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, metronidazole 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 x 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 cytotoxic-
ity, 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-
dissolving ability of Ca\((\text{OH})_2\). They were also able to show that pretreatment with Ca\((\text{OH})_2\) enhanced the tissue-dissolving ability of NaOCl. METZLER AND MONTGOMERY\(^6\) evaluated the effectiveness of 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, Ca\((\text{OH})_2\) has a hemostatic effect and can terminate persistent bleeding after pulp extirpation. The antibacterial effect of 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, Ca\((\text{OH})_2\) showed germicidal activity against \textit{P. anaerobius}, \textit{B. melaninogenicus}, \textit{B. oralis} and \textit{V. alcalescens}. Because of the alkaline pH of Ca\((\text{OH})_2\) solution

<table>
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<th>TIME</th>
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MN: Metronidazole  
+ : Positive growth  
− : Negative growth

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

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<td>48 Hours</td>
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+ : Cytopathological effect  
− : No cytopathological effect

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

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<thead>
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+ : Cytopathological effect  
− : No cytopathological effect
Table 4  The CPE of dilutions of NaOCl on HEP II cells at 5 minutes—48 hours

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+ : Cytopathological effect  
- : No cytopathological effect 

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

and the stringent pH requirements of cells growing in a synthetic medium, the Ca (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 Ca (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, Ca (OH)$_2$ showed a cytopathologic effect at all dilutions beginning from the fifth minute. This effect can be explained by the fact that when Ca (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 periodontal 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 Ca (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

Ca (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.
References


