested on an HEP-II continuous cell culture, which was provided by the Virology Department of the Faculty of Veterinary Medicine, University of Ankara.

The HEP-II cell culture was passaged to roller tubes (Grainer, GmbH Nutringen, Germany), following the formation of an adequate monolayer in the tissue culture bottle. Then the cell culture medium was removed and the cell surface was washed gently with phosphate-buffered saline minus (PBS-M). PBS-Versen-Trypsin solution was added to the cell culture bottle in order to dissociate the cells from each other and/or the bottle surface. After completion of the washing procedure, the aliquot was centrifuged at 1000 rpm for 10 min, followed by incubation of the cell culture for 10 min at 37°C.

Preparation of Cell Cultures in Tubes: The cell pellets obtained from centrifugation were resuspended at 3×10^5 cells/ml in Eagle's minimum essential medium (Eagle's MEM: Gibco, Paisley, Scotland) supplemented with 2% fetal bovine serum (FBS: Paesel GmbH and Co., Frankfurt, Germany), 100 IU/ml penicillin, 10 µg/ml streptomycin and 50 µg/ml kanamycin. Two milliliters of cell suspension was inoculated into each of a series of roller tubes and incubated in 5% CO₂ at 37°C for two days.

Antibacterial Agents: Ten-times serially diluted Ca (OH)₂, NaOCl, and metronidazole with PBS-M were prepared and checked to contain the minimum antibacterial doses. The prepared samples were stored at -20°C. Following the attainment of confluent monolayer cell growth in

the roller tubes, the media were removed, and fresh medium containing 10% of each antiseptic dilution prepared beforehand was placed into four roller tubes. Then medium containing PBS-M at the same percentage was added to the four tubes as a control. The cell culture tubes containing each of the antiseptic dilutions were examined at regular intervals with a tissue culture microscope (Olympus, Tokyo, Japan), and the observed cytotoxicity data were recorded in the terms of CPE.

Results

The superior lethal coefficients of metronidazole and NaOCl were found to be 10 mg/ml and 2/100, respectively. However, in order to provide acceptable bactericidal levels and cytotoxicity, a 0.5% concentration of NaOCl has been recommended^[1]. Therefore, the effect of 0.5% NaOCl solution on the obligate anaerobic microorganisms was examined. The activities of the disinfectants on *B. melaninogenicus*, on the basis of their superior lethal coefficients, are shown in Table 1.

Metronidazole at its suitable dilution was fully effective against *P. anaerobius* and *B. oralis* in both the 5- and 10-min tests at 24, 48 and 72 h of incubation, but ineffective against *V. alcalescens*, and destroyed *B. melaninogenicus* only in the 10-min test at 24, 48 and 72 h of incubation (Table 2).

Ca(OH)₂ destroyed *V. alcalescens* and *B. oralis* in both the 5- and 10-min tests at 24, 48 and 72 h, but *P. anaerobius* and *B. melaninogenicus* were destroyed only in the 10-min test (Table 3).

NaOCl diluted adequately was ineffective against *V. alcalescens* in the 5-min test at the end of 48 h of incubation, but was effective in the 10-min test (Table 4). The control paper points, placed directly into thioglycolate tubes after being contaminated with the bacterial cultures, all produced positive growth. However, the contaminated control paper points placed into the thioglycolate tubes did not produce any growth. The results of these control procedures were an indication of the effectiveness of the anaerobic growth methods.

The CPE of the appropriate dilutions of calcium hydroxide, sodium hypochlorite and metronidazole are shown in Tables 2, 3 and 4, respectively. Apart from metronidazole, all dilutions of Ca(OH)₂ and NaOCl were cytotoxic against HEP-II cells starting from the fifth minute. However, the CPE of metronidazole was evident after 48 h.

Discussion

The toxicity of the different materials and medicaments that have been used in irrigation, canal disinfection, and obturation procedures in endodontics has been evaluated by both *in vivo* and *in vitro* methods since 1948.

The purpose of the present investigation was to determine the germicidally effective dilutions of some currently used intra-canal irrigants and disinfectants such as Ca(OH)₂, NaOCl and metronidazole, and to correlate their antimicrobial effects with their toxicity against HEP-II cell cultures.

NaOCl has been the most commonly used root canal irrigant at a concentration of 5%. However, *in vitro* studies of its toxicity on cultured cells suggest that this concentration may be too high for clinical use^[2]. A 0.5% concentration was found to produce germicidal effects on the tested microorganisms, and more dilute concentrations were shown to have cytopathologic effects on HEP-II cells. Even the more dilute germicidally effective concentrations of NaOCl were shown to have undesirable cytopathologic effects on HEP-II cells.

It is known that Ca(OH)₂ has several uses in different areas of endodontics. STEVENS AND GROSSMAN^[3] were able to demonstrate the disappearance of bacteria in canals treated with Ca(OH)₂. CVEK et al.^[4] showed that calcium hydroxide was able to eliminate necrotic debris which acted as a substrate for bacteria in the incisors. HASSELGREN et al.^[5] demonstrated the tissue-

Table I The effects of $\text{Ca}(\text{OH})_2$, NaOCl and metronidazole on obligate anaerobic microorganisms at 24, 48 and 72 hours of incubation

		<i>B. melaninogenicus</i>		<i>B. oralis</i>		<i>V. alcalescens</i>		<i>p. anaerobius</i>	
TIME	MINUTE	5	10	5	10	5	10	5	10
	Hour								
Ca(OH ₂)	24	+	—	—	—	—	—	+	—
	48	+	—	—	—	—	—	+	—
	72	+	—	—	—	—	—	+	—
MN	24	+	—	—	—	+	+	—	—
	48	+	—	—	—	+	+	—	—
	72	+	—	—	—	+	+	—	—
NaOCl	24	—	—	—	—	—	—	—	—
	48	—	—	—	—	+	—	—	—
	72	—	—	—	—	+	—	—	—

MN : Metronidazole

+ : Positive growth

— : Negative growth

Table 2 The CPE of dilutions of metronidazole on HEP II cells at 5 minutes—48 hours

CONTACT TIME	DILUTIONS		
	1 : 100	1 : 1000	1 : 10000
5 Minutes	—	—	—
10 Minutes	—	—	—
15 Minutes	—	—	—
24 Hours	—	—	—
48 Hours	+	+	+

+ : Cytopathological effect

— : No cytopathological effect

Table 3 The CPE of dilutions of $\text{Ca}(\text{OH})_2$ on HEP II cells at 5 minutes—48 hours

CONTACT TIME	DILUTIONS		
	1 : 40	1 : 400	1 : 4000
5 Minutes	+	+	+
10 Minutes	+	+	+
15 Minutes	+	+	+
24 Hours	+	+	+
48 Hours	+	+	+

+ : Cytopathological effect

— : No cytopathological effect

dissolving ability of $\text{Ca}(\text{OH})_2$. They were also able to show that pretreatment with $\text{Ca}(\text{OH})_2$ enhanced the tissue-dissolving ability of NaOCl . METZLER AND MONTGOMERY^[6] evaluated the effectiveness of $\text{Ca}(\text{OH})_2$ as an aid in cleaning the root canal system, and found that it helped to remove pulp tissue debris. In addition, $\text{Ca}(\text{OH})_2$ has a hemostatic effect and can terminate persistent bleeding after pulp extirpation. The antibacterial effect of $\text{Ca}(\text{OH})_2$ is due to its high pH of 12. Calcium hydroxide in the form of a supernatant liquid or slurry has a pH of 11.8. In our investigation, $\text{Ca}(\text{OH})_2$ showed germicidal activity against *P. anaerobius*, *B. melaninogenicus*, *B. oralis* and *V. alcalescens*. Because of the alkaline pH of $\text{Ca}(\text{OH})_2$ solution

Table 4 The CPE of dilutions of NaOCl on HEP II cells at 5 minutes—48 hours

CONTACT TIME	DILUTIONS			
	1 : 50	1 : 500	1 : 5000	1 : 50000
5 Minutes	+	+	+	+
10 Minutes	+	+	+	+
15 Minutes	+	+	+	+
24 Hours	+	+	+	+
48 Hours	+	+	+	+

+ : Cytopathological effect

— : No cytopathological effect

and the stringent pH requirements of cells growing in a synthetic medium, the $\text{Ca}(\text{OH})_2$ solution was mixed with the cell culture medium to ensure that the volumes used would not significantly alter the pH level. TORNECK et al.^[7] investigated the effect of $\text{Ca}(\text{OH})_2$ on porcine pulp fibroblasts and demonstrated that it increased the ratio of DNA synthesis in the culture medium. We used human embryonic placenta cells (HEP-II), which are more sensitive than fibroblasts. Probably for this reason, $\text{Ca}(\text{OH})_2$ showed a cytopathologic effect at all dilutions beginning from the fifth minute. This effect can be explained by the fact that when $\text{Ca}(\text{OH})_2$ solution is used in direct contact with vital cells, the OH^- ion concentration in the solution may suppress cell activity and arrest vital processes.

SUNDQVIST^[8] showed that metronidazole has a wide spectrum of bactericidal action against obligate anaerobe microorganisms. LOESHE et al.^[9] and LEKOVIC et al.^[10] suggested that metronidazole decreases anaerobe populations in human periodantal pockets. HOSHINO et al.^[11,12] demonstrated that metronidazole had an antibacterial effect against microorganisms of carious dentin *in vivo* and *in vitro*.

In our study, metronidazole showed a bactericidal effect on all obligate anaerobe microorganisms except for *V. alcalescens*. However, the bactericidal mechanism of metronidazole is still controversial. It is believed to act by inhibition of protein synthesis^[13,14]. This drug has the advantage of eliminating pathogenic anaerobes, without disturbing the protective commensal aerobic flora. In addition, resistance to the drug does not appear to develop among anaerobes^[10]. We found that metronidazole had a lower CPE than $\text{Ca}(\text{OH})_2$ and NaOCl. This confers another advantage to the drug besides its other properties.

In vitro conditions are more preferable in cytotoxicity experiments because such studies are more controllable and repeatable. At the same time, they are quicker and less expensive than animal experiments, and easier to standardize. However, correlation of the results of *in vitro* studies and *in vivo* cytotoxicity tests performed on cell cultures are very sensitive. Even the more diluted concentrations of the same medicament can be used safely without producing any reactions in patients under clinical conditions. Besides this, bacterial contamination increases the periapical tissue toxicity of the material. Preparation of more dilute solutions of antimicrobial agents in order to decrease their toxicity will have a negative effect on antimicrobial activity.

Conclusion

$\text{Ca}(\text{OH})_2$, metronidazole and NaOCl are all effective antimicrobial agents against obligate anaerobic microorganisms. The CPE of metronidazole was found to be the lowest among the disinfectants tested. We suggest that in persistent cases of infection, especially those due to anaerobic microorganisms, the clinical use of metronidazole is advisable for achieving a maximum antimicrobial effect and a minimum cytotoxic effect.

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