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ORIGINAL ARTICLE

Antibacterial Effect of Cupral® on Oral Biofilms - An *In-Vitro* Study

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ABSTRACT

Objective: This study aimed to assess the efficacy of Cupral®, a $Ca(OH)_2$ and Cu^{2+} based materials used in endodontics, against biofilms of the oral species Streptococcus oralis, Streptococcus gordonii and Aggregatibacter actinomycetemcomitans at different maturation stages.

Methods: Biofilms of the bacterial target species were grown in brain heart infusion (BHI) medium for 1 and 5 days on titanium disks (titanium, grade 4) to collect microbial communities at different stages of biofilm maturation. Biofilms were subjected to different Cupral® concentrations (4-, 15- and 50-fold dilution) to assess the antimicrobial- and biofilm dissolving effect. 0.2% chlorhexidine gluconate (CHX) solution was used as a positive control. Biovolume and antibacterial efficacy were analyzed by live/dead staining in combination with confocal laser scanning microscopy (CLSM) to quantify biofilm detachment and antibacterial efficacy.

Results: All tested Cupral® concentration showed a strong antibacterial effect on tested bacterial species at all biofilm maturation stages. Efficacy of biofilms detachment was concentration dependent, i.e. higher Cupral® concentrations generally led to increased biofilm detachment. The antibacterial efficacy of tested Cupral® concentration was at least equal to CHX treatment (P=0.03).

Conclusion: Cupral® shows a strong anti-biofilm efficacy and may be applied for oral biofilm treatment and control in dental disciplines other than endodontics.

Keywords: Antibacterial, antibiofilm, biofilm, biofilm lysis, biofilm detachment, Cupral®, oral infection, treatment

HIGHLIGHTS

- Oral Biofilms are one of the most difficult challenge in dentistry which can be responsible for tooth loss.
- Cupral can be a new medicinal product with biocidal potential to fight against pathogenic oral Biofilms and resulting infections.

INTRODUCTION

Oral diseases are the most prevalent chronic infections in the world (1); in most cases tooth decay and periodontitis are reasons for that (2, 3). Most of the oral disorders are bacterial biofilm driven. Biofilm formation is a natural occurring process in the oral cavity and so far more than 700 different bacteria have been shown to cooperate in oral microbial communities (4). In sessile microbial communities, the cells are embedded in a matrix of self-secreted polymeric substances, such as DNA, proteins or polysaccharides which forms a diffusion barrier against antimicrobial substances, predation and host immune response (5), and adapt metabolic activity to the biofilm lifecycle. As a consequence, antibiotic resistance may increase by several hundred folds and renders drug treatment often ineffective (6, 7).

These days, the use of copper as an antibacterial compound has gained increasing attention (8-13), e.g. copper and copper alloys have been registered as the first solid antimicrobial material by U.S. Environmental Protection Agency in 2010 (8). Several mechanisms of antibacterial actions of copper ions have been proposed: a) formation of hydroxyl radicals (14), b) oxidative crosslinking of thiol residues in proteins (8) and c) competition between copper- and other metal ions for protein binding sites (15, 16).

The use of calcium hydroxide as a disinfectant agent in dentistry was introduced in the 1920 by B.W. Hermann (17). Since then, it has become one of the major antiseptic preparations in endodon-

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tics. In aqueous formulations $Ca(OH)_2$ dissociates into calcium ions and hydroxyl ions: $Ca(OH)_2 -> Ca^{2+} + 2OH^-$

The release of hydroxide ions in aqueous solutions generates a strong alkaline (micro-) environment; saturated $Ca(OH)_2$ solution has a pH of 12.4 (18). The strong alkaline environment induces DNA strand separation also as proteins undergo conformational changes due to deprotonation of acidic residues (19). In both cases, biomolecules are denatured and lose their biological function. Limited solubility of $Ca(OH)_2$ in water of 1.7 g/l (20) causes a slow and gradual ions release of saturated formulations (21) and suspensions are thus suited to be applied as interappointment dressings (22-24). Also, its activity against biofilms is reported to be limited (25, 26).

The germicidal efficacy of the medicinal product Cupral® is based on combined bactericidal activity of copper ions/complexes and its strong alkalinization potential through release of hydroxide ions from $Ca(OH)_2$ and $Cu(OH)_2$. Although Cupral® is used in endodontics since more than a decade and has proved to be effective, only a few studies have analyzed its biocidal potential in more detail (27) and studies targeting efficacy against oral biofilm formers are so far missing. Therefore, the aim of this study was to evaluate the efficacy of Cupral®® against biofilms of the oral commensal species Aggregatibacter actinomycetemcomitans, Streptococcus oralis and Streptococcus gordonii at different maturation stages and substance concentrations.

MATERIALS AND METHODS

Ethical approval for the current study was given by the local ethics committee (No. 4348, Hannover Medical School, Germany).

Bacterial strains and culture conditions

The bacterial strains Aggregatibacter actinomycetemcomitans (A. ac.) DSM 1123, Streptococcus oralis (S. oralis) DSM 20627 and Streptococcus gordonii (S. gordonii) 20568 were acquired from the German Collection of Microorganisms and Cell Cultures (DSMZ). The bacteria were routinely propagated in Brain Heart Infusion (BHI) medium at 37 °C both under aerobic (streptococci) and anaerobic conditions (A. ac.). Precultures were grown overnight (streptococci) or for 72 h (A.ac.) under agitation.

Biofilm formation und dynamic growth conditions

Biofilms were grown on titanium (grade 4) specimens as they were used in pre-studies as good biofilm formers (28, 29). These were disc-shaped, had a height of 1.8 mm and were 12 mm in diameter. Specimens were glued to glass cover slips using Silagum Light (DMG, Hamburg, Germany) which were subsequently placed in a glass staining-rack (Fig. 1). Precultures were diluted to an optical density (OD_{600}) of 0.03 and were used for inoculation of 1.5 l Brain Heart Infusion medium (BHI; Oxoid, Hampshire, UK). The racks with fixed titanium samples were immersed in the bacterial suspension and cultivated at 37 °C under continuous stirring at 200 rpm using a magnetic stirrer system (Cimarec™ i Compact; Thermo Fisher, Waltham, USA). Culture medium was changed every 48 h, where applicable. Experiments were performed in triplicate as independent biological replicates.

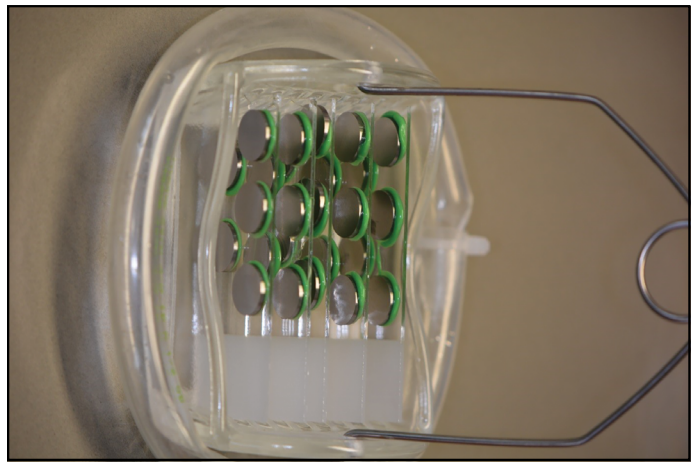

Figure 1. Technical set-up to induce biofilm formation on titanium specimens. Titanium disks are glued to microscopic slides and placed in glass staining rack before immersion in nutrient broth and cultivation for 1 to 5 days

Cupral® treatment of biofilms

Cupral® was diluted 1:4 (v/v; referred to as C4 in the following), 1:15 (v/v referred to as C15 in the following) and 1:50 (v/v; referred to as C50 in the following) with ddH_2O to give the test suspensions. Titanium disks with attached biofilms were transferred to petri-dishes and submerged in the three different Cupral® dilutions for 24h at 37 °C. As controls, biofilms were incubated in Dulbecco's phosphate buffered saline (negative control, Biochrom GmbH, Berlin, Germany) and 0.2% CHX solution (positive control; Meridol med 0.2% CHX, CP GABA, Hamburg, Germany). After the incubation step, the biofilms were washed twice by gentle immersion in ddH_2O to rinse off any attached solid Cupral® components. Cupral® supernatants from the petri-dishes were subjected to bacterial growth evaluation by plating 100 µl suspension on BHI agar plates followed by an incubation step at 37 °C for at least 24 h.

Evaluation of microbial viability in biofilms and supernatants

Bacteria were stained live/dead (BacLight® Bacterial Viability Kit; Life Technologies, Carlsbad, USA) with the fluorescence dyes Syto9 and propidium iodide (PI). Both dyes were mixed by equal volumes and diluted 1:1000 in PBS. Biofilms were incubated in the staining solution for 30 min in the dark, washed in PBS and subsequently fixed in 2.5 % (v/v) glutardialdehyde. In short, Syto9 passes bacterial membranes by diffusion and intercalates into genomic DNA. PI instead, cannot penetrate intact bacterial membranes. If cell membrane integrity is impaired, PI intercalates into genomic DNA and displaces SYTO9. Using fluorescence microscopic examination, both cell vitality states can be distinguished: vital cells (membrane-intact) appear green fluorescent, non-vital cells (membrane-impaired) red fluorescent. Biofilms were analyzed by Confocal Laser Scanning Microscopy (CLSM; Leica SP2, Wetzlar, Germany). z-stack images (10x magnification, 3 µm step size) were acquired at three randomized positions (center, up, down) on an imaginary line through the center of the titanium specimen.

Quantification of the antibacterial effect of Cupral® treatment

3D reconstructions of biofilms were processed from z-stack images using the IMARES (Version 5.0; Bitplane AG, Zürich Schweiz) Software package and spatial parameters of the microbial agglomerates were calculated. A minimal intensity value of 12.52 was preset to compensate for background fluorescence by unspecific attachment of Syto9 to $Ca(OH)_2$ particles. Based on the volume of red fluorescent (non-vital) and green fluorescent (vital) cells, a live/dead ratio was created for each z-stack image. Mean biofilm height was calculated from biovolume and size of picture section and expressed as percentage ratio relative to the untreated biofilm control. The following outcomes were regarded as antibacterial a) biofilm detachment from the surface due to chemically-induced degradation and b) increased percentage of membrane impaired (dead) cells within treated biofilms compared to untreated controls.

Statistical analysis

All statistical analysis was performed using the SPSS statistical software package, version 24.0 (IBM Coorperation, Armonk, USA). The correlations between biofilm height and treatment procedure, and cell vitality and treatment procedure were analyzed using the Kruskal-Wallis omnibus test. The experimental results were evaluated using the following null hypotheses: "Distribution of relative biofilm height is the same across all categories of treatment" and "Distribution of percentage avital cells is the same across all categories of treatment" with post-hoc group comparison. Significance values were adjusted by Bonferroni correction for multiple tests; a P-value<0.05 was regarded as statistically significant.

RESULTS

Streptococcus gordonii

The biofilm formation on the titanium specimen surfaces was reproducible throughout the independent biological replicates. The suspensions C4 and C15 detached microbiological agglomerates from the surfaces the most effectively. C50 showed the lowest biofilm detachment potential. Biofilm detachment was generally reduced on day 3 old biofilms (Fig. 2a upper box-plot). The differences in biofilm height were statistical significant between the different treatment categories: C4-CHX (P<0.001), C15-CHX (P<0.001) and C50-CHX (P=0.03). No statistical significance was observed, when comparing relative biofilm heights pairwise for the different biofilm maturation stages and treatment regimes. The supernatants of Cupral® treated biofilm preparations were analyzed for viable planktonic cells. For C15 and C50 treatments, no viable cells were detected by cultivation on solid nutrient agar. For C4 preparations no colony formation was detected, however the high content of solid $Ca(OH)_2$ particles aggravated clear bacterial colony detection.

CHX treated biofilms reproducibly showed an increased biovolume compared to Cupral® treated samples. The amount of biofilm on the surface was at least two fold higher for the maturation stages day 1, day 2, day 3 and day 5 compared to the untreated controls. Only for day 4 old biofilms, CHX treatment resulted in a 20% reduction of biovolume.

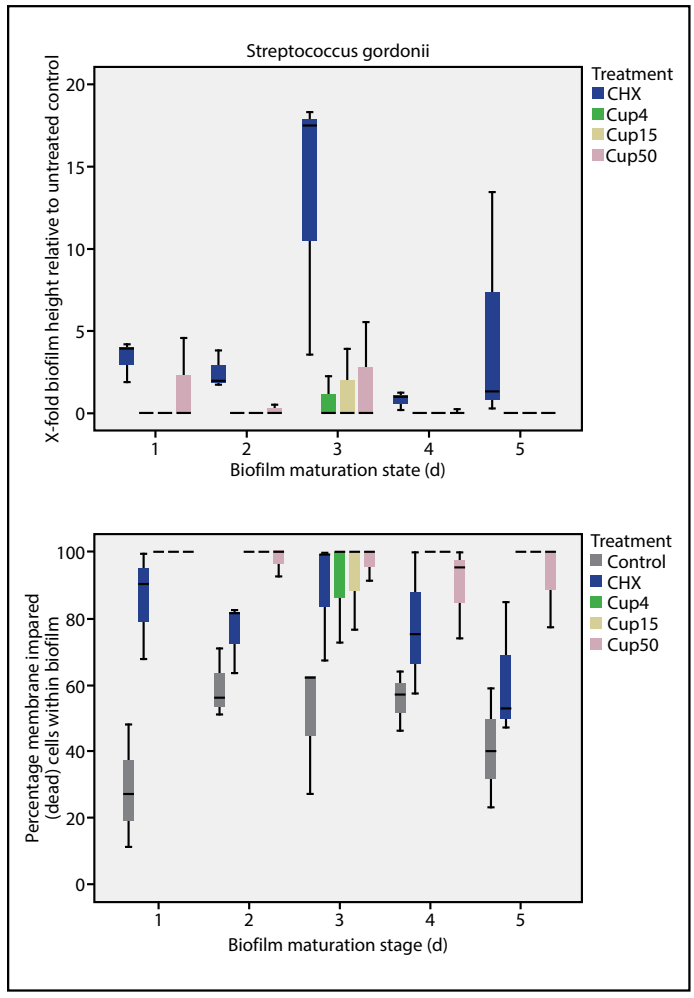

Figure 2. Diagrams depict the effect of Cupral treatment on biofilm height and cell survival of S. gordonii biofilms at different maturation stages; upper box-plot: Change of mean biofilm height compared to the untreated control in response to Cupral treatment for 24 h; lower box-plot: Relative proportion of dead cells within biofilms after 24 h incubation in Cupral suspension

Remaining cell agglomerates on the surfaces were only observed for C50 treatment (Fig. 2 lower box-plot); CHX treatment resulted in no observable biofilm detachment. CHX treatment resulted in a lower cell damage rate compared to C50 treatment. However this difference was not statistically relevant. C4 and C15 solutions were not considered in the statistical analysis at this point, due to lack of data through detachment of biofilms from the surfaces.

Streptococcus oralis

Biofilm formation throughout the biological replicates was reproducible; however with increasing biofilm maturation state, the percentage of membrane compromised cells increased. All tested Cupral® suspensions were highly effective against attached biofilms at all maturation stages. The Cupral® suspensions C4, C15 and C50 almost completely detached biofilms from the surfaces, with a significantly higher efficacy compared to CHX treated samples (P<0.001 for C4, C15 and C50). However, after C15 and C50 treatment of two days old biofilms, minor bacterial agglomerates remained attached to the surfaces (Fig. 3 upper box-plot).

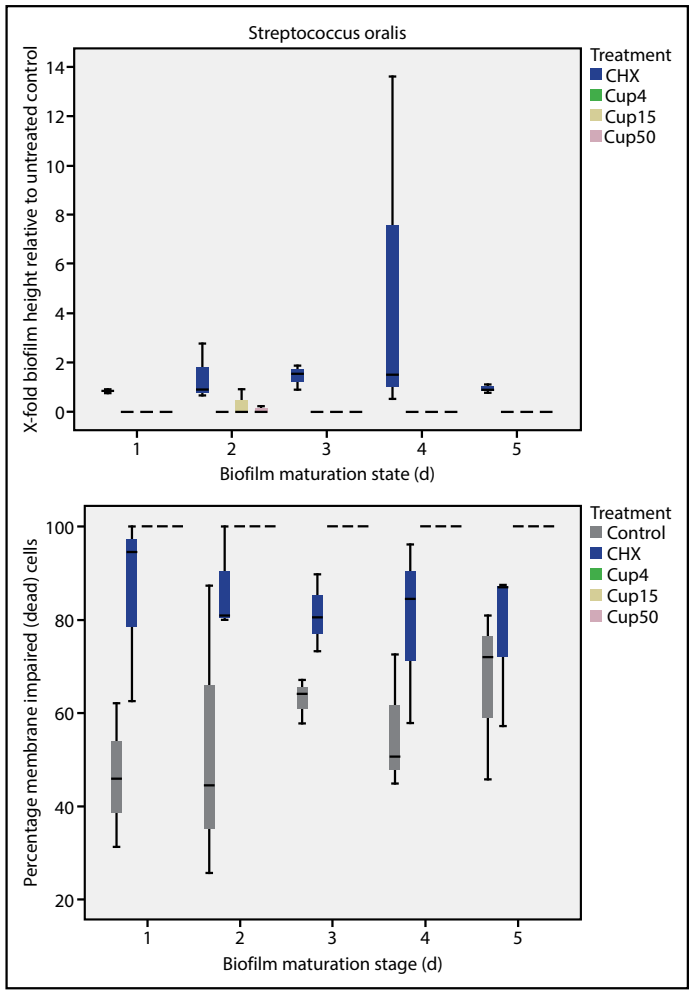

Figure 3. Diagrams depict the effect of Cupral treatment on biofilm height and cell survival of S. oralis biofilms at different maturation stages; upper box-plot: Change of mean biofilm height compared to the untreated control in response to Cupral treatment for 24h; lower box-plot: Relative proportion of dead cells within biofilms after 24 h incubation in Cupral suspension

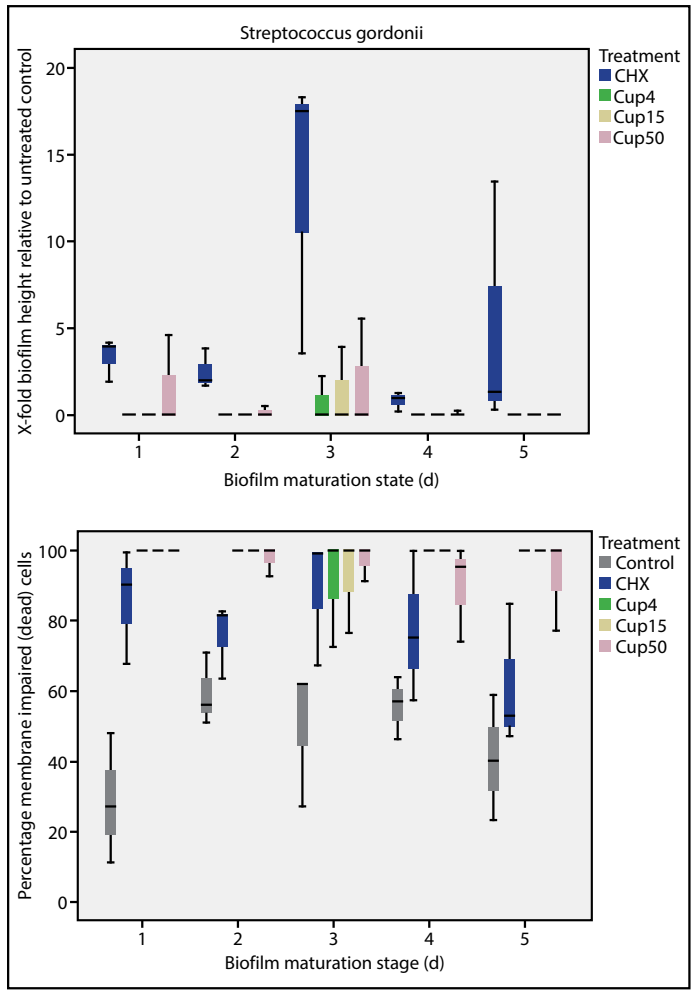

Figure 4. Diagrams depict the effect of Cupral treatment on biofilm height and cell survival of A. actinomycetemcomitans biofilms at different maturation stages; upper box-plot: Change of mean biofilm height compared to the untreated control in response to Cupral treatment for 24h; lower box-plot: Relative proportion of dead cells within biofilms after 24 h incubation in Cupral suspension

Bacterial cells in the supernatants of Cupral® treated biofilms were not replication-competent/viable on solid nutrient medium - no colony formation was detected. For the C4 suspension undissolved $Ca(OH)_2$ particles, equivalent in size to bacterial colonies, complicated visual inspection. CHX treatment induced membrane damage in 57-94 % of biofilm cells (Fig. 3 lower box-plot). With exception for 2 d old biofilms, all Cupral® suspensions completely detached biofilms from surfaces. Remaining cells after Cupral® treatment showed an overall viability <1 %, as assessed by CLSM analysis.

Aggregatibacter actinomycetemcomitans

Biofilm formation on specimen surfaces varied between the three biological replicates. Either biofilm detachment in late biofilm maturation stages (day 4 and 5) or delayed onset of biofilm formation was observed and the anti-biofilm effect could not be analyzed as biological triplicates for all biofilm maturation stages. However, C4 and C15 treatment resulted in complete detachment of biofilms at all tested maturation stages, whereas cell agglomerates remained on the surfaces after C50 treatment of day 2, 3 and 4 old biofilms (Fig. 4 upper box-plot). Again, CHX treatment resulted in minor or no biofilm detachment. The difference in the percentage of dead cells for C50 treatment and CHX treatment was not statistically significant (Fig. 4 lower box-plot).

DISCUSSION

On a medical perspective, biofilm treatment becomes increasingly challenging at later maturation stages, as biofilm architecture has completely evolved and offers strong protection from external threats (30). Therefore, the antiseptic effect of Cupral® was evaluated at various biological conditions *in vitro*, to determine its efficacy against biofilms at different maturation stages and the effective concentration window. For a realistic test procedure, biofilms were prepared in a way that they morphologically resembled those found in the oral cavity. Flow chamber models are widely accepted for this purpose (29, 31-33) as fluid flow is considered to have substantial influence on (oral) biofilm formation processes. The underlying mass transport is a key driver for nutrient transport and waste removal processes and an essential influencing factor for microbial growth in the

oral cavity. For example, shear stress induces compact biofilm morphology (34), and tight adhesion forces to the substratum surfaces (35). In terms of the experimental reproducibility, unspecific detachment effects, e.g. induced through experimental handling, can simply be avoided by choosing an appropriate *in vitro* model. Furthermore, the described experimental design precludes unspecific bacterial sedimentation on the surfaces, as mainly actively adhering (vital) bacterial cells take part in the biofilm formation process. Accordingly, increased accumulation of dead cells was precluded that may had been biased the observed biological effects.

The collection of biofilms at different maturation stages was successfully performed for all tested bacterial species. However, obvious differences between biofilm development among the tested species was microscopically observable. For both streptococci, the biofilm formation was relatively uniform and reproducible throughout the biological replicates. However, the oral pathogen A. actinomycetemcomitans showed either a prolonged lag-phase or extensive biofilm detachment on day 4 and day 5. A possible explanation for the latter effect could be accumulation of acidic metabolic waste products. A. actinomycetemcomitans is known to be sensitive to acidic environmental conditions (36). With increasing biomass on the surfaces at later maturation stages, available nutrients are metabolized faster resulting in a pH downshift as buffer capacity of the nutrient broth may have been exceeded. Both tested streptococci species are tolerant to acidic environments (37), as many species of the genus Streptococcus are directly involved in caries formation or can reside in close proximity to acidogenic species. Accordingly, pH changes may not have influenced streptococci growth but have negatively affected proliferation of A. actinomycetemcomitans and caused biofilm detachment at prolonged cultivation for this species.

Cupral® treatment of biofilms was effective at all tested concentrations in terms of biofilm detachment capacity and killing of biofilm cells. At concentrations C4 and C15, the antibacterial intracanal dressing showed the highest biofilm detachment effect. The efficacy of both active ingredients, Cu^{2+} and $Ca(OH)_2$, are well known (13, 38-40). Cell lysis as a consequence of the highly alkaline microenvironment has frequently been reported; however for complete detachment of cells and EPS matrix, this it is not the case. Beeton et al. (41) reported antibiofilm activity of organic copper (II) complexes on mature biofilms. This effect was attributed to a nuclease activity of the metal-organic complex that targets compounds of the EPS matrix. However, this effect has not been described for $Cu(OH)_2$ treatment. The observed effects may be attributed to a combined effect of a strong alkaline environment and a high Cu^{2+} concentration. These findings are supported by the effect of low concentrated Cupral® suspensions (C50) where biofilm detachment has been observed to much lesser extent.

As a result of prolonged CHX treatment, a considerable increase of biofilm volume was observed. Previous studies of Lee et al. (42), Wang et al. (43) and Ariaz-Moliz et al. (44) provided no evidence for the observed effect. However, treatment durations were either in the range of several minutes (42-44) or considerably exceeded 24 h (42). It is likely that increased biovolume is a stress-related reaction. We observed that it was highest for mature streptococcal biofilms at an age of day 3 and at longer time interval. Biofilm formation is a multi-stage process (5, 45) ending up with a mature multi-layered bacterial community. Major growth limiting step is the nutrient supply and accumulation of metabolic waste products in the lower part of the biofilm. In this situation, bacterial metabolic activity in deeper biofilm layers can be reduced (46, 47). As for both streptococci and A. actinomycetemcomitans the highest biovolumes were observed between day 3 and day 5, CHX treatment may have targeted a completely evolved microbial community. According to Hoffman et al. (48) a biomass increase may be triggered by a stress related reaction to antimicrobials. It is, therefore, can be concluded that massive biomass increase may be due to a stress-related response of bacteria in deeper biofilms layers. Those were better protected from CHX exposure and may have triggered massive EPS production. However, due to long treatment duration, bacteria have been killed at a later time point during CHX exposure which is in accordance with the low cell survival rate.

CONCLUSION

In this study we demonstrated the antiseptic efficacy of Cupral® dressing on *in vitro* formed biofilms of oral commensals. The disinfection capacity, i.e. rate of bacterial killing, of Cupral® was comparable to CHX. However, disintegration of biofilm structures, i.e. removal of extrapolymeric substances resulting from biofilm formation, was only observed after Cupral® treatment. Based on the experimental results, the application of Cupral® solutions as antibacterial rinsing and endodontic irrigation solutions seems to be a promising approach to fight pathogenic oral biofilms and resulting infections.

Disclosures

Conflict of interest: The authors certify that they have no conflict of interest.

Ethics Committee Approval: Ethical approval for the current study was given by the local ethics committee (No. 4348, Hannover Medical School, Germany).

Peer-review: Externally peer-reviewed.

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