Antimicrobial and antibiofilm efficacy of a copper/calcium hydroxide-based endodontic paste against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*

Aida METO¹, Bruna COLOMBARI², Arianna SALA², Eva PERICOLINI², Agron METO⁴, Samuele PEPPOLONI² and Elisabetta BLASI²,³

¹ School of Doctorate in Clinical and Experimental Medicine, University of Modena and Reggio Emilia, Modena, Italy
² Department of Surgical, Medical, Dental and Morphological Sciences with interest in Transplant, Oncological and Regenerative Medicine; University of Modena and Reggio Emilia, Modena, Italy
³ School of Specialization in Microbiology and Virology, University of Modena and Reggio Emilia, Modena, Italy
⁴ Department of Therapy, Faculty of Dental Medicine, Aldent University, Tirana, Albania

Corresponding author, Elisabetta BLASI; E-mail: elisabetta.blasi@unimore.it

Endodontic biofilm is a microbial community, enclosed in a polymeric matrix of polysaccharide origin where are found pathogens, like bacteria and opportunistic fungi responsible for various endodontic pathologies. As clinical importance is the fact, that biofilm is extremely resistant to common intracanal irrigants, antimicrobial drugs and host immune responses. The aim of this study was to evaluate the *in vitro* efficacy of a Cu/CaOH₂-based endodontic paste, against bacteria and fungi, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. We found that such compound significantly reduced microbial replication time and cell growth. Moreover, biofilm formation and persistence were also affected; treated biofilms showed both a reduced number of cells and levels of released pyoverdine. This study provides the first evidence on effectiveness of this endodontic compound against microbial biofilms. Given its wide range of action, its use in prevention and treatment of the main oral biofilm-associated infections will be discussed.

Keywords: Biofilm, Cupral, Antimicrobial activity, Antibiofilm activity

INTRODUCTION

In human body, one of the most complex and heterogeneous microbial communities occurs in the oral cavity, where adhesion of planktonic microbes to a surface, either biotic or abiotic, is followed by co-aggregation, growth, production of an extracellular matrix and maturation of a sessile structure, the so call oral biofilm⁵. The relevance of such biofilm in health and disease, both locally and distally, is receiving increasing attention.

The main etiological factor in the emergence of apical periodontitis is an infection by oral microbes and their products, that once reaching the pulp and periapical tissues, locally produce tissue damage and necrosis in about 90% of cases⁶. Accordingly, special efforts are aimed at counteracting the microbial agents present in an infected pulp system⁷; nevertheless, it is very difficult to guarantee their complete elimination. Increasing literature documents the presence of facultative anaerobic bacteria, such as Gram-positive cocci (*Enterococcus faecalis* and *Staphylococcus aureus*) and Gram-negative bacteria (*Pseudomonas aeruginosa*) as pathogenic for dental pulp condition⁴.⁶ Another opportunistic fungal pathogen, *Candida* spp has occasionally been isolated from root canal periapical lesions, granulomas and necrotic pulp tissue as well⁷.⁸.⁹

Microbial agents tend to penetrate tooth structure and accumulate in dentinal canals, at a considerable depth, where they likely produce endodontic biofilms and are hardly reached by endodontic instruments and irrigants⁸.¹⁰, especially in cases of complicated anatomy, lateral canals and apical ramifications¹¹-¹³. *Enterococcus faecalis, Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans* (the most commonly isolated fungal specie in the oral cavity)⁶.¹⁰, are frequently observed inside pulp system⁴,¹² and they are one of the multiple factors responsible for the failure of endodontic therapy. An indispensable condition for success in endodonicy is not only the treatment of the main root canal, but also the elimination of microbial cells commonly localized in lateral canals, apical deltas and dentinal tubules; only in this way, a permanent sterility of the treated dental element may be guaranteed¹⁰. Despite improvement in endodontic techniques, the percentage of failure remains still high, because of intra-radicular bacteria persistence¹⁷. For this reason, it is necessary to use antimicrobial agents, that besides being not toxic for the patients, are capable to deeply penetrate into dentinal system, and, in turn, efficiently eliminate bacteria, possibly even when structured in biofilm. Notoriously, once structured as biofilm, microbial agents have enhanced resistance to antibiotics and disinfectants, given their complex and heterogeneous arrangement as a microbial sessile population embedded into an extracellular, minimally

---

Color figures can be viewed in the online issue, which is available at J-STAGE.

Received Aug 2, 2018: Accepted Oct 31, 2018
permeable polymeric matrix\textsuperscript{18-20}. Among the numerous soluble factors involved in biofilm formation/maturation, quorum sensing molecule, including pyoverdines\textsuperscript{21,22} and eDNA\textsuperscript{23,24}, have been described. Because of such a complex scenario, antibiotic therapy as well as intracanal irritants treatment have to be focused towards the eradication of root canals infections and possibly endodontic biofilm disruption\textsuperscript{19}.

Innovative endodontic techniques have become widely available over the last years\textsuperscript{25,26} in particular, a copper-calcium-hydroxide (Cupral) treatment has been carefully regarded given its potent antimicrobial properties\textsuperscript{27}. Not only vegetative forms, but also bacterial spores and viruses were affected, underlying the wide-range efficacy such system\textsuperscript{27}. Initial evidence exists on spores and viruses were affected, underlying the wide-range efficacy such system\textsuperscript{27}. Initial evidence exists on its performance in clinical practice, where it is used twice by centrifugation at 3,500 rpm for 10 min, counted saline (PBS; EuroClone, Whethereby, UK), washed in inoculating loop, suspended in phosphate buffered saline (PBS; EuroClone, Whethereby, UK), washed twice by centrifugation at 3,500 rpm for 10 min, counted Colony Forming Units (CFU)/mL. Bacteria from frozen stocks were placed into Tryptic Soy Broth (TSB: OXOID, Milan, Italy) and placed to grow at 37°C overnight. Then, by sterile loops, 10 µL of each broth were seeded onto Tryptic Soy Agar (TSA) plates (OXOID) and allowed to grow for 24 h. Isolated colonies were then collected (1 or 2 for each species), added to 10 mL of TSB and allowed to grow overnight at 37°C with gentle shaking. Bacterial concentrations were then assessed by a McFarland standard curve and diluted to the working strength concentration of $1 \times 10^{5}$ Colony Forming Units (CFU)/mL. C. albicans cultures were maintained by biweekly passages onto Sabouraud Dextrose Agar (SDA) plates (OXOID). The day before each experiment, fresh cultures were seeded onto SDA plates and incubated at 37°C. After the overnight incubation, fungal cells were harvested by a sterile inoculating loop, suspended in phosphate buffered saline (PBS; EuroClone, Whethereby, UK), washed twice by centrifugation at 3,500 rpm for 10 min, counted by Burker’s chamber and suspended at $1 \times 10^{5}$ yeast cells/mL in YPD (OXOID) prior to be used in the experiments. For long-term storage, C. albicans was maintained as frozen stocks at −80°C, in glycerol solution 20% (v/v).

**Endodontic paste**

A commercially available compound (Cupral, HUMANCHEMIE, Alfeld, Germany), containing highly dispersed calcium hydroxide [Ca(OH)\textsubscript{2}], copper sulphate (II) (CuSO\textsubscript{4}), calcium sulphate (CaSO\textsubscript{4}), copper hydroxide (II) [Cu(OH)\textsubscript{2}], methylcellulose [\(\text{C}_{6}\text{H}_{13}\text{O}_{7}(\text{OH})\times(\text{OCH}_{3})\)] and distilled water was used. A starting solution of Cupral (weight/volume) was prepared by diluting 1 g of compound in 4 mL of distilled water (25%); serial dilutions (1:10) were then prepared to obtain 2.5% and 0.25% final solutions. Cupral preparations were sterilized by autoclave prior to be used in the study. Furthermore, in our experiments Cupral’s solutions were diluted 1:1 with culture medium (controls) or microbial cultures; the new concentrations were 12.5, 1.25 and 0.125% into the wells.

**Microbial growth assays**

Microbial suspensions ($1 \times 10^{5}$ CFU/mL; 100 µL/well) in 96-well plates (Sarstedt, Nümbrecht, Germany) were exposed or not to Cupral (100 µL/well, at the concentrations above indicated); blank wells (distilled water or Cupral without bacteria) were also included in the assay. The plates were then incubated at 37°C in presence or absence of 5% CO\textsubscript{2} for various times, as detailed elsewhere. Then, microbial growth was assessed measuring optical density (OD) or CFU in Cupral-treated and untreated groups, as follows. The absorbance was measured by the SunRise Microplate Reader (Sunrise, Tecan, Salzburg, Austria) at 595 nm wavelength, at the following times: 0, 3, 6, 24 and 48 h. The results were expressed as ΔOD, by subtracting the blank OD values from the OD of the experimental samples. The CFUs were evaluated at 0, 24 and 48 h, according to standard protocols by appropriated diluting and plating microbial 100 µl of each microbial suspensions on TSA (bacterial groups) or SDA (fungal groups) plates. After 48 h of incubation, the pH of each microbial culture was measured using the litmus papers. In selected experiments, the doubling time (DT) was determined in control and Cupral-treated microbial cells. Briefly, cultures in exponential growth phase were (90–120 min) exposed or not to Cupral and further incubated at 37°C; then, the ODs were measured at various time points and converted to CFU, using the McFarland curve for bacterial cells and an in house reference growth curve for C. albicans. The DT was calculated using the following formula, considering time 0 (initial CFU) and various time points (final CFU):

\[
\text{Doubling time} = \frac{\text{time} \times \log(2)}{\log(\text{final CFU}) - \log(\text{Initial CFU})}
\]

The results of DT were expressed in min.

**MATERIALS AND METHODS**

**Microbial strains**

The following American Type Culture Collection (ATCC) strains were used in this study: Staphylococcus aureus ATCC 6538, Pseudomonas aeruginosa ATCC 9027 and Candida albicans SC5314.

**Microbial and fungal cultures conditions**

Bacteria from frozen stocks were placed into Tryptic Soy Broth (TSB: OXOID, Milan, Italy) and placed to grow at 37°C overnight. Then, by sterile loops, 10 µL of each broth were seeded onto Tryptic Soy Agar (TSA) plates (OXOID) and allowed to grow for 24 h. Isolated colonies were then collected (1 or 2 for each species), added to 10 mL of TSB and allowed to grow overnight at 37°C with gentle shaking. Bacterial concentrations were then assessed by a McFarland standard curve and diluted to the working strength concentration of $1 \times 10^{5}$ Colony Forming Units (CFU)/mL. C. albicans cultures were maintained by biweekly passages onto Sabouraud Dextrose Agar (SDA) plates (OXOID). The day before each experiment, fresh cultures were seeded onto SDA plates and incubated at 37°C. After the overnight incubation, fungal cells were harvested by a sterile inoculating loop, suspended in phosphate buffered saline (PBS; EuroClone, Whethereby, UK), washed twice by centrifugation at 3,500 rpm for 10 min, counted by Burker’s chamber and suspended at $1 \times 10^{5}$ yeast cells/mL in YPD (OXOID) prior to be used in the experiments. For long-term storage, C. albicans was maintained as frozen stocks at −80°C, in glycerol solution 20% (v/v).
**Disk-diffusion test**

The disk-diffusion assay was performed according to “EUCAST disk diffusion antimicrobial susceptibility testing method” (http://www.eucast.org EUCAST European Committee on Antimicrobial Susceptibility Testing Breakpoint tables for interpretation of MICs and zone diameters Version 8.0 2018). Briefly, a sterile cotton swab was dipped in *Staph. aureus* stock suspension (1×10⁵ bacterial cells/mL) and inoculated on Mueller-Hinton agar (MHA) plates (OXOID). The plates were allowed to dry for 3 to 5 min. Then, sterile and neutral filter paper disks (6 mm) were placed onto the MHA surface and embedded with Cupral 12.5, 1.25 and 0.125% (10 µL/disk) or gentamicin (10 µg/10 µL/disk), used as positive control. The plates were then incubated at 37°C, examined after 24 or 48 h and the growth inhibition haloes around each disk were measured. The results were expressed as halo diameters in mm.

**Confocal microscopy analysis of *P. aeruginosa* biofilm**

*Pseudomonas* was allowed to produce biofilm onto coverslips inserted into 6-microplate (Sarstedt); then, the fluorescent property21,22 of *Pseudomonas* spp was exploited to perform confocal microscopy imaging, as detailed elsewhere20. Briefly, bacterial suspensions (1×10⁵ bacterial cells/mL in TSB) were seeded on coverslips (1,000 µL/well), and treated or not with Cupral 1.25%. The plate was incubated for 24 h at 37°C and 5% CO₂. After incubation, the coverslips were washed twice with PBS and fixed with 4% paraformaldehyde (PFA: Sigma-Aldrich, Darmstadt, Germany) for 30 min at 4°C, washed twice with PBS and then analyzed by confocal microscope Leica TCS SP8 (Wetzlar, Germany), at 492/517 nm wavelength excitation/emission as recommended by the distributor.

**Pyoverdine production by *P. aeruginosa* biofilm exposed or not to Cupral**

After 24 h of culture with *Pseudomonas*, the supernatants were collected, centrifuged twice at 10,000 rpm for 15 min in order to remove the remaining bacteria. To further, ensure that the supernatants were devoid of bacteria, 50 µL of the supernatants were inoculated onto TSA plates and incubated for 48 h at 37°C under aerobic conditions. No bacterial colony formation on TSA plates was observed. Pyoverdine release was quantified in 100 µL of culture supernatants and fluorescence emission was quantified with a multi-well fluorescence plate reader (Synergy HTX, BIOTEK, Winooski, VT, USA) (excitation/emission: 360/460), according to a standard protocol29. The measured amounts of pyoverdine were plotted as mean±SEM (Standard Error Mean) of relative fluorescence units (RFU).

**Assay for live/dead microbial evaluation**

Biofilm formation was performed in a black 96 well microplate. The microbial cells (1×10⁵ bacterial cells/mL and 1×10⁶ fungal cells/mL) were seeded on TSA plates and incubated for 24 h at 37°C in presence of CO₂. Biofilms were then treated with diluents (controls) or Cupral 1.25% and incubated for further 6 and 24 h. After incubation, the samples were stained with the “live/dead cells stain kit” (Thermo Fisher Scientific, Waltham, MA, USA), based on 5(6)-carboxyfluorescein diacetate (CFDA) to label alive cells (30 min) and propidium iodide (PI) binds to DNA and labels the dead cells (15 min). The staining was conducted according to manufacturer's instructions. After a total 30 min of incubation at 37°C, the samples were washed twice with PBS and the fluorescence emission (CFDA excitation/emission: 485/528; PI excitation/emission: 528/645) was analyzed using a multi-well fluorescence plate reader (Synergy HTX, BIOTEK). The results were expressed as RFU.

**Statistical analysis**

Data depicted in Figs. are the mean±standard error (SEM) from replicate samples of 2–3 different experiments. Statistical analysis was conducted using GraphPad Prism 7.0 software and carried out with one-way ANOVA with Bonferroni’s post-hoc test. Statistical significance was set at p<0.05, while p<0.0001 was indicative of highly statistically significant differences.
RESULTS

Evaluation of Cupral pH values under different experimental conditions
The pH value is a crucial parameter for microbial survival and growth. Thus, we initially assessed the pH of Cupral at different working dilutions. Results in Table 1 show that, a Cupral solution in distilled water at 25% (v/v) had a pH value of 14, which progressively decreased to 13 and 11, when it was diluted at 2.5 and 0.25%. When Cupral was diluted in TSB or YPD media (a condition used throughout the study to set up all the experiments), the pH values remained very high at concentrations of 12.5 and 1.25% Cupral, while it decreased to a value close to neutrality at 0.125%. When selected samples were maintained in the presence of 5% CO₂ for 48 h, the pH decreased to values between 9 and 7, depending upon Cupral dilutions. Finally, pH values were measured in microbial cultures (Staph. aureus, P. aeruginosa, C. albicans) exposed or not to Cupral and then incubated for 48 h in the presence or absence of CO₂. We found that, in the presence of CO₂, the pH values were lower than those measured in the absence of CO₂, at all the conditions tested; such reduction was particularly evident at the Cupral 12.5% solution. The culture media alone showed pH values close to 7 regardless of the presence or not of CO₂ (data not shown).

Effect of Cupral on microbial growth
The antimicrobial activity of Cupral was evaluated in Staph. aureus, P. aeruginosa and C. albicans, by the CFU assay and the measurement of OD₅₉₅. Briefly, microbial cultures were exposed to Cupral at different concentrations (namely, 12.5, 1.25 and 0.125%) or diluent (controls) and then incubated, in the presence or absence of CO₂, up to 48 h. At time zero and at various time points, we measured the OD₅₉₅ and the CFU, as detailed above. The results in Figs. 1 and 2 show the ΔOD kinetic values (left panels) and CFU/mL (right panels) of Staph. aureus, P. aeruginosa and C. albicans, treated with scalar doses of Cupral and incubated in the presence (Fig. 1) or absence (Fig. 2) of CO₂ for different times. We found that Cupral greatly affected both bacterial and fungal cells growth in a concentration-dependent manner; the phenomenon was evident with all the strains tested and mainly occurred at 24–48 h of incubation, both in presence and absence of CO₂. Particularly, in the presence of CO₂ (Fig. 1), Staph. aureus, P. aeruginosa and C. albicans, exposed to Cupral 0.125% behaved similarly to untreated controls, with a time-dependent growth occurring mostly from 24 h. Moreover, when using the 12.5% Cupral solution, the residual microbial load, evaluated either by OD or CFU assay, dropped to almost undetectable levels, irrespective of the time points assessed. The 1.25% Cupral solution caused intermediate, yet significant inhibitory effects against all the microbial species tested. Figure 2 shows the results observed in the absence of CO₂. Once again, Cupral 0.125%-treated and untreated controls showed similar trends of growth, although some statistically significant differences were detected. On the other hand, the two Cupral concentrations (12.5 and 1.25%) strongly affected both bacterial and fungal growth, down to values close or even below the control.

According to the "EUCAST disk diffusion antimicrobial susceptibility testing method", we next assessed the effects of Cupral, by using the halo inhibition test. Figure 3 shows a representative experiment with Staph. aureus, in which gentamicin (10 µg/10 µL/disk) was used as positive control. An inhibition halo of about

Table 1  pH values of Cupral under different experimental conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pH values</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Distilled H₂O</td>
<td>Cupral 25%</td>
<td>Cupral 2.5%</td>
<td>Cupral 0.25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>14</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>TSB or YPD+CO₂</td>
<td>Cupral 12.5%</td>
<td>9</td>
<td>8.5</td>
<td>7</td>
</tr>
<tr>
<td>TSB or YPD–CO₂</td>
<td>Cupral 1.25%</td>
<td>13</td>
<td>12</td>
<td>7.5</td>
</tr>
<tr>
<td>S. aureus+CO₂</td>
<td>Cupral 0.125%</td>
<td>8.5</td>
<td>8.5</td>
<td>7.5</td>
</tr>
<tr>
<td>S. aureus–CO₂</td>
<td></td>
<td>13.5</td>
<td>9</td>
<td>8.5</td>
</tr>
<tr>
<td>P. aeruginosa+CO₂</td>
<td></td>
<td>8.5</td>
<td>8</td>
<td>7.5</td>
</tr>
<tr>
<td>P. aeruginosa–CO₂</td>
<td></td>
<td>12</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>C. albicans+CO₂</td>
<td></td>
<td>8</td>
<td>7.5</td>
<td>6.5</td>
</tr>
<tr>
<td>C. albicans–CO₂</td>
<td></td>
<td>12</td>
<td>8</td>
<td>7</td>
</tr>
</tbody>
</table>

*pH values of three different concentrations of Cupral in distilled water, *pH values of Cupral diluted in the growth media or in the presence of microbial cells and incubated for 48 h in presence or absence of CO₂.
13 mm was observed at the highest Cupral concentration (12.5%), while gentamicin returned a halo of 16 mm. No effects were observed with Cupral 1.25 and 0.125%.

Finally, the antimicrobial effect of Cupral was assessed by the evaluation of microbial cell duplication time (DT), operationally working during the exponential growth phase (within 90–120 min). Figure 4 shows the DT values, expressed in minutes, of microorganisms exposed or not to Cupral. Our results show that Staph. aureus and C. albicans exposed to Cupral 12.5 and 1.25% failed to complete their cell replication cycle; under these same experimental conditions, P. aeruginosa DT was approximately 11,000 min. Lastly, the DT values of microbial cultures exposed to Cupral 0.125% returned values comparable to those obtained in their respective controls.

**Effect of Cupral on P. aeruginosa biofilm formation**

We investigated the ability of Cupral to interfere with biofilm formation through confocal microscopy, exploiting the fluorescent property of Pseudomonas spp. Figure 5 (left panel) shows a horizontal view of a control sample (24 h-old biofilm), characterized of cellular aggregates (green fluorescence areas) reaching a medium thickness of 50±11 µm, as established by multiple acquisitions. Lateral views indicate multicellular layers abundantly
Fig. 2  Cupral effects on microbial growth in the absence of CO₂.
Growth curves of Staph. aureus, P. aeruginosa and C. albicans were performed in the absence (diluent •) or presence of Cupral at 12.5% (■), 1.25% (▲) and 0.125% (×). Microbial cultures were incubated for 0, 3, 6, 24 and 48 h in the absence of CO₂. Then, the ODs were assessed and the values of ΔOD were calculated as difference between the OD of samples and their respective controls, ±SEM (left panels). In parallel groups, microbial cultures were incubated for 0, 24 and 48 h in the absence of CO₂; then, the CFU/mL were evaluated (right panels). The depicted values represent the average of 6 replicates of 2 independent experiments. T: time (h). *p ≤ 0.05, **p ≤ 0.005, ***p ≤ 0.001, ****p ≤ 0.0001.

distributed along the Z axis. Also, dispersed areas likely consisting of nutrient and water transport channels were detectable (dark areas). Figure 5 (right panel) is representative of P. aeruginosa bacteria exposed to Cupral 1.25% for 24 h; under this condition, only few scattered cells were evident with no tridimensional structure or measurable biofilm thickness.

Pyoverdine production by P. aeruginosa biofilm exposed or not to Cupral
To further investigate Cupral effects on biofilm, we assessed the release of pyoverdine, an important pigment for in vivo iron gathering and virulence expression in P. aeruginosa and thus promotes the formation of biofilm. As shown in Table 2, Cupral significantly impaired the pyoverdine production at 12.5 and 1.25% concentrations. In contrast, the pyoverdine levels in 0.125% Cupral solution were similar to control sample (biofilm cells); even in planktonic cells the pyoverdine production was very low.

Effect of Cupral on biofilm persistence
We investigated the effect of Cupral on pre-formed biofilm, using the three microbial strains; quantification of total (untreated controls) and residual (following Cupral treatment) biomass was evaluated, by CV assay
Antibacterial activity of Cupral was assessed by disk diffusion assay. a) Staph. aureus cells (1×10^5 cells) were inoculated on plates of Muller-Hinton agar. Neutral filter paper disks were placed onto the plates surfaces and embedded with Cupral (10 µL/disk: 12.5, 1.25 and 0.125%) or gentamicin, as positive control (10 µg/10 µL/disk). The plate was examined after 24 h of incubation at 37°C in absence of CO_2; the disk diffusion haloes were measured and the diameters (mm) reported in lower panel (b).

Inhibitory effects of Cupral on cell growth was calculated as DT of exponentially growing microbial cells exposed or not to Cupral and tested by CFU assay. The DT expressed in minutes were calculated using the formula described in materials and methods. N.A.: no assessable.

(Fig. 6) and optical microscope morphological analysis (Fig. 7). Briefly, microbial cells were incubated for 24 h in 96 well plates in CO_2 to allow biofilm formation; then, medium or Cupral was added and a further 24 h incubation performed. As shown in Fig. 6, Cupral significantly reduced microbial biomass; the effect was concentration-dependent and similar trends were observed in all the 3 strains tested. In particular, the percentages of biomass reduction ranged from 47 to 94 % for Staph. aureus, from 28 to 95 % for P. aeruginosa and...
Fig. 5  Confocal microscopy of *P. aeruginosa* exposed or not to Cupral. *P. aeruginosa* biofilm treated or not with Cupral was observed through confocal microscope, at 10× magnification, exploiting the auto-fluorescent property of *Pseudomonas* spp. The main images illustrate two representative horizontal sections (X and Y axes) of the biofilms, while the thin images (laterally and below each main image) indicate biofilms sections observed along the Z axis. The left panel refers to 24 h control biofilm and the right panel to *P. aeruginosa* exposed to Cupral 1.25% for 24 h.

### Table 2  Pyoverdine production by *P. aeruginosa* biofilm treated or not with Cupral

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Levels of pyoverdine</th>
<th>p values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated biofilm (diluent)</td>
<td>631,475±4152</td>
<td>—</td>
</tr>
<tr>
<td>Cupral 12.5%</td>
<td>202,586±7446</td>
<td>≤0.00001</td>
</tr>
<tr>
<td>Cupral 1.25%</td>
<td>547,174±18389</td>
<td>0.06</td>
</tr>
<tr>
<td>Cupral 0.125%</td>
<td>764,866±1850</td>
<td>ns</td>
</tr>
<tr>
<td>Untreated planktonic cells (diluent)</td>
<td>188,963±7211</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Pyoverdine production (RFU) in *P. aeruginosa* untreated planktonic or biofilm-associated cells exposed for 24 h, to three different concentrations of Cupral. Statistical analysis was performed by comparing the levels of pyoverdine produced by Cupral-treated *vs.* untreated biofilm cells (diluent).

Untreated biofilm *vs.* untreated planktonic cells were also statistically evaluated; ns: non-significance.

from 27 to 75% for *C. albicans*. Moreover, Fig. 7 shows representative microscopy images of 24 h-old biofilm of *Staph. aureus*, *P. aeruginosa* and *C. albicans*, in the presence of CO₂, exposed or not to Cupral 1.25% for additional 24 h. As expected, control microbial biofilms (left panels), showed abundant sessile structure; conversely, in parallel samples exposed to Cupral 1.25%, a thin layer of damaged microbial cells was detectable with scant and heterogeneous debris, probably Cupral-derived aggregates (right panels, arrows).

**Effect of Cupral on alive/dead cells embedded in microbial biofilm**

Finally, we investigated the impact of Cupral on viability of preformed microbial biofilms (Fig. 8). In detail, 24 h-old microbial biofilms were exposed to Cupral 1.25% for additional 6 (Figs. 8a and b) or 24 h (Figs. 8c and d) and then stained with CFDA and PI, to discriminate living from dead cells directly inside biofilms. Cupral treatment reduced the number of alive bacterial cells embedded into biofilms, in all the cases, although reaching significant differences only in *Staph. aureus* at 6 (Fig. 8a) and in *C. albicans* at 24 h (Fig. 8c). Accordingly, when expressing the data as live/dead percentage, we found that the percent of dead cells was consistently higher in Cupral-treated samples than in control groups, at both time points, both in *Staph. aureus* and *C. albicans* biofilms (Figs. 8b and d); as opposed, *P. aeruginosa* biofilm showed higher dead cell percent in the diluent samples than in Cupral-treated samples, at both times (Figs. 8b and d).
Fig. 6  Cupral effects on biofilm persistence. The effects of Cupral were assessed on pre-formed biofilms by *Staph. aureus*, *P. aeruginosa* and *C. albicans* by CV colorimetric analysis. Two wavelengths were used: 570 nm for microbial biofilms and 540 nm for fungal biofilm. The biomass reduction in Cupral-treated groups was expressed as % of residual biofilm compared to controls (100%). The values represent the average of 6 replicates of 2 independent experiments ±SEM. *p≤0.05, **p≤0.005, ***p≤0.001, ****p≤0.0001.

Fig. 7  Morphology of preformed biofilm treated or not with Cupral.

*Staph. aureus, P. aeruginosa* and *C. albicans* were allowed to produce biofilm by incubation 24 h on coverslips in the presence of CO₂; then biofilms were exposed or not to Cupral 1.25% for additional 24 h. Morphology was evaluated by optical microscope. The left panels show representative images of 48 h microbial biofilms in medium. The right panels illustrate biofilms exposed to Cupral 1.25%. The bars in Fig. indicate 50 µm. The arrows indicate aggregates/debris.
Fig. 8 Cupral effects on biofilm vitality.
Left panels: Relative Fluorescence Units (RFU) mean of live (dark grey) and dead (light grey) cells was determined in Staph. aureus, P. aeruginosa and C. albicans biofilms (24 h), treated or not with Cupral 1.25% for additional 6 h (a) and 24 h (c). Right panels: percent of live and dead cells in each group at 6 h (b) and 24 h (d). The data shown are representative of two experiments with three replicates. The dotted lines refer to the dead cells, while the continuous lines refer to the live cells. \*p \leq 0.05, \**p \leq 0.01.

DISCUSSION
In the present study, the antimicrobial capacity of Cupral has been assessed using microorganisms, such as Staph. aureus (Gram+), P. aeruginosa (Gram−), and C. albicans (fungus), known to commonly colonize human oral cavity, where they produce biofilm onto biotic and abiotic surfaces. Moreover, such microorganisms are considered among the most resistant species detectable in infected root canals, thus being often associated with endodontic treatment failures.

The formation and development of biofilm is a process that begins with adhesion of microorganisms as planktonic forms, followed by growth, extracellular polymeric matrix production, detachment and re-adhesion, in a dynamic and continuous reorganization that leads to the formation of a sessile structure. Of clinical importance is the fact that, once produced, biofilm is extremely resistant to common disinfectants, antimicrobial drugs and host immune defenses. In particular, endodontic failures often occur when permanent sterility cannot be achieved inside treated root canals especially if biofilm has been produced. Accordingly, a crucial aim of root irrigation and disinfection with antimicrobial products is the elimination of microbial cells, even if organized as biofilm, particularly in intra-canals surfaces not easily reached by endodontic instruments. Sodium hypochlorite (NaOCl) is a frequently used as irrigating solution in endodontics because of its ability to dissolve necrotic tissues as well as its potent antimicrobial action; yet, caustic and toxic effects to vital tissues are often noted. Other compounds, such as chlorhexidine (CHX) and cetrimide, are less effective than NaOCl in eradicating for instance Enterococcus faecalis biofilm; nevertheless, CHX has the ability to inhibit bacterial adhesion to dentin. Furthermore, the use of calcium hydroxide as an intracanal medication has been associated with periradicular healing and high antibacterial efficacy, this basic formulation in clinical practice can be associated with other antimicrobial molecules such as iodoform, propolis, royal jelly or copper hydroxide, as in Cupral composition. Cupral is a suspension based on copper-calcium-hydroxide, with bactericidal effects due to alkaline proteolysis, as reported by Knappwost; on these bases, it is expected that, in vivo, when entering into contact with irrorated tissues, it reacts with buffer solution of carbonic acid/hydrogen carbonate of the
blood and forms a calcium carbonate mineral membrane that increases overtime in thickness to the borderline of blood-supplied tissues. In turn, this membrane prevents the entry of OH\(^-\) ions into the tissues, thus neutralizing the acid environment of the inflamed site providing analgesic effects\(^{27}\). Furthermore, it is likely that calcium hydroxide, present in Cupral paste, reacts with copper sulphate in alkaline environment to form copper hydroxide and possibly depending on pH variation, the reaction and dissociation between calcium hydroxycuprate and copper hydroxide may occur\(^{27}\). Our present study provides the first evidence on the in vitro efficacy of Cupral on growth of planktonic microorganisms and on their ability to produce and persist as biofilm. In particular, by spectrophotometric and CFU assay, we demonstrate the antimicrobial effects of Cupral against bacterial and fungal planktonic cells. Interestingly, the efficacy of Cupral has been demonstrated in the presence and absence of CO\(_2\). The rational for including CO\(_2\) in our study derives from the fact that such a weak dioxide acid is expected to reduce the pH, by reacting with copper and calcium hydroxide present in Cupral. Thus, we assume that, because of such acidic property, CO\(_2\) tends to mimic the in vivo environment where microorganisms grow releasing acidic products; under such conditions, Cupral has been found to exert its antimicrobial activity.

We demonstrate that Cupral drastically affects microbial cells interfering with their cell cycle in a dose-dependent manner; indeed, the time of microbial cell replication drastically augments, irrespective of the microorganisms investigated, particularly, Staph. aureus and C. albicans exposed to Cupral 12.5 and 1.25% become unable to complete their cell-cycle, while Paeruginosa appears somehow less affected. These results are in line with what observed in terms of microbial cell growth and biofilm formation, namely both CFU and OD values are significantly reduced in microorganisms treated with Cupral with respect to their relative controls, again, the observed trends are dose-dependent and have been detected in the presence or absence of CO\(_2\), thought to a different extent. CO\(_2\) partially reduces Cupral efficacy; indeed, when used at 1.25%; the CFU and OD levels of Cupral-treated C. albicans are higher in the presence than in the absence of CO\(_2\). Thus, under acidic conditions closely mimicking the in vivo situation (addition of CO\(_2\)), Cupral happens to be not as effectiveness; yet, growth of microbial cells exposed to 10 times diluted Cupral (1.25%) still remains much below the control levels. The antimicrobial activity of Cupral has also been demonstrated by the disk diffusion test, where the inhibition halo of Staph. aureus exposed to Cupral 12.5% is very close to that obtained with gentamicin (15 vs. 16 mm), used as a positive control. We are aware that the disk diffusion may not be the most appropriate assay to assess Cupral, that, as a gelatinous formulation, diffuses with difficulty and in an uneven manner within constituted MH agar meshes (culture medium used in the assay); yet, the row data produced strengthen the efficacy of Cupral as antimicrobial compound.

By a previously established model\(^{41}\), here we provide initial evidence on the efficacy of Cupral against preformed microbial biofilms. A marked inhibitory effect has been observed with respect to each of the three microorganisms tested, as shown by the significant and dose-dependent reduction of microbial biomass. It should be noted that Staph. aureus biofilm is still affected at the lowest concentration of Cupral (0.125%), when pH is close to neutrality. On this basis, we can speculate that Cupral acts not only via its high alkalinity, but also because of the presence of elements such copper, known to exert antimicrobial activity\(^{42}\). In fact, copper is an essential nutrient for both human and microbial cells, while, at high doses, it can exert toxic effects. The exact mechanisms of copper action is not clear, yet; many hypotheses have been formulated, including a) loss of potassium or glutamate through the outer cell membrane, b) interference in the osmotic equilibrium, c) binding to proteins that do not require copper ions, d) oxidative stress induction with hydrogen peroxide generation\(^{42,43}\). It is worth noting that the Cupral effective dose of 1.25% contains approximately 350 µg/mL of copper ions, a value very close to the MIC previously established for copper against Staph. aureus\(^{44}\); to our opinion, this strengthens the hypothesized antimicrobial role of such ion in our model.

Old data\(^{27}\) ascribe to Cupral some cytotoxicity in vitro (undiluted paste onto murine 3T3 fibroblasts, for 24 h); yet, no clinical evidence exists on its in vivo toxic or side effects. An obvious advantage of using our in vitro system is the possibility of easily testing serial dilutions. The demonstration that 10 and 100 fold diluted Cupral still retains relevant antimicrobial effects opens to novel formulations, that optimizing the dosage of the active principles, may implement clinical treatment.

Confocal microscopy of a 24 h-old P. aeruginosa biofilm shows that Cupral 1.25% prevents biofilm formation, as demonstrated by the absence of a three-dimensional structure, while cellular aggregates and water channels are expected and easily detectable in control samples. Also, optical microscopy of preformed biofilm of Staph. aureus, P. aeruginosa and C. albicans, treated with Cupral 1.25% for additional 24 h, points out major differences in morphology between treated and untreated biofilm, with lack of a three-dimensional architecture and little residual cells in Cupral-treated samples. In line with these results, further data, obtained by a “live/dead cell” staining, reveal that Cupral 1.25% reduces the number of alive cells embedded in biofilms, both at 6 and 24 h of treatment, no matter whether considering bacterial or fungal biofilms. In parallel, the number of dead cells is higher in Cupral-treated samples as compared to their respective control groups, both in Staph. aureus and C. albicans; on the contrary, P. aeruginosa biofilm treated with Cupral has less dead cells as compared to its control. This last result can be interpreted considering the contribution of extracellular DNA (eDNA). Notoriously, eDNA is abundantly accumulated during P. aeruginosa biofilm formation by lysis of a subpopulation of bacteria\(^{23,24}\).
by mechanisms dependent on acyl homoserine lactone (autoinducer 1) and quinolone signalling during quorum sensing attainment\textsuperscript{40}. Thus, eDNA may initially facilitate bacterial adhesion and cell-to-cell aggregation; then, as an important component of biofilm matrix, eDNA enhances stability of the sessile community and strengthens its resistance to antibiotics or detergents\textsuperscript{40}. We can envisage that the increased PI RFU observed in \textit{P. aeruginosa} biofilm may be due to eDNA accumulation, in turn possibly protecting cells from Cupral’s action. Another quorum sensing-mediated phenomenon, in \textit{P. aeruginosa} biofilm, is pyoverdine production. Our study shows that pyoverdine levels in untreated planktonic cells result very low in comparison with those observed in biofilm cells; interestingly, the latter are inhibited in a dose-dependent fashion by Cupral. Taken together, these findings add further evidence on the ability of Cupral to prevent \textit{Pseudomonas} production and persistence biofilm.

CONCLUSION

Currently, Cupral is an endodontic paste used in root treatment and, more recently, also in gingivitis and indirect covering of dental pulp, with the aim of obtaining a clinical improvement. Here, we provide in vitro novel insights on the antimicrobial efficacy of Cupral, demonstrating its ability to affect \textit{Staph. aureus}, \textit{P. aeruginosa} and \textit{C. albicans} as well; growth of planktonic cells is impaired, biofilm formation is prevented and preformed biofilm is damaged. Thus, our results suggest that Cupral, a promising clinically used compound, may be a good candidate for treatment of oral infections, including biofilm-associated ones, irrespective of the fact that a Gram+, Gram− or fungal cell maybe the causative agent.

ACKNOWLEDGMENTS

The authors would like to acknowledge the staff members of the Centro Grandi Strumenti, University of Modena and Reggio Emilia, for the precious support in microscopy analysis.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

REFERENCES