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Antimicrobial activity of silver nitrate against periodontal pathogens

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Metal ions were evaluated as potential antimicrobial agents suitable for local delivery in the oral cavity for the treatment of periodontitis. Silver nitrate, copper chloride, and zinc chloride were tested for antimicrobial activity in *in vitro* killing assays conducted in phosphate buffered saline with a series of oral bacteria including gram-negative periodontal pathogens and gram-positive streptococci. Copper and zinc salts failed to exhibit strong and consistent activity against periodontal pathogens. In contrast, silver at a concentration of 0.5 μg/mL produced a 3 $\log_{10}$ reduction in colony forming units (CFU)/mL or greater against all periodontal pathogens tested including *Porphyromonas gingivalis, Prevotella intermedia, Prevotella denticola, Bacteroides forsythus, Fusobacterium nucleatum vincentii, Campylobacter gracilis, Campylobacter rectus, Eikenella corrodens*, and *Actinobacillus actinomycetemcomitans*. In comparison, substantially higher concentrations of silver nitrate failed to kill oral streptococci. A silver nitrate concentration of 25 μg/mL produced $\log_{10}$ reductions in CFU/mL of 3.5–5 in killing assays performed in human serum against *P. gingivalis*, demonstrating the ability of silver to retain activity in a biological medium similar to that encountered *in vivo* in the periodontal pocket. These results identify silver nitrate, an antimicrobial that may possess advantages over traditional antibiotics, as a potential agent for controlled release local delivery in the oral cavity for the treatment of periodontitis.

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Periodontitis is an infectious disease of bacterial origin (1). In recent years, considerable progress has been made as to the identification of the bacterial species responsible for the disease and to their impact on systemic health (2, 3). The traditional treatment for periodontitis, scaling and root planing, is still regarded as a critical component of therapy (4). However, antibiotic therapy is commonly administered along with scaling and root planing to further reduce the bacterial load and the likelihood of recurrent infection (5, 6). Treatment by local drug delivery has become increasingly attractive as it provides for the generation of high drug concentrations at the site of infection with considerably lower levels established elsewhere in the body (6).

Although the adjunctive use of antibiotics is at least moderately effective (7–9), problems with this approach remain. A significant number of periodontal infections do not respond to scaling and root planing alone or in combination with antibiotic therapy (10). In addition, the ever-increasing problem of antibiotic resistance has prompted a reevaluation of adjunctive antibiotic therapy in the treatment of periodontal disease (11). Interestingly, in spite of the lower systemic drug concentrations afforded by local delivery, antibiotic resistance can still result (6). Although the effect was transient in many cases, with the number of resistant bacteria dropping to pretreatment levels in the months following therapy, the potential for development of resistance should not be dismissed. Recent studies have documented significant increases in the populations of antibiotic resistant flora in the oral cavity compared with levels found a decade earlier (11). For these reasons, an

antimicrobial that is not a standard antibiotic may be less likely to induce resistance and may be suitable for long-term use. We sought the identification of a novel compound for the treatment of periodontitis, suitable for local controlled release drug delivery in the oral cavity. In particular, our goal was to identify a compound with an activity spectrum selective for periodontal pathogens.

The antimicrobial properties of silver and copper were identified in antiquity (12). More recent investigation has identified antimicrobial properties resident in zinc (13, 14). All three metals are components of amalgams and alloys in dental materials (15). Copper and zinc salts have been incorporated into mouthwashes as antimicrobial agents for the treatment of gingivitis (14). Silver is commonly used as a treatment for suppurating lesions and burns, in which silver sulphadiazine is the active ingredient. Silver, and to a lesser extent copper, also find application as antimicrobial components of catheters (16–18). Until recently, eye infections in the newborn were routinely treated with silver, in the form of silver nitrate eye drops (19). The present day uses of these metals underscore both their antimicrobial activity and low potential for toxicity in local delivery applications.

As a first step in the development of a sustained release formulation for the treatment of periodontitis, we have investigated the *in vitro* activity of silver nitrate, copper chloride, and zinc chloride against a panel of oral bacteria. We find that silver nitrate has an antimicrobial spectrum that suggests its use in an oral cavity sustained release formulation.

Material and methods

Silver nitrate was obtained from Aldrich Chemical Company, St. Louis, MO, USA. Copper chloride, zinc chloride, human serum, hemin, menadione, sodium formate and disodium fumarate were purchased from Sigma Chemical Company, St. Louis, MO, USA. Agar plates were purchased from Binax/NEL Inc. Waterville, Maine, USA. Brain heart infusion agar and broth were purchased from DIFCO Laboratories, Detroit, MI, USA.

Cultivation of oral bacteria

Bacteria were cultured in an anaerobic atmosphere composed of 80% nitrogen, 10% carbon dioxide, and 10% hydrogen atmosphere, or in an aerobic atmosphere containing 5–7% carbon dioxide.

Growth on solid media – *Actinobacillus actinomycetemcomitans*, *Eikenella corrodens*, *Fusobacterium nucleatum*, *Prevotella denticola*, *Prevotella intermedia*, *Streptococcus gordonii*, *Streptococcus mitis*, *Streptococcus mutans*, and *Streptococcus sobrinus*, were cultured on TSBY agar, tryptic soy agar supplemented with sheep blood, 50 mL/L; hemin, 5 mg/L; and yeast extract, 10 g/L; as previously described (20). *Bacteroides forsythus* was cultured on TSBY agar supplemented with N-acetylmuramic acid, 10 mg/L (20). *Campylobacter rectus* and *Campylobacter gracilis* were cultured on brain heart infusion agar supplemented with sheep blood, 50 mL/L; hemin, 5 mg/L; yeast extract, 10 g/L; sodium formate, 2 g/L; and disodium fumerate, 4.1 g/L (20). *Porphyromonas gingivalis* was cultured on TSBY agar supplemented with menadione, 0.5 mg/L (20). *Actinomyces viscosus* was cultured on brain heart infusion agar.

Growth in liquid media – *S. gordonii*, *S. mitis*, *S. mutans*, and *S. sobrinus*, were grown in Todd Hewitt broth supplemented with 0.5% glucose (21). *A. viscosus* was cultured in brain heart infusion broth. All other bacteria were grown in liquid media containing the ingredients listed for solid media, from which agar and sheep blood were omitted. *A. actinomycetemcomitans* was grown in tryptic soy broth supplemented with yeast extract, 10 g/L.

Bacterial cell killing assays performed in buffer

P. gingivalis, *P. intermedia*, *P. denticola*, *B. forsythus*, *F. nucleatum vincentii*, *C. gracilis*, *C. rectus*, *E. corrodens* and *S. gordonii* were cultured under anaerobic conditions as were plated bacteria derived from cell killing assays. *S. mitis*, *S. mutans*, *S. sobrinus* DS-1 and *A. viscosus* were cultured under aerobic conditions as were plated bacteria derived from cell killing assays (Table 1). *A. actinomycetemcomitans* and *S. sobrinus* 6715 were cultured, and bacteria from cell killing assays were incubated under each atmospheric condition (Table 1). In Table 1, where cell killing assay data derived from the two different atmospheric conditions for one bacterium are shown, the upper and lower sets of data are the values obtained after aerobic and anaerobic incubation, respectively. The cell killing assays identified in Table 1 were performed in PBS (phosphate buffered saline). Exceptions were *S. gordonii* which was assayed in 25% PBS and *S. mutans* and *S. sobrinus* DS-1, which were assayed in 10 mM MES (2-[N-Morpholino] ethanesulfonic acid), 20 mM NaCl, pH 5.2.

Cell killing assays with anaerobic and facultative oral bacteria were carried out by dilution of mid-log liquid cultures into the appropriate assay buffer (Tables 1, 2) to a concentration of approximately 2×10^5 colony forming units (CFU)/mL.

Table 1. Antimicrobial activity of silver nitrate, copper chloride, and zinc chloride against oral bacteria

Oral bacteria	Antimicrobial agent	Log$_{10}$ Reduction in CFU/mL					
		50 μg/mL	25 μg/mL	5 μg/mL	0.5 μg/mL	0.05 μg/mL	0.005 μg/mL
P. gingivalis A7A1-28	$AgNO_3$	nd	nd	nd	4.2[a], 3.1	3.0, 3.1	0
P. gingivalis ATCC 33277	$AgNO_3$	nd	nd	4, 4.2	4, 4.2	2.9, 3.3	0.2, 0
P. denticola ATCC 33185	$AgNO_3$	nd	nd	4.1, 3.8	4.1, 3.8	nd	nd
P. intermedia ATCC 25611	$AgNO_3$	nd	nd	nd	3.8, 3.3	3.8, 3.3	0.2, 0.4
B. forsythus ATCC 43037	$AgNO_3$	nd	nd	nd	4.2, 3.2	0.3, 0	0.2
C. gracilis ATCC 33236	$AgNO_3$	nd	nd	3.7, 3.4	3.4, 3.4	nd	nd
C. rectus ATCC 33238	$AgNO_3$	nd	nd	nd	4.1	1.4	nd
E. corrodens ATCC 23834	$AgNO_3$	nd	nd	3.0, 3.4	0.8, 3.1	nd	nd
E. corrodens 558	$AgNO_3$	nd	nd	4.3, 3.1	3.9, 3.1	nd	nd
F. nucleatum ATCC 49256	$AgNO_3$	nd	nd	nd	3.4, 3.8	0, 1.6	0
A. actinomycetemcomitans ATCC 29523	$AgNO_3$	nd	nd	3.7, 3.8, 3.0, 3.9	3.0, 3.9 3.7, 3.8	nd	nd
S. gordonii 51656	$AgNO_3$	nd	0, 0	nd	nd	nd	nd
S. mitis JK 195	$AgNO_3$	0.2, 0.2	nd	0.2, 0.3	0.2, 0.3	nd	nd
S. mutans SJ32	$AgNO_3$	0, 0	nd	0, 0.1	0, 0	nd	nd
S. sobrinus DS 1	$AgNO_3$	nd	0	0.2	0	nd	nd
S. sobrinus 6175	$AgNO_3$	0, 0.6, 0	nd	0, 0, 0	0, 0, 0	nd	nd
A. viscosus 15987	$AgNO_3$	3.7	3.7	3.7, 2.7	3.7, 2.7	nd	nd
P. gingivalis A7A1-28	$ZnCl_2$	0	0.1	0.2, 0	0.1, 0	nd	nd
P. gingivalis A7A1-28	$CuCl_2$	nd	4.2	4.2, 4.0	4.2, 4.0	1.5	nd
E. corrodens 558	$CuCl_2$	nd	nd	0.6	0.3	nd	nd
A. actinomycetemcomitans ATCC 29523	$CuCl_2$	1.2	nd	0.9	0.9	nd	nd
S. mitis JK 195	$CuCl_2$	3.4, 3.0	nd	4.4, 3.1	0.3, 0.2	nd	nd
S. mutans SJ32	$CuCl_2$	3.5, 3.6	nd	3.5, 3.6	2.5, 3.6	nd	nd
A. viscosus ATCC 15987	$CuCl_2$	3.7	nd	2.7	2.8	nd	nd

[a] numbers represent the Log$_{10}$ Reduction in CFU/mL obtained in an independent experiment.
nd = not done.
P. gingivalis = *Porphyromonas gingivalis; P. denticola* = *Prevotella denticola; P. intermedia* = *Prevotella intermedia; B. forsythus* = *Bacteroides forsythus; C. gracilis* = *Campylobacter gracilis; C. rectus* = *Campylobacter rectus; E. corroders* = *Eikenella corrodens; F. nucleatum* = *Fusobacterium nucleatum; A. actinomycetemcomitans* = *Actinobacillus actinomycetemcomitans; S. gordonii* = *Streptococcus gordonii; S. mitis* = *Streptococcus mitis; S. mutans* = *Streptococcus mutans; S. sobrinus* = *Streptococcus sobrinus; A. viscosus* = *Actinomyces viscosus.*

Table 2. Antimicrobial activity of silver nitrate against *P. gingivalis* ATCC 33277 in human serum.

Time of incubation	Log$_{10}$ Reduction in CFU/mL						
	100 μg/mL	50 μg/mL	25 μg/mL	12.5 μg/mL	6.3 μg/mL	3.1 g/mL	1.6 μg/mL
1 hour 10^8 CFU/mL	5.0[a] 4.8 4.8	5.0 4.8 4.8	2.4 2.4 2.6	0 0.3 0	0	nd	nd
1 hour 10^5 CFU/mL	4.4 4.4	nd	3.5 4.4	nd	0 0.1	nd	0.1 0.2
24 hours 10^8 CFU/mL	4.9 4.8	4.9 4.8	4.9 4.8	4.9 1.7	0.9 0.1	0.4 0.1	0

[a] numbers represent the Log$_{10}$ reduction in CFU/mL obtained in an independent experiment.
nd = not done.

B. forsythus was not grown in liquid culture but resuspended into assay buffer from newly grown plates. The resulting bacterial suspensions were combined with an equal volume, 200 μL, of the same buffer containing silver nitrate, copper chloride, or zinc chloride, and incubated anaerobically at 35°C for one hour. Following incubation, the assay suspensions were inoculated onto agar plates, with or without prior dilution, for determination of CFU. Determination of silver nitrate, copper chloride and zinc chloride mediated killing was made by comparison to control assays in which

metal salts were omitted. The $\log_{10}$ reduction in CFU/mL was determined by subtracting the $\log_{10}$ transformed counts of viable bacteria in metal salt containing assays from the $\log_{10}$ transformed counts of viable bacteria in control assays.

Bacterial cell killing assays performed in human serum

Cell killing assays with *P. gingivalis* in pooled human serum were carried out as described for the bacterial cell killing assays, with the following changes (Table 2). *P. gingivalis* mid-log liquid cultures were centrifuged at 5,000 rpm in a table-top microfuge for 5 min and the supernatant culture media was removed. The bacterial pellets were resuspended in pooled human serum. An absorbance reading of one pellet, resuspended in PBS, was used to identify the amount of serum required to achieve a bacterial cell concentration of 1×10^5 or 1×10^8 CFU/mL. 395 μL of the *P. gingivalis* serum suspension were combined with 5 μL of aqueous silver nitrate solution or 5 μL of sterile, deionized water (controls), and the resulting suspensions were incubated anaerobically for 1 h or for 24 h. Cells were plated and killing was determined as described above.

Results

Antimicrobial activity of silver nitrate against periodontal pathogens and gram positive oral bacteria

The antimicrobial activity of silver nitrate against periodontal pathogens was determined by cell killing assay (Table 1). The periodontal pathogens *P. gingivalis*, *P. intermedia*, *P. denticola*, *B. forsythus*, *F. nucleatum*, *C. gracilis*, *C. rectus*, *E. corrodens*, and *A. actinomycetemcomitans* were suspended in PBS, combined with silver nitrate, incubated for 1 h and plated. In all cases, a concentration of 0.5 μg/mL silver was sufficient to reduce bacterial concentrations by at least 3 orders of magnitude. Two of the species tested, *P. gingivalis* and *P. intermedia*, were equally sensitive at 0.05 μg/mL silver. To test the effect of anaerobic *versus* aerobic metabolism on silver sensitivity, *A. actinomycetemcomitans* was cultured and tested under both conditions (Table 1). No difference in silver susceptibility was found.

The gram-positive oral bacteria *S. gordonii*, *S. mitis*, *S. mutans*, *S. sobrinus*, and *A. viscosus* were tested against silver nitrate by cell killing assay (Table 1). *A. viscosus*, a gram-positive bacillus, demonstrated sensitivity to silver nitrate similar to that obtained with anaerobic, gram-negative periodontal pathogens. In contrast, all of the oral streptococci tested displayed little or no sensitivity to silver nitrate. This was seen at 5, 25 and 50 μg/mL silver, concentrations 10–100 higher than those effective against the panel of periodontal pathogens tested. These results demonstrate that silver is consistently active against a diverse group of periodontal pathogens but not against oral streptococci.

The antimicrobial activity of copper chloride and zinc chloride against oral bacteria

P. gingivalis, *E. corrodens*, *A. actinomycetemcomitans*, *S. mitis*, and *S. mutans* were examined for sensitivity to copper chloride by cell killing assay and *P. gingivalis* was examined for sensitivity to zinc chloride (Table 1). *P. gingivalis* and *S. mutans* were very sensitive to copper chloride and demonstrated $\log_{10}$ reductions in viability of 2.5 to 3.6 at copper concentrations down to 0.5 μg/mL. *S. mitis* displayed similar sensitivity to copper chloride, down to a concentration of 5 μg/mL copper. In contrast, both *E. corrodens* and *A. actinomycetemcomitans* failed to produce $\log_{10}$ reductions in viability greater than 0.3–0.9 at copper concentrations of 5 and 0.5 μg/mL. A $\log_{10}$ reduction in viability of 1.2 was obtained with *A. actinomycetemcomitans* at a concentration of 50 μg/mL copper. Zinc chloride was not active against *P. gingivalis* at concentrations of up to 50 μg/mL zinc. These results suggest that neither metal salt would be active against a wide range of periodontal pathogens.

The antimicrobial activity of silver nitrate in human serum

The composition of gingival crevicular fluid is similar to that of serum during periodontal infection (4). In addition, the concentration of bacterial pathogens in an infected periodontal pocket can easily exceed the concentrations utilized in standard cell killing assays. In an attempt to examine the activity of silver against a periodontal pathogen under conditions as close as possible to those encountered *in vivo*, cell killing assays were performed with *P. gingivalis* ATCC 33277 and silver nitrate in human serum. *P. gingivalis* was incubated with or without silver nitrate for periods of 1 and 24 h, with bacterial concentrations of 10^5 and 10^8 CFU/mL (Table 2). Assays performed with 10^5 CFU/mL *P. gingivalis* resulted in $\log_{10}$ reductions in viability of 4.4 at 100 μg/mL and 3.5–4.4 at 25 μg/mL. Assays performed with 1 hour incubations of 10^8 CFU/mL *P. gingivalis* and silver nitrate produced a 5 $\log_{10}$ reduction of viability at silver concentrations of 100 and 50 μg/mL, and a 2–3 $\log_{10}$ reduction in CFU/mL, at 25 μg/mL. Increasing the time of incubation to 24 hours,

with assays performed with 10^8 CFU/mL, accentuated the $\log_{10}$ reductions in CFU/mL to about 5 at 25 μg/mL silver and produced strong killing at 12.5 μg/mL silver.

Discussion

In view of the issues associated with the use of antibiotics to treat the chronic infection associated with periodontitis, a search for non-antibiotic antimicrobial agents with strong activity against the gram-negative anaerobes implicated in periodontitis and amenable to local delivery in the oral cavity was conducted. Water soluble salts of silver, copper and zinc were selected for *in vitro* testing in view of their known antimicrobial properties and low potential for toxicity when delivered locally. An additional advantage is that these compounds lack the side effects and resistance issues associated with conventional antibiotics.

0 Zinc chloride failed to demonstrate activity against *Porphyromonas gingivalis* and was not tested further (Table 1). Copper chloride was quite active against *P. gingivalis*. However, it failed to produce strong activity against other periodontal pathogens (Table 1). In contrast, silver nitrate demonstrated strong activity against the periodontal pathogens *P. gingivalis*, *P. denticola*, *P. intermedia*, *B. forsythus*, *C. gracilis*, *C. rectus*, *E. corrodens*, *F. nucleatum*, and *A. actinomycetemcomitans*. In all cases, a silver concentration of 0.5 μg/mL, and in some cases a silver concentration of 0.05 μg/mL, was sufficient to produce a 3 $\log_{10}$ reduction in viability or better in cell killing assays, under ionic conditions similar to those encountered in the periodontal pocket. Thus, the activity spectrum of silver nitrate includes the pathogenic species consistently associated with periodontal disease. Interestingly, despite its strong effect against periodontal pathogens, silver was not active against oral streptococci. Silver concentrations of 25 μg/mL and 50 μg/mL (fifty to one hundred times higher than those effective against periodontal pathogens) were inactive against *S. gordonii*, *S. mutans* and *S. sobrinus* and only very weakly active against *S. mitis* (Table 1).

A series of *P. gingivalis* cell killing assays, performed with pooled human serum as the assay medium, demonstrated that a strong killing effect was maintained down to silver concentrations of 25–12.5 μg/mL (Table 2), a result that is particularly noteworthy. In response to infection, the composition of gingival crevicular fluid approaches that of serum (4). Thus, the *in vitro* activity of silver in serum demonstrates its ability to maintain activity when present at relatively low concentration in a complex biological fluid which closely approximates the composition of the serum exudate found in the periodontal pocket. A recent phase 1 human clinical study examined the safety and microbiological effects of controlled silver release from an erodible wafer in the periodontal pocket (22, 23). Average crevicular fluid silver concentrations remained at or above 25 μg/mL for a period of 21 days. In correlation with this, a statistically significant relationship between the levels of silver delivered and the reduction in anaerobic bacteria was observed. Thus, a silver concentration of 12.5–25 μg/mL, identified as effective in the *in vitro* serum killing assays performed with *P. gingivalis*, may represent a threshold concentration required for *in vivo* activity. This result is especially encouraging as periodontal pathogens reside primarily within a subgingival biofilm, whereas the cell killing assays presented here are performed with bacterial suspensions. Although cell killing assays performed in serum do not allow a direct comparison to activity against bacteria present in biofilms, the relatively small size of the silver ion, compared to other antimicrobials and antibiotics, may facilitate its penetration into biofilms. Experiments to examine the activity of silver against periodontal pathogens within the context of a biofilm are in progress.

A clear understanding of the mechanism(s) underlying the antimicrobial activity of silver and copper has yet to be identified (12). There is, however, evidence to suggest that the antimicrobial activity of silver, copper and zinc may result from their ability to bind to essential enzyme sulfhydrals (12, 24). Interestingly, the only metal in the present study that failed to demonstrate activity against *P. gingivalis*, zinc, also has the lowest affinity for sulfhydrals of the three metals tested (25).

An important consideration with regard to the use of silver as an antimicrobial is the potential for the development of resistance. Silver resistance, mediated by plasmid and transposon mechanisms, is found in both gram-positive and gram-negative bacteria (12). However, the continuing effectiveness of silver in the treatment of burns and wounds and the more recent success found with newly developed silver catheters is an indication that induction of silver resistance is uncommon (17, 26). In addition, although cross-resistance between conventional antibiotics is frequently encountered, silver resistance genes afford resistance only to silver and do not alter susceptibility to conventional antibiotics (26, 27). Long-term use of silver can, in some cases, produce a localized discoloration of tissue (12). In the clinical study cited above, four of nine subjects exhibited silver related staining of tissue.

which was described as mild (22). All such staining either disappeared spontaneously by the end of the study or was reversible with tooth polishing post treatment.

In conclusion, we have demonstrated through antimicrobial testing, the strong and selective activity of silver nitrate against periodontal pathogens. This result, together with its continuing effectiveness in local delivery applications and low potential for toxicity, suggests silver may be a valuable antimicrobial for sustained release local delivery in the treatment of periodontitis.

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