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Anticariogenic and Antibacterial Properties of a Copper Varnish Using an *In Vitro* Microbial Caries Model

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Clinical Relevance

The topical application of an antibacterial agent may have the potential to decrease the severity of existing root caries lesions or prevent the development of new lesions.

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SUMMARY

The antimicrobial and anticariogenic properties of a copper varnish (experimental mixture of Doc's Best Red Copper cement and Copalite varnish, Cooley and Cooley, Ltd, Houston, TX, USA: designated in this study as "Copper Seal") on the root surface were evaluated in an *in vitro* microbial caries model. Fifty-six human root specimens were prepared from anterior teeth and randomly divided into four groups: Groups 1 and 3—Copper Seal; Group 2—chlorhexidine varnish, the positive control (Cervitec, Ivolcar Vivadent, Schaan, Liechtenstein) and Group 4—a negative control that received no treatment. The varnishes were painted in Groups 1, 2 and 3, then visually removed after 24 hours in Group 1. The specimens were demineralized in a microbial caries model for five days. Plaque was collected from the specimens to obtain bacterial colonization numbers, then the specimens were sectioned and analyzed for lesion extent using Confocal Laser Scanning microscopy. There were no significant differences ($p>0.05$) among the four groups in

terms of bacterial count. Regarding caries lesion development, the group with copper varnish visually removed (Group 1) and the non-treated group (Group 4) had significantly greater total area caries lesions and total lesion fluorescence than the copper varnish without removal group (Group 3) and the chlorhexidine group (Group 2). Therefore, it was concluded that copper and chlorhexidine varnishes have anticariogenic effects on root surfaces, as tested in this model.

INTRODUCTION

Dental caries has become a serious oral health problem for older adults. This is partially due to the increasing longevity of the population and the increases in tooth retention within this age group.[1] The prevalence of root caries in the US increases with age (18-75+) from 7% to 56%.[2] A root caries lesion is a soft, irregularly-shaped lesion either totally confined to the root surface or involving the undermining of enamel at the cemento-enamel junction, but clinically indicating a lesion was initiated on the root surface.[3] The development of root caries lesions is similar to the development of enamel caries, with periods of demineralization and remineralization.[4] However, demineralization is approximately twice as rapid on the root surface as on enamel, because the root has half as much mineral as enamel and demineralization can occur at a higher pH.[5] Delivering antimicrobial agents to the root surface can affect root caries development.

Copper ions have been reported to have an antibacterial effect both *in vitro*[6-11] and *in vivo*.[12] Copper reduces the number of bacteria on tooth surfaces. The suggested mode of the action of copper is the limitation of bacterial growth and the inhibition of glycolysis, leading to a decrease in acid production.[13-14] Copper has also been found to interfere with glucan formation by glucosyl transferase. Such a process may contribute to reduced plaque accumulation.[15]

Foley and others[16-17] suggested the use of copper cement as a liner under a less soluble material to take advantage of copper cement's cariostatic properties. Afseth and others[18] found a reduction in caries development in rats after adding 65 ppm copper in the drinking water. Rosalen and others[13] found that copper, which was co-crystallized with sucrose was an effective cariostatic agent in rats.

In another *in vivo* study, Foley and Blackwell[12] compared the effect of copper cement with glass ionomer cement (GIC) on carious dentin that remained under restorations. These authors sampled the dentin microbiologically at one and six months and found that copper cement demonstrated a significant effect on the total anaerobic bacterial count over one month. Over six months, copper cement caused a significantly greater reduction in *mutans streptococci* than GIC.

Chlorhexidine (CHX) is currently the most potent chemotherapeutic agent against *mutans streptococci* and dental caries. The positively charged chlorhexidine component binds to the negatively charged bacterial cell wall and extracellular complexes. This binding alters the cell's osmotic equilibrium, allowing low molecular weight substances to leak out. At higher concentrations, bacterial cytoplasm precipitates, resulting in cell death. CHX varnishes have been studied *in vitro*[19-21] and *in vivo*[22-25] and have been found to be antibacterial.

Cooley and Cooley (Houston, TX, USA), the manufacturer of copper cement (Doc's Best Red Copper cement) and copalite varnish, have recently proposed mixing copper cement powder with copalite varnish (Copper Seal) to serve as an antibacterial varnish to be painted on tooth surfaces. This preparation could have many potential uses, especially considering that there is no approved antibacterial varnish currently on the US market. The topical application of an antibacterial agent may have the potential of decreasing the severity of existing root caries lesions or preventing the development of new lesions.

This study investigated the antimicrobial and anticariogenic properties of a copper varnish (Copper Seal) on root surfaces and compared it to a CHX varnish (Cervitec), using an *in vitro* microbial caries model.

METHODS AND MATERIALS

Specimen Preparation

The *in vitro* microbial caries model experiment involved four groups. A total of 14 specimens were prepared for each group: two specimens were used for baseline confocal microscopy measurements and 12 specimens for *in vitro* demineralization. Clinically sound anterior teeth, stored in 0.1% thymol, were selected. The teeth were required to have apical closure and root surface areas without any visible damage or demineralization. The lingual sides were flattened so that one-half the labial-lingual width of the tooth remained. The incisal two-thirds of the crown and the apical third of the root were cut and discarded. An acid resistant varnish (red fluoride-free nail varnish) was applied, leaving an approximate 4 mm x 4 mm window on the buccal root surface (about 2 mm apical to the CEJ). The flattened root surface was affixed with a cyanoacrylate adhesive to one end of a 1.5 cm long Plexiglas rod. The specimens were numbered with a two-digit number, randomly divided into treatment groups and mounted onto acrylic plates made to fit tightly on the stirring magnet of the caries-forming vessel. Each caries vessel had a total of 12 specimens, three from each treatment group (Figure 1).

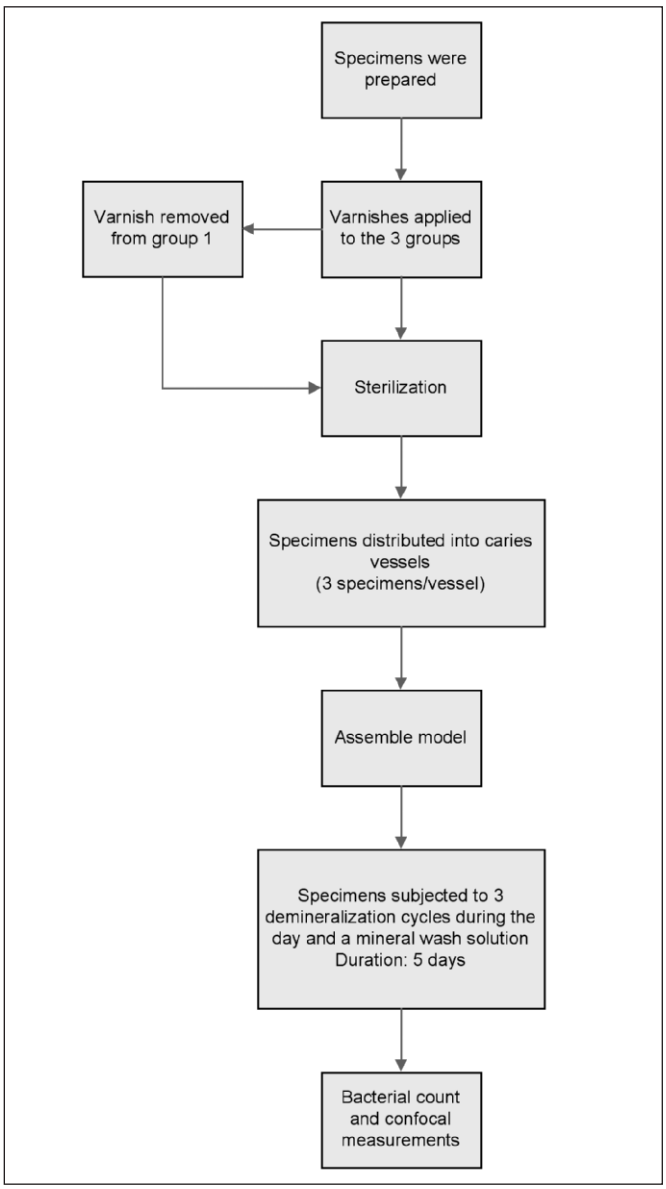

Figure 1. *Diagram showing how specimens were subjected to the experiment.*

Intervention

Prior to varnish application, the teeth from all four groups were air dried. The copper varnish (Group 1 and Group 3) and 1% chlorhexidine varnish (Group 2) were placed following the manufacturer's instructions, while Group 4 received no treatment. The specimens were kept in humid conditions for 24 hours at 37°C, then the varnish was removed from Group 1 (peeled off using a scalpel). The removal technique had been previously tested in a small pilot study. Two methods were compared in the pilot study: a scalpel vs a toothbrush. The scalpel removed all of the visually remaining varnish, but microscopy demonstrated copper varnish residue on plugs inside the dentinal tubules, while the toothbrush was not able to remove any of the varnish.

Figure 2. In-vitro *microbial caries model.*

Sterilization

The plastic tubing and flasks were labeled according to their respective group and autoclaved. Autoclave sterilization was done using a general-purpose steam sterilizer (AMSCO Renaissance Series Steam Sterilizer Model #3201, Erie, PA, USA), with the liquid cycle at 250-254°F and 17-22 psi for 45 minutes. Gas sterilization of the specimens mounted on acrylic bases was done using a 3M Healthcare, Model #8-XL sterilizer (3M ESPE, St Paul, MN, USA). The gas was composed of 100% ethylene oxide, Steri-Gas, an EO gas cartridge and item #8-170 from 3M at a temperature of 131°F for one hour. Total cycle time was 5 hours and 30 minutes and the humidity was 35% to 80%.

Inoculum Preparation

A vial of frozen *Streptococcus mutans* TH16 (serotype c) was thawed at room temperature and transferred to a TSBS (tripticase soy broth supplemented with 5% sucrose) tube, and the organisms were grown in 5% CO_2 at 37°C for 24 hours. The bacteria were plated on a TSA-trypticase Soy Agar Plate to check for lack of contamination. After 24 hours, the TSBS tube was removed from the incubator and centrifuged for 20 minutes at 3000 rpm. After discarding the supernatant, the

pellet was diluted in approximately 20 ml of fresh TSBS, then vortexed. The OD of the inoculum was 0.323 at 540 nm, as determined by spectroscopy using fresh TSBS as the control.

All groups were inoculated once at the beginning of the experiment on both of the specimens' surfaces and placed in 5 ml of TSBS fluid on the bottom of the vessel. Following inoculation, the specimens were incubated at 37°C for two hours to allow the bacteria to adhere to the tooth structure before beginning normal cycling of the microbial system.

Circulating Fluid

For each vessel, the specimens were exposed to circulating TSBS for 30 minutes three times a day at 37°C for five days and to a mineral washing solution (MW) for a total of 22.5 hours per day.[26] The MW contained 0.25 ppm F. The circulating fluids were delivered to and removed from the treatment vessels by peristaltic pumps regulated by timers. The TSBS was intended to reproduce nutrient intake three times a day, while the MW represented an artificial saliva buffer solution. Every group had its own TSBS bottle. Groups 1 and 2 were connected to one MW bottle, and Groups 3 and 4 to another MW bottle (Figure 2). The drainage containers for all four groups in each experiment were changed daily, and the drainage fluid was monitored for pH, bacteria viability and lack of contamination.

Analyses

Upon completion of the experiment, the following analyses were conducted:

1. pH Determination

   The pH of the fluid remaining in the caries-vessels after the last sucrose cycle, the remaining drainage-fluid, TSBS media and MW media were all monitored for pH.

2. Bacterial Quantification

   Upon termination of the experiment, all specimens were aseptically removed and placed in 5 ml of sterile saline. The specimens were vortexed (20 seconds) and sonicated (20 seconds) to disrupt plaque from the tooth surface. The dislodged bacteria were diluted 1:1000 and 1: 10,000 and double-plated in *Mitis Salivarius* supplemented with bacitracin and sucrose (MSSB) for *S mutans* and Trypticase Soy Agar (TSA) to check for lack of contamination. An automated spiral plater (Whitley Automatic Spiral Plater, DW Scientific; Shipley, UK) was used for plating. The plates were incubated for 48 hours at 37°C, 5% CO_2. Counting bacterial colonies was performed using Protocol (Synoptic LTD, North Cambridge, UK). The device is a combined hardware and software system for automatically counting colonies in colony-forming units (CFU/ml).

3. Confocal Laser Scanning Microscope (CLSM) Analysis

   CLSM was used to determine the extent of demineralization. A resin (SNAP, Parkell Bio-Materials Division, Farmingdale, NY, USA) was applied on top of each specimen and allowed to polymerize for 10 minutes. Each specimen was then cut in half perpendicular to the demineralized window using a Silverstone-Taylor hard tissue microtome (Scientific Fabrications Laboratory, Lafayette, CO, USA). One half of each specimen was stained overnight with a 0.1 mM solution of Rhodamine B (Aldrich Chemical Co, Milwaukee, WI, USA). The cut, stained surface of each section was kept moist (to avoid dentin shrinkage) for analysis using a confocal microscope (LSM510-META, Carl Zeiss Microimaging, Inc, Thornwood, NY, USA) to determine the extent of the lesions.[27] After the specimens were illuminated with the He/Ne laser (20%) using a 543 nm excitation wavelength and a 580-633 nm filter, live images were brought into focus (using a 10x Zeiss Plan Neofluar objective, NA 0.3), and digital images were obtained from each specimen (four frames per image). The confocal pinhole was set at 82.3 µm. The images were analyzed using Metamorph (version 5.0) software (Universal Images Corp, West Chester, PA, USA). For each section, measurements (lesion area and total fluorescence) were made in one area 250 µm in length at the center of the lesion.

Table 1: *Final pH Measurements of Model Components*

	Vessel A1	Vessel A2	Vessel B1	Vessel B2
Drainage fluid	4.11	4.00	4.05	4.07
TSBS	7.09	7.03	7.06	7.08
Caries vessel	4.80	4.78	4.89	4.74
MW	6.50	6.50	6.52	6.52

Table 2: *Bacterial Quantification*

Group	N	Mean, cfu/ml	Standard Deviation
Copper removed	12	2.14×10^8 [a]	1.03×10^8
Chlorhexidine	12	2.27×10^8 [a]	1.04×10^8
Copper—not removed	11*	2.49×10^8 [a]	1.82×10^8
No treatment	12	2.31×10^8 [a]	1.43×10^8

[a] *Groups were not statistically significant (p>0.05).*

* *Specimen was lost during removal from the caries vessel.*

Table 3: *Confocal Microscopy Results*

Group	N	Mean Area of the Caries Lesion µm (SD)	Mean Total Fluorescence of the Caries Lesion (SD)
Copper removed	11*	14901 (5499)[a]	527027 (338265)[c]
No treatment	12	14082 (4850)[a]	397968 (152536)[c]
Copper—not removed	12	4672 (6091)[b]	85312 (120443)[d]
Chlorhexidine	12	4203 (5650)[b]	57356 (75866)[d]

Values with same letters showed no statistical significance (p>0.05).

**A specimen was lost.*

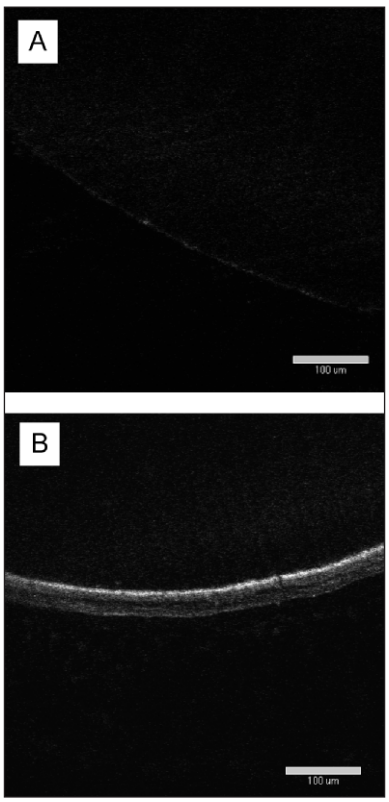

Figure 3. *A: CLSM image of a specimen from the copper varnish group; B: CLSM image showing a lesion in a tooth from the varnish-removed group.*

Statistical Analysis

Comparisons among the four groups for differences in confocal measurement and bacterial count were performed using analysis of variance (ANOVA) models. The ANOVAs included a random effect for vessel to account for within- and between-vessel variability. The analyses were performed using the ranks of data to satisfy the ANOVA assumptions.

RESULTS

The pH of the fluid remaining in the caries vessels after the last sucrose cycle, as well as the remaining drainage fluid, TSBS media and MW media were measured (Table 1). There were no major differences in pH values among the four vessels for each fluid.

Bacterial results are shown in Table 2. No significant difference in bacterial counts ($p>0.05$) was found among the four groups. For confocal measurements, the group with copper removed (Group 1) and the not treated group (Group 4) had significantly greater total lesion area and total fluorescence than the copper varnish without removal group (Group 3) and the chlorhexidine group (Group 2) (Table 3).

DISCUSSION

After five days of demineralization in the bacterial caries model, no significant difference was found among the different groups in *S mutans* plaque growth. This may be a result of multiple factors. First, the copper and CHX varnishes were applied on a small area 4 mm x 4 mm only at the beginning of the experiment. Second, each caries vessel had a representative number of specimens from each group. There was a continuous flow in the vessel, and the CHX or copper varnish was not expected to affect the specimens from the other two groups; however, it is possible that there was some carry over effect, especially at the beginning. Third, the effectiveness and duration of action of a varnish depended on both the concentration of the active agent and the number of applications. Copper varnish is still new, and the manufacturer has not specified how many applications are needed for this particular use. In the current study, only one application was used. Also, the amount of copper released was not specified by the manufacturer. It is possible that the amount released was not sufficient to demonstrate a prolonged antibacterial effect after five days.

Duguid[7] measured the bacterial inhibition effect of different copper concentrations and found that 10^{-3} M copper inhibited the rate of growth, whereas 10^{-4} M and lower concentrations had little or no effect. In a more recent study, Foley and Blackwell[10] measured the amount of copper released from copper cements at 2, 7 and 28 days and at six months and found that the highest amount was released after two days, with the majority of the decrease measured at day seven. Thus, it is possible that the varnish had an initial effect on *S mutans* numbers, but the effect was lost after longer incubation. A similar explanation may apply to the CHX varnish.

In 1996, Van Loveren and others[21] compared the effect of different CHX varnishes in a bacterial demineralization model and found CHX to be antibacterial

and, in their experiment, the varnishes were applied adjacent to the specimens. The experiment extended over three serial 22-hour demineralization periods with fresh *S mutans* suspensions used for each period. The bacterial count in their study was higher in the second and the third applications of the bacterial suspension when compared to the first 22 hours. Therefore, it is possible that both CHX and copper varnishes did have an antibacterial effect at the early stages of the experiment, but that the measurable effect was lost after five days. In the current study, although most of the copper varnish was still at the tooth surface after completing the experiment, the active ingredient could have been released. This is in agreement with Foley and others.[17,28]

In the current study, the cariogenic property of copper was evaluated using the CLSM. The intact copper and chlorhexidine varnish groups had significantly less total fluorescence and total lesion area compared to the copper-removed and no-treatment groups (Figure 3). There was no significant difference between the intact copper group and the chlorhexidine groups for all caries lesion parameters measured. Both lesion area and total fluorescence correlate well with microradiography ΔZ (mineral loss calculated as the integrated area [vol% μm] between sound and demineralized enamel or dentin). It is believed that fluorescence is related to the porosity of the lesion, so that the more demineralization, the higher the total lesion fluorescence.[27] In addition, laminations were seen in lesions which were similar to those seen in *in vivo* lesions, reflecting the occurrence of demineralization-remineralization as a result of the different cycles of TSBS and MW, and they provided evidence that the model adequately simulated the oral environment.[26]

In the current study, due to the lack of bacterial data, the authors cannot conclude whether the anticariogenic effect observed was due to mechanical coverage of the tooth structure or an initial release of antimicrobial agents from the copper and CHX varnishes. However, the data indicate that, as long as the varnish stays in place, caries development is significantly slowed down. The antibacterial effect of the varnish depends on the release of copper ions to the media; if the amount released was not sufficient or did not last long enough to kill most of the bacteria, then the growth of the bacteria nourished regularly would overcome the effect of the varnish. Most of the published data about copper cement discussed the germicidal effect but not the availability of the active ingredient for the media or the mechanism release of the ions.[6,10,29-30] The data from Group 1 demonstrates that small plugs remaining after removal of most of the varnish material is, however, not sufficient to interfere with the caries process.

CONCLUSIONS

Chlorhexidine and copper varnishes have anticariogenic effects on root surfaces. However, based on the results of the current study, it was unclear whether the effect was due to the release of antibacterial agents and/or to the mechanical coverage of tooth structure. As long as the varnish stayed on the surface, caries development was slowed down. The application regimen and long-term caries effects of a copper varnish need further investigation before clinical protocols can be recommended.

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