Insights into the bacterial and fungal ecology of endodontic infections
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Antimicrobial effect of a modified vanadium chloroperoxidase on *Enterococcus faecalis* biofilms at root canal pH

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Ship’s bow of the *Doggersbank* in Groningen, the Netherlands.

Fresh antifouling paint should prevent the buildup of barnacles and algae on undersea surfaces of ships. Similarly, modified VCPO was developed to prevent similar buildup, but may also have antimicrobial activity in the root canal system.
ABSTRACT

AIM
Previous research showed an antimicrobial effect of vanadium chloroperoxidase (VCPO) on in vitro Enterococcus faecalis biofilms. The current study aimed to optimize the use of this enzyme at the root canal pH using a modified VCPO (mVCPO) that was adapted to function at a higher pH and to explore the biocompatibility of mVCPO.

METHODOLOGY
The activity of the original and modified VCPO was assessed using the monochlorodimedone assay. For antimicrobial assessment, 48-hour biofilms of E. faecalis OS-16 were incubated 5 or 30 minutes with mVCPO, bromide and hydrogen peroxide; and colony-forming units were determined. A metabolic activity assay was used to evaluate the cytotoxic effect of mVCPO on oral fibroblasts.

RESULTS
Reaction products generated by mVCPO at a root canal pH of 7.7, significantly inactivated the biofilm after 5 minutes and even more after 30 minutes (Mann-Whitney U test; \( P < 0.05 \)). The mVCPO reaction products showed less cytotoxic effects than control solutions and 0.5% sodium hypochlorite (Kruskal-Wallis test; \( P < 0.05 \)).

CONCLUSION
The incubation of mVCPO in the presence of its substrates with in vitro E. faecalis biofilms showed a significant antimicrobial effect at the root canal pH. Also, cytotoxicity tests showed preliminary biocompatibility. Therefore, an interappointment dressing containing mVCPO could aid in improving current endodontic treatment through continuous and local generation of antimicrobials.
INTRODUCTION
Apical periodontitis is the inflammatory reaction of periapical tissues to a polymicrobial biofilm infection of the root canal system (Fabricius et al. 1982; Ricucci & Siqueira 2010). Treatment aims at inactivation and removal of these microorganisms and their irritants. So far, it has been impossible to completely disinfect the root canal system because of its elaborate anatomy (Vertucci 1984), the biofilm configuration (Chávez de Paz et al. 2010), and medicament inactivation by dentin (Margental et al. 2013). Current antimicrobial therapy is unable to overcome all these limitations and fully penetrate the root canal system. Therefore, even after extensive chemomechanical treatment, microorganisms persist (Byström & Sundqvist 1985) and are able to maintain or replicate into pathogenic numbers, causing persistent or recurrent apical periodontitis (Molander et al. 1998).

Previous research has shown the antimicrobial effect of the reaction products of the fungal Curvularia inaequalis enzyme vanadium chloroperoxidase (VCPO) on planktonic and biofilm bacteria (Renirie et al. 2008; Hoogenkamp et al. 2009), and specifically on in vitro biofilms of Enterococcus faecalis (Persoon et al. 2012). This bacterium is associated with apical periodontitis in endodontically treated teeth (Molander et al. 1998). Using low concentrations of hydrogen peroxide ($H_2O_2$) under slightly acidic conditions (equation 1), VCPO catalyses the oxidation of halides, such as bromide or chloride, into its corresponding hypohalous acid. In a spontaneous reaction, singlet oxygen ($^1O_2$) is generated (equation 2). Above a pH of 7, a major portion of the hypohalous acid dissociates to hypohalite. Hypohalous acid, hypohalite and singlet oxygen all have antimicrobial activity (Pellieux et al. 2000; Mercade et al. 2009).

\[
\begin{align*}
X^- + H_2O_2 + H^+ &\rightarrow HOX + H_2O \quad \text{(equation 1)} \\
HOX + H_2O_2 &\rightarrow ^1O_2 + H_2O + X^- + H^+ \quad \text{(equation 2)}
\end{align*}
\]

Vanadium haloperoxidases are very stable enzymes and are resistant towards inactivation by high temperature, organic solvents and singlet oxygen (Van Schijndel et al. 1994; Renirie et al. 2003). High enzyme stability and continuous production of antimicrobials allow for many promising applications, such as hard surface antifouling, surface sanitization and disinfection of medical equipment and contact lenses (Barnett et al. 1995; Hansen et al. 2003). The enzyme could also be used in a prolonged and continuously active interappointment dressing for complementary disinfection of infected root canals.
Many antimicrobial dressings for endodontic treatment have proven to be very effective in in vitro studies. Unfortunately, many of them show little efficacy when incubated with dentin (Haapasalo et al. 2000). Dentin has a strong buffering effect and buffers any enclosed medicaments to a neutral pH (Wang & Hume 1988; Camps & Pashley 2000). The original VCPO enzyme has a pH optimum of 5.5 (Van Schijndel et al. 1993) and, thus, like other endodontic medicaments, will not function to its optimal extent at the neutral pH of the root canal (Haapasalo et al. 2000). To overcome this limitation and further disinfect the root canal system, we examined the use of a modified variant of the VCPO enzyme (mVCPO), which is more active at a neutral pH and a slightly alkaline pH (Hasan et al. 2006). Before testing a possible application within the root canal, not only the compatibility with the tooth, but also with the surrounding soft tissues has to be established.

Therefore, this study aimed to optimize the use of a modified VCPO for complementary disinfection during endodontic treatment. First, we studied the antimicrobial potential of mVCPO on in vitro Enterococcus faecalis biofilms at the root canal pH. Second, we explored the cytotoxicity of mVCPO on oral fibroblast cells.

**METHODOLOGY**

**pH adaptation and enzymatic activity**

For this experiment both the original VCPO, initially found in the fungus C. inaequalis, and mVCPO were used. This enzyme was obtained using directed evolution in which the VCPO gene was randomly mutated to generate many gene variants, and mutants with the best VCPO activity were combined. The resulting triple mutant enzyme had three amino acid substitutions, resulting in an enzyme with much higher activity at a mildly alkaline pH. The enzymes were prepared as previously described (Hasan et al. 2006).

We have determined that a pH of 7.7 mimics the root canal system, when a solution is buffered by dentin (data not shown). Kinetic analyses were performed at a pH of 5.5, 7.7 or 8.3 using monochlorodimedone (MCD: 2-chloro-5,5-dimethyl-1,3-dimedon; Alfa Aesar, Heysham, UK) as a scavenger for hypobromous acid. VCPO reaction products halogenate MCD, causing a decrease in absorbance at 290 nm (Hager et al. 1966). In a kinetic assay (Spectramax Plus 384; Molecular Devices, Sunnyvale, CA), the brominating activity of VCPO and mVCPO was measured in quartz cuvettes containing 100 mM citrate or Tris-acetate buffer, 100 µM vanadate (Sigma-Aldrich, St. Louis, MO), 1 mM H₂O₂ (Merck, Darmstadt, Germany), 1 or 5 mM potassium bromide (Sigma-Aldrich), 50 µM MCD and 1 µM VCPO or mVCPO.
Antimicrobial activity

*E. faecalis* strain OS-16, an oral isolate (Sedgley et al. 2005), was used for this experiment. Bacteria were routinely cultured in Brain-Heart Infusion (BHI) medium supplemented with 1.5% Agar (BD, Sparks, MD) at 37°C under anaerobic conditions (10% H₂, 10% CO₂ in N₂). Biofilms were grown in modified semi-defined biofilm medium (BM) (Persoon et al. 2012).

Biofilms were grown on polystyrene pegs as previously described (Persoon et al. 2012). Cultures inoculated from freezer stocks were grown overnight at 37°C under anaerobic conditions in BM supplemented with 0.36% glucose. Overnight cultures were adjusted to a final optical density of OD₆₂₀ = 0.02 in BM with 0.2% sucrose, to enhance biofilm formation. For each inoculum, 200 µL per well was distributed into a 96-well microtitre plate (NUNC, Roskilde, Denmark). An Immuno TSP PolySorp lid with 96 corresponding polystyrene pegs (NUNC) was positioned on the plate for active attachment of the biofilm. Biofilms were grown for 48 hours under anaerobic conditions at 37°C, and BM supplemented with 0.2% sucrose was refreshed after 8, 24 and 32 hours. Biofilms were washed three times with buffered peptone water (Oxoid Ltd, Basingstoke, UK) to remove nonadherent bacteria.

Biofilms were treated with VCPO as previously described (Persoon et al. 2012). Pegs with biofilms were inserted into another 96-well microtitre plate with 180 µL incubations of 50 mM Tris-acetate buffer at a pH of 7.7, 1 µM mVCPO and 5 mM potassium bromide; 10 mM H₂O₂ per well initiated the reaction. Incubations without mVCPO served as the negative controls. Biofilms were incubated for 5 and 30 min, after which biofilms were washed three times with buffered peptone water to remove any active agents.

To determine the antimicrobial effect of mVCPO reaction products on the biofilms, pegs were processed as previously described (Persoon et al. 2012). The peg with the biofilm was removed from the lid and put into 1 mL cysteine peptone water. Samples were vortexed and sonicated 30 times for 1 s at 40 Hz (VC130 Ultrasonic processor, Sonics & Materials Inc., Newtown, CT) and then serially diluted in cysteine peptone water and spiral plated (EddyJet; IUL instruments, Barcelona, Spain) onto BHI agar plates. Plates were incubated under anaerobic conditions at 37°C for 48 hours, after which the colony-forming units (CFUs) per biofilm were determined. The experiment was performed six times in triplicate.

Cytotoxicity assay

Fibroblasts were obtained from erupted third molars of healthy donors after extraction (Scheres et al. 2010). Donors gave written informed consent. The collection
and use of these cells has been approved by the Medical Ethical Committee of the VU University Medical Center (#2011/328). Fibroblasts were isolated as previously described (Scheres et al. 2010). Cells were cultured in Dulbecco Minimal Essential Medium (DMEM; Gibco BRL, Paisley, Scotland) supplemented with 2% antibiotics (antibiotic antimycotic solution: 100 U/mL penicillin, 100 µg/mL streptomycin and 250 ng/mL amphotericin B, Sigma–Aldrich) and 10% fetal calf serum (HyClone, Logan, UT) in a humidified atmosphere with 5% CO₂ in O₂ at 37°C.

Cells were seeded into a 96-well microtitre plate (Cellstar, Greiner Bio-One, Alphen aan de Rijn, the Netherlands) at 3 x 10⁴ cells per well in DMEM with 10% fetal calf serum and incubated in a humidified atmosphere with 5% CO₂ in O₂ at 37°C. After 24 hours, cells were washed twice with DMEM without serum because serum has an inhibitory effect on VCPO (Hansen et al. 2003). Cells were incubated with suspensions of 1 µM mVCPO in DMEM, 1 µM mVCPO and 10 mM H₂O₂ in DMEM or 10 mM H₂O₂ in DMEM. The culture medium already contained 120 mM chloride, so no further halides were added. Pure DMEM served as a negative control, and 5% Triton X-100 (Merck) and 0.5% sodium hypochlorite (NaOCl) in DMEM served as positive controls. After an additional 24 hours of incubation, suspensions were replaced with 100 µL DMEM and 10 µL WST-1 (Roche Diagnostics Nederland BV, Almere, the Netherlands) per well. Metabolically active cells convert the tetrazolium salt WST-1 into formazan; this was quantified by measuring the absorbance at 450 nm (Spectramax Plus 384), which is directly correlated to the number of viable cells (Ishiyama et al. 1993). Reference measurements at 680 nm were subtracted to correct for any variance of the microtitre plate. The experiment was performed three times in quadruplicate.

**Statistical analysis**

Data were analysed using the Statistical Package for Social Sciences (version 18.0; SPSS, Chicago, IL). Statistical analyses were performed on log₁₀ converted data for the CFUs; the nonparametric Mann-Whitney U test and Kruskal-Wallis test were used. The level of significance was set at α = 0.05.

**RESULTS**

Determination of the activity of the two enzymes at three pH levels showed that the original enzyme was very active at the optimum pH level of 5.5 (Table 7.1). However, the activity decreased a thousand-fold at the root canal pH. The VCPO modified to function at higher pH values showed considerable activity at a pH of 7.7 and 8.3. The enzymatic efficacy of mVCPO was further optimized when the halogen
TABLE 7.1. Enzymatic activity of VCPO and mVCPO. Rate of the reactions is expressed as Δ decrease in the absorbance of monochlorodimedone per minute at a concentration of 0.05 µM enzyme for VCPO and mVCPO at different pH levels and concentrations of bromide. ND stands for not-determined values.

<table>
<thead>
<tr>
<th>pH</th>
<th>VCPO 1 mM Br</th>
<th>mVCPO 1mM Br</th>
<th>mVCPO 5mM Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>1.72</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>7.7</td>
<td>5 x 10^-4</td>
<td>0.64</td>
<td>1.05</td>
</tr>
<tr>
<td>8.3</td>
<td>ND</td>
<td>0.59</td>
<td>ND</td>
</tr>
</tbody>
</table>

TABLE 7.2. Bacterial inactivation by mVCPO treatment. Average colony-forming unit (CFU)-counts and percentages of bacterial inactivation of antimicrobial products of mVCPO at a concentration of 5 mM bromide.

<table>
<thead>
<tr>
<th>Treatment time</th>
<th>5 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative controls without mVCPO (CFUs ± sd)</td>
<td>3.29 x 10^6 ± 1.09 x 10^6</td>
<td>3.38 x 10^6 ± 3.38 x 10^6</td>
</tr>
<tr>
<td>Enzymatic treatment with mVCPO (CFUs ± sd)*</td>
<td>8.91 x 10^3 ± 2.45 x 10^4</td>
<td>4.23 x 10^2 ± 9.90 x 10^1</td>
</tr>
<tr>
<td>Bacterial inactivation (%)</td>
<td>99.73</td>
<td>99.99</td>
</tr>
</tbody>
</table>

* Significant difference between 5- and 30-minute treatment calculated on log10 converted data (P = 0.016)

FIGURE 7.1. Gingival and periodontal ligament (PDL) fibroblast cell survival after exposure to mVCPO and its reaction products. Results are displayed in percentage absorbance (A$_{450nm}$ - A$_{680nm}$) and normalized according to negative control values of the DMEM group. Used concentrations: 1 µM mVCPO, 10 mM H$_2$O$_2$, 5% Triton X-100 and 0.5% NaOCl.
concentration was raised to 5 mM bromide, which is in agreement with previous experiments (Hasan et al. 2006). Despite the different pH, mVCPO functioned with similar efficacy at the root canal pH as the original VCPO at its optimum pH of 5.5.

The mVCPO significantly inactivated *E. faecalis* for both short and long incubation times when compared with the negative controls without mVCPO (Mann-Whitney U test; *P* < 0.001; Table 7.2). The 30-minute treatment provided significantly more bacterial reduction than the 5-minute treatment (Mann-Whitney U test; *P* = 0.016).

A cytotoxicity assay showed full compatibility of the mVCPO reaction products with the fibroblast cells (Figure 7.1). The survival of fibroblasts is expressed in percentage absorbance; DMEM served as a negative control and was used to normalize the results. Statistical analysis on survival percentages showed a difference between the tested groups (Kruskal-Wallis test; *P* = 0.021). Because of the small group sizes, statically significant differences could not be specified in post hoc tests. Cells treated with just mVCPO stay active after 24 hours of exposure. Cells also stay active after treatment with the active reaction suspension (i.e., mVCPO with H$_2$O$_2$). Most cells did not survive exposure to the Triton X-100. Cells treated with 0.5% NaOCl and 10 mM H$_2$O$_2$ experienced a similar adverse effect.

**DISCUSSION**

The root canal system environment determines the effect of inserted endodontic medicaments (Morgental et al. 2013), the pH level is one of the most obvious effects. Dentinal calcium phosphates have a substantial buffering effect, thereby altering the pH of fluids introduced into the root canal system up to neutral levels (Wang & Hume 1988; Camps & Pashley 2000). Furthermore, the bacterial biofilm in an infected root canal can play a role in altering the pH of introduced fluids. Bacteria in caries lesions are constantly supplied with carbohydrates and consequently produce acids, which lower the pH (Marsh 2003). However, in the inflamed periodontal pocket few carbohydrates are available for metabolism. Bacteria can then use proteins from the gingival crevicular fluid. This increases the level of amino acids and as a result the pH (Marsh 2003). A similar process may occur in the root canal system, where dentinal fluid, pulp remnants and inflammatory exudates are the source of nutrition. Necrosis of the pulp tissue can also influence the pH; necrotic pulps are shown to have a pH of 6.4 - 7.0 (Tronstad et al. 1981). Thus, dentinal phosphates, proteolytic bacteria and necrotic tissue cause the intracanal pH to be slightly alkaline. VCPO showed maximal activity at a pH of 5.5, but at the root canal pH of 7.7 its activity is decreased a thousand-fold. For this reason, we studied mVCPO which was optimized for operating at a more alkaline pH range. Endodontic therapy with this enzyme can
be the next step because it can exert a continuous and local antimicrobial effect in the root canal.

mVCPO seems suitable as an endodontic disinfectant because it inactivated 99.73% of the bacteria in 5 minutes. Despite the lower activity of mVCPO at a pH of 7.7 compared with the original VCPO functioning at a pH of 5.5, our results were as good as observed in previous experiments using the original VCPO (a 99.99% bacterial inactivation of *Streptococcus mutans* (Hoogenkamp et al. 2009) and a 99.78% bacterial inactivation of *E. faecalis* (Persoon et al. 2012)), and 2% NaOCl (a 99.99% bacterial inactivation of *E. faecalis* (Persoon et al. 2012)). This could be explained by the enhanced generation of singlet oxygen under alkali conditions (Kanofsky 1984), which may be more bactericidal than hypohalous acid (Pellieux et al. 2000). Additionally, the broadened enzymatic activity range of mVCPO allows for bacterial inactivation from pH 5 - 8 (Hasan et al. 2006), which can be relevant in case of pH values varying after the use of acidic medicaments or in caries lesions. Moreover, the antimicrobial efficacy of mVCPO was further increased by using bromide instead of chloride because this yields more effective antimicrobial products at a pH of 5.5 (Persoon et al. 2012) and by raising the halide concentrations (Hasan et al. 2006). Finally, increasing the incubation time resulted in an additional antimicrobial effect. Its clinical significance may be small or even absent, but so far the number of remaining microorganisms, which is still compatible with healing, is undetermined (Peters & Wesselink 2002). Even the slightest additional disinfection could mean the difference between a persisting apical periodontitis and its healing. Moreover, the contact time in this study was merely 30 minutes, so for an actual interappointment dressing of several days, the contact time and consequent antimicrobial effect are anticipated to be larger.

The results of this study suggest that mVCPO can be used as an antimicrobial interappointment dressing, and it has several advantages. The enzyme, together with necessary substrates, can be applied in the root canal system, after current chemomechanical cleansing procedures. The enzyme itself is stable and shows resistance towards wide pH and temperature ranges, organic solvents and singlet oxygen (Van Schijndel et al. 1994; Renirie et al. 2003). The reaction mechanism recycles bromide, so only a low concentration is required. However, if the introduced halide source is exhausted, bodily fluids can supplement it. Bromide is present in saliva and serum at concentrations of 0.17 mM and 0.06 mM, respectively (Michigami et al. 1989), and chloride at concentrations of 16 mM and 32 mM, respectively (Rehak et al. 2000). Also, oral bacteria (Carlsson et al. 1983) and even *E. faecalis* (Huycke et al. 1996) are able to produce H$_2$O$_2$. This means that all necessary substrates already are, or can easily be introduced in the root canal, allowing an extended working period and an
antimicrobial effect deep into the elaborate root canal system.

Moreover, our results showed that mVCPO is fully biocompatible. An intracanal dressing and its reaction products can come into contact with vital tissue via the apical foramen; therefore, any medicament has to be biocompatible. The current standard for endodontic disinfection is NaOCl (Byström & Sundqvist 1985). Cytotoxicity tests confirm serious adverse effects, even at low concentrations of NaOCl (Simbula et al. 2010). The current standard for interappointment medication is calcium hydroxide, but this kills 100% of the fibroblasts (Badr et al. 2011). Results from our cytotoxicity assay show that NaOCl and just H₂O₂ were considerably harmful towards fibroblasts. In contrast, mVCPO and its reaction products at antimicrobial concentrations were mild towards fibroblasts. The current antimicrobial therapy of choice has more adverse effects and limitations. Thus, mVCPO functioning at the root canal pH seems biocompatible for use in contact with human tissues.

CONCLUSION
The reaction products of mVCPO showed substantial antimicrobial activity towards in vitro E. faecalis biofilms at the pH found in the root canal system. Also, fibroblast cells remained vital after mVCPO treatment. Further research should explore means to insert this enzyme into the root canal system and test its efficacy and biocompatibility within in vivo infected root canals. When this is substantiated, interappointment dressing with mVCPO may provide an effective complementary procedure to current chemomechanical disinfection.

ACKNOWLEDGEMENTS
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AUTHOR CONTRIBUTIONS
Conceived and designed the study: IFP, MAH, PRW, WC. Performed the study: IFP, MAH, AB, AFH, RW. Analysed the data: IFP, MAH. Drafted the manuscript: IFP, MAH. Critically revised the manuscript: AB, PRW, AFH, RW, WC. All authors accepted the final version of the manuscript.