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In vitro bactericidal activity of a carbohydrate polymer with zinc oxide for the treatment of chronic wounds

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Abstract

Introduction. Biological adhesives and effective topical therapeutic agents that improve wound healing are urgently required for the treatment of chronic ulcers. A biodegradable adhesive based on a carbohydrate polymer with zinc oxide (CPZO) was shown to possess anti-inflammatory activity and enhance wound healing, but its bactericidal activity was unknown.

Aim. To investigate the bactericidal activity of CPZO against bacteria commonly present as infectious agents in chronic wounds.

Methodology. We examined the bactericidal activity of CPZO against three biofilm-producing bacteria (*Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*) through three strategies: bacterial suspension, biofilm disruption and *in vitro* wound biofilm model.

Results. In suspension cultures, CPZO had direct, potent bactericidal action against *S. aureus* within 24 h, whereas *E. coli* took 7 days to be eliminated. By contrast, *P. aeruginosa* survived up to 14 days with CPZO. CPZO had biofilm disruption activity against clinical isolates of *S. aureus* in the anti-biofilm test. Finally, in the *in vitro* wound biofilm model, CPZO dramatically reduced the bacterial viability of *S. aureus* and *P. aeruginosa*.

Conclusions. Together with its previously shown anti-inflammatory properties, the bactericidal activity of CPZO gives it the potential to be a first-line therapeutic option for chronic various ulcers and, possibly, other chronic ulcers, preventing or controlling microbial infections, and leading to the healing of such complicated chronic ulcers.

INTRODUCTION

Chronic varicose venous ulcers (CVUs), diabetic ulcers (DUs) and pressure ulcers are chronic wounds characterized by a prolonged or excessive inflammatory phase, persistent infections and the inability of the dermis to respond to reparative stimuli [1]. CVUs may affect ~1% of people in their lifetimes with an annual cost of USD $2.5–5 billion [2]. Successful healing of a wound by primary intention requires a balanced combination of growth factors, cytokines and chemokines, which participate in a complex process involving different types of cells and signalling pathways [3]. As wound infections lead to poor healing, their successful treatment depends on proper antimicrobial control. Although wound debridement somehow decreases bacterial growth in chronic wounds [4], treatment usually requires local antimicrobial preparations, topical antiseptics and systemic antibiotics [5]. As yet, there is no conclusive evidence of the effectiveness or superiority of one agent over another to control infection and reduce the time taken for healing [6]. Biological adhesives have been shown to improve wound healing and prevent anastomotic leaks. The most commonly used adhesives are composed of fibrin-based products and/or collagen patches, which have several disadvantages, such as hypersensitivity reactions, promotion of infection and a high cost [7]. Pebisut is the commercial name in Mexico of a biodegradable adhesive carbohydrate polymer with ZnO (CPZO), which enhances healing and possesses anti-inflammatory activity in patients with CVUs [8]. Although preliminary clinical data suggest that this adhesive mixture decreases infection rates in CVUs, its bactericidal activity remains unknown to date [9–15]. Therefore, we examined the bactericidal activity of CPZO

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Abbreviations: c.f.u., coloby-forming units; CPZO, carbohydrate polymer with zinc oxide; CVU, chronic varicose venous ulcer; DU, diabetic ulcer; LB, Luria broth; TSA, Trypticase sodium agar; TSB, Trypticase sodium broth.

Table 1. Bacterial strains used in this study

Strain	Characteristics	Source
S. aureus subsp. *aureus* ATCC 25923	Slime producing, *icaA+/icaD+*	Clinical isolate
S. aureus subsp. *aureus* ATCC 43300	Slime producing, *icaA+/icaD+, mecA+, MRSA SCCmec: type II*	Clinical isolate
E. coli ATCC 25922	Biofilm producing, csgABC operon	Clinical isolate
P. aeruginosa ATCC 27853	Biofilm producing, *pilQPONM*	Blood culture
P. aeruginosa PAO1	Biofilm producing, *AcrB/Mex*-type RND	Wound

against Gram-positive and Gram-negative bacteria, all of which frequently complicate chronic wounds.

METHODS

Cpzo

CPZO – a medical device II – is a biodegradable, adhesive-containing zinc oxide combined with a polymer of naturally occurring carbohydrates. CPZO is commercially available as Pebisut. Due to its thixotropic properties, its use requires external mechanical or thermal energy for 25–40 s with a clean instrument.

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1 and were grown in trypticase soy broth (TSB) (BD, Franklin Lakes, NJ, USA) at 37 °C. When necessary, media were supplemented with CPZO 50% (diluted in isotonic saline solution) in 2× TSB and aliquoted. Bacterial stock cultures were maintained at −80 °C in TSB with 30% (vol/vol) glycerol for future experiments.

Bactericidal activity of CPZO in bacterial suspensions

The initial evaluation of the bactericidal activity of CPZO against the bacteria under study was conducted *in vitro*. Stocks of ATCC 25923, ATCC 25922 and ATCC 27853 strains were streaked on liquid Luria broth (LB) agar and were incubated overnight at 37 °C. One colony of each strain was inoculated in 10 ml of LB broth and cultured under agitation overnight at 200 r.p.m. and 37 °C, after which cultures were diluted in cold isotonic saline solution and adjusted to 10^7 colony-forming units (c.f.u.) using a McFarland nephelometer. In order to confirm the bacterial concentration, appropriate bacterial culture dilutions were spread onto LB agar plates and cultured in triplicate for an additional 24 h for c.f.u. ml^{-1} counting. Previously adjusted bacterial suspensions were inoculated to tubes of 10 ml 50/50% CPZO/normal saline and cultured at 37 °C for 14 days. Culture viability was evaluated by enumerating c.f.u. by means of the calibrated loop technique on Tripticase sodum agar (TSA). Each assay was performed in triplicate.

Anti-biofilm activity of CPZO against clinical isolates of *S. aureus*

In order to identify potential biofilm-producing strains, prior to examine the anti-biofilm activity of CPZO, 17 clinical isolates of *S. aureus* were subjected to phenotypic (biofilm production capacity) and genetic (detection by PCR of *icaA* and *icaD* gene) characterization. For the anti-biofilm activity, a starting inoculum of 10^5 c.f.u. in TSB with 1% glucose was distributed in 96-well flat-bottomed microtitre plates covered with a lid (Nunc Immuno-TSP, Thermo Scientific) and incubated statically for 24 h at 37 °C to allow mature biofilm formation in the wells. Plates were then rinsed thrice in 1× sterile phosphate-buffered saline (PBS) to remove planktonic bacteria, placed in a new plate containing 100 µl 50% CPZO and cultured for 24 h at 37 °C. Cell viability was evaluated in a new plate containing 200 µl fresh TSB and sonicated at 45–60 Hz for 10 min. colony-forming units (c.f.u.) ml^{-1} were obtained daily for 3 days using the Miles and Misra method [16, 17].

In vitro wound biofilm model for assessing the effectiveness of CPZO

In vitro wound biofilm model experiments were performed as described by Hammond *et al.* [18], with minor changes. Briefly, biofilm-producing bacterial strains (*Pseudomonas aeruginosa* PAO1 (PAO1) and *Staphylococcus aureus* subs. *aureus* ATCC 43300 (*Staph*) were grown overnight in LB broth at 37 °C in a shaker. One ml from each culture was pelleted, washed and resuspended in 1 ml isotonic saline solution, and serially diluted to adjust to ~10^3 c.f.u. 10 μl^{-1}. To examine the effectiveness of CPZO, gauzes with or without CPZO (400 mg) were placed over sets of two disks each containing the mature biofilms of PAO1 or *Staph*. For each assay, two sterile nitrocellulose 0.22 µm disks (Millipore, Bedford, MA, USA) were placed on the surface of LB agar plates and aliquots of 10 µl (containing ~10^3 c.f.u.) per disk were added to disks containing treatment (400 mg CPZO) or control (saline), which were cultured at 37 °C for 24, 48 and 72 h. Prior to control or CPZO treatments, inoculated disks were preincubated for 24 h at 37 °C to allow the formation of mature biofilms, immediately after which plates were incubated at 37 °C for 24, 48 and 72 h.

Detection of surviving bacteria after CPZO treatment

Following the removal of the gauzes (after 24, 48 or 72 h of incubation), disks were transferred onto 1.5 ml microtubes containing 1 ml PBS. Tubes were vortexed vigorously in order to disrupt the biofilms mechanically and detach all bacteria from the disks. Bacteria were pelleted and resuspended in 100 µl PBS, after which they were serially diluted. One hundred microlitres of each dilution were spread on triplicate LB agar plates and incubated at 37 °C for 24 h. The colony count for each length of microfilm culture was registered and logarithmic reduction values (LRVs) were calculated by means of the following equation: $L=10_{10}(A) - \log_{10}(B)$, where L is the LRV, A is the number of viable bacteria without treatment and B the number of viable bacteria after treatment. Additionally, the percentage reduction values (PRVs) were calculated by means of the following equation: PRV= $[(1–10^{-L}) (100)]$ [16].

Statistical analysis

Graphs were drawn with GraphPad software version 8.0, and statistics were obtained from the same software using the 'Multiple *t* tests of two-way ANOVA with RM by column function' with correction for multiple comparisons using the Bonferroni–Dunn method and a value of $P<0.05$ as statistically significant.

RESULTS

CPZO inhibits bacterial growth in suspension

To initially examine the antimicrobial activity of CPZO, three different bacteria at concentrations of 10^5, 10^6 and 10^7 c.f.u. were cultured in suspension in the presence of 50% CPZO. We found that CPZO inhibited growth of *S. aureus* subsp. *aureus* ATCC 25923 as early as at 24 h of exposure and up to day 14. Gram-negative bacilli were slightly more resistant to CPZO, as reduction of the number of colonies started at day three, and total absence of viable *E. coli* ATCC 25922 required 1 week of exposure. In the case of *P. aeruginosa* ATCC 27853, the number of colonies remained unchanged, even after 2 weeks (Fig. 1a). This phenomenon was similar at all the bacterial concentrations tested.

Anti-biofilm activity of CPZO against clinical isolates of biofilm-producing *S. aureus* strains

As an initial approach to examine the effects of CPZO on biofilm production, we evaluated the effect of the CPZO at 24, 48 and 72 h against the planktonic cells of 17 clinical isolates of *S. aureus*, all of them positive for at least 1 of the *ica* genes (not shown). Fig. 2a shows that at 24 h CPZO had anti-biofilm activity, as 40% (8/17) of *S. aureus* clinical isolates has a total absence of living bacteria (0 c.f.u. ml^{-1}) and 60% (9/17) had a 2–3 log reduction. Moreover, at 48 h, 100% (17/17) of the isolates had a complete absence of biofilm (Fig. 2b), with identical results at 72 h. This indicates that CPZO possesses bactericidal activity against *S. aureus* from different clinical isolates. Although the reduction of

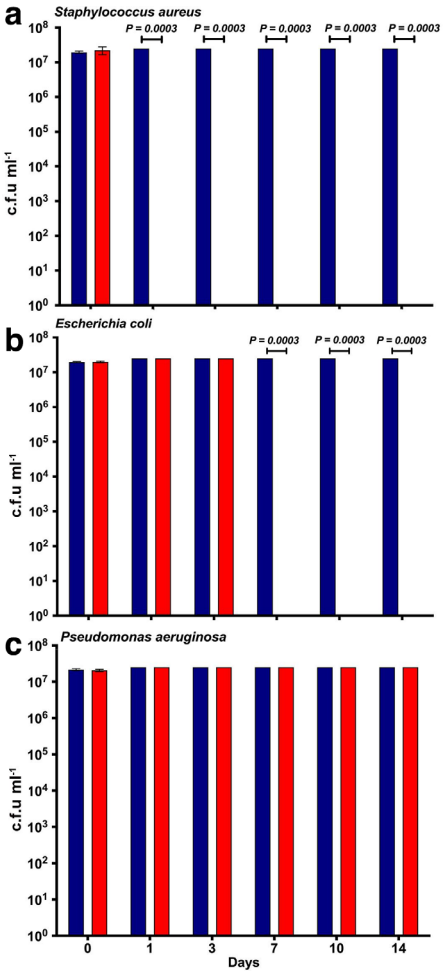

Fig. 1. Bactericidal activity of 50% CZPO against three bacteria cultured in suspension for 14 days. (a) *S. aureus* subsp. *aureus* ATCC 25923, (b) *E. coli* ATCC 25922 and (c) *P. aeruginosa* ATCC 27853.

biofilm by CPZO was only statistically significant in a few of the isolates at 24 h, and none at 48 h, this was due to the high variability of the numbers obtained in the control cultures, but the relevance of the inhibition by CPZO was evident in the marked reduction of bacterial growth in its presence and its total inhibition at 48 h.

CPZO reduces bacterial viability in an *in vitro* wound biofilm model

For the next experiments, classical microbiological culture of biofilms was achieved with and without CPZO treatment in an *in vitro* wound biofilm model in agar plates. A significant difference between non-treated and treated biofilms was observed for both *Staph* and PAO1 at all times (24, 48 and 72 h) (Fig. 3). In the case of *Staph*, it was significantly reduced after 24 h ($P<0.01$) and completely eliminated after 72 h CPZO contact *in vitro*, as determined by the wound biofilm model, with log reduction values of 2.91, 4.43 and 7.29 LRV, respectively; and reductions (PRV values) of 99.87 (24 h), 99.99 (48 h) and 100 % (72 h) of bacterial

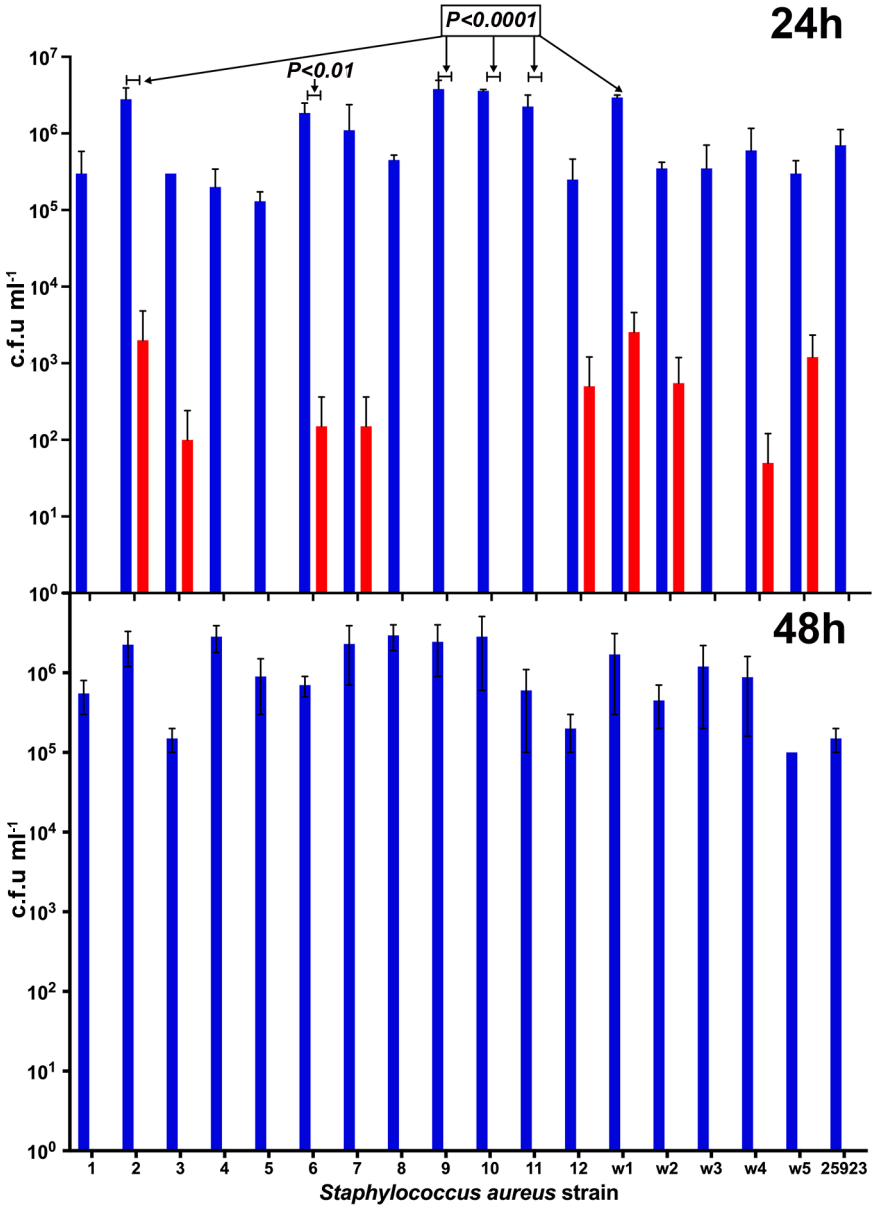

Fig. 2. Antibiofilm activity of CPZO against different clinical isolates of *S. aureus* at 24 (a) and 48 (b) h. Comparison between CPZO treatment (red) and untreated control (blue). For isolates with no red bars no bacterial growth (c.f.u.) was recovered in the CPZO-treated cultures.

viability (Fig. 3a). On the other hand, for PAO1, the LRVs were 2.43, 2.76 and 5.96 at 24 (P<0.01), 48 and 72 h, respectively (Fig. 3b). After 72 h of CPZO exposure, there were still some viable bacteria remaining (PRV value of 99.99 %).

DISCUSSION

The present study was conducted to examine whether CPZO, which is employed as an agent to improve the healing of chronic wounds, possesses anti-bacterial properties under different conditions. The main findings were that CPZO is able to suppress the growth of *S. aureus* (wild-type and clinical isolates), *E. coli* and *P. aeruginosa* in at least three *in vitro* models. The finding that a non-antibiotic, locally applied mixture has *in vitro* bactericidal activity against bacterial pathogens frequently complicating chronic wounds could be of clinical relevance for the treatment of these conditions and of help to prevent the excessive and indiscriminate use of antibiotics.

Antimicrobial resistance is a major burden of today's world. Bacteria such as *S. aureus, Enterococcus* spp., *P. aeruginosa, Acinetobacter* spp. and *Neisseria gonorrhoeae* lead the list of a rising number of multidrug-resistant pathogens that constitute

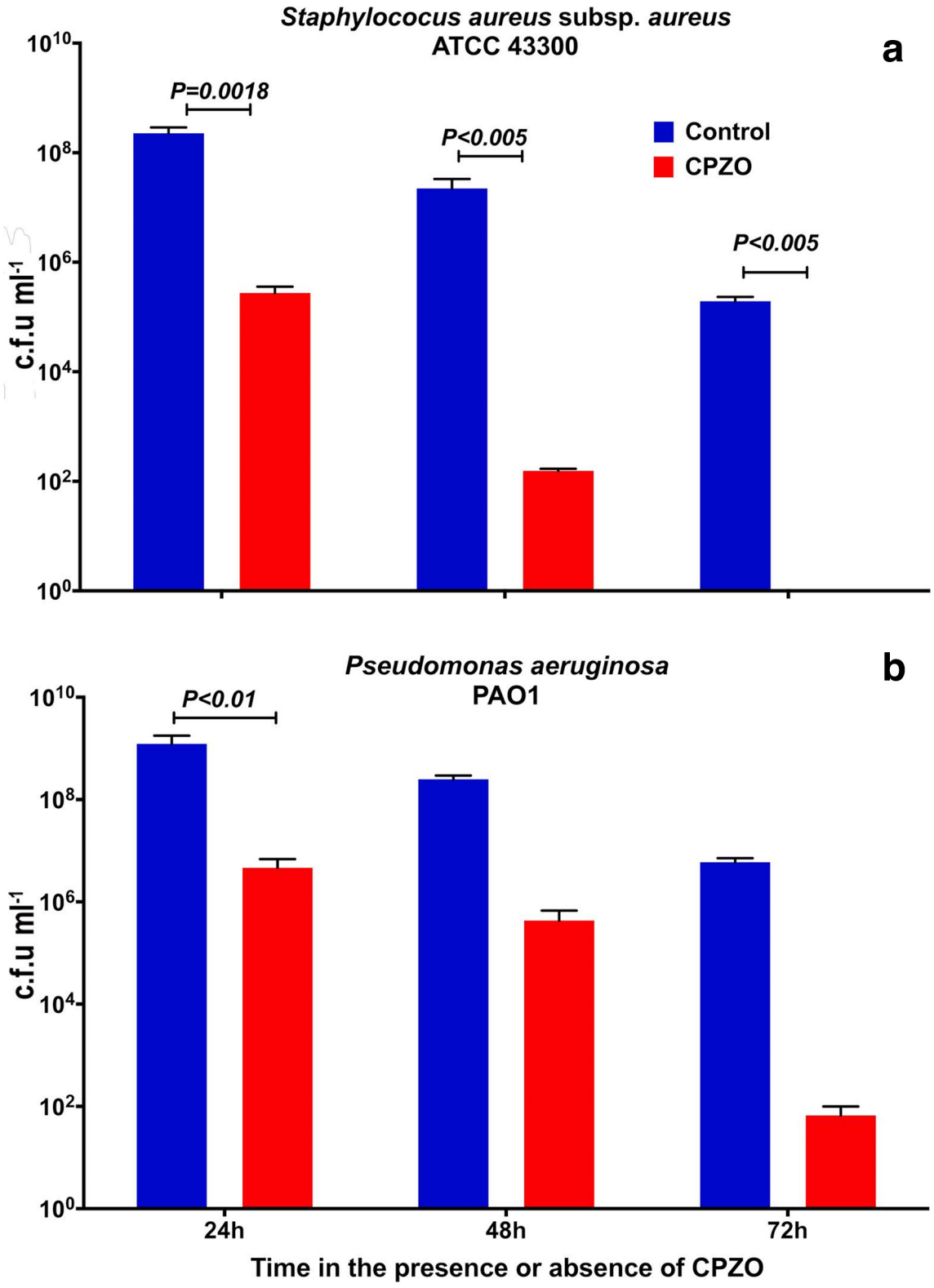

Fig. 3. Disruption of mature biofilms formed by (a) *S. aureus* subsp. *aureus* ATCC 43300 and *P. aeruginosa* PAO1 (b) in an *in vitro* wound model before and after CPZO-treatment at 24, 48 and 72 h. Numbers of c.f.u. per biofilm were determined by plating serial dilutions on LB agar.

a major threat to our survival as a species [17, 19, 20]. These same bacteria, plus other Gram-positives (*Corynebacterium* spp., *Clostridium* spp.), non-fermenting Gram-negative bacilli and fungi, are commonly isolated from chronic wounds [21, 22]. Although the majority of wounds seen in medical practice heal by primary intention, when they become chronic ulcers, bacterial entry and colonization are unavoidable [23]. Moreover, ongoing inflammation contributes to the colonization of chronic wounds by infectious agents [12]. Treatments for these ulcers include local products to prevent and control inflammation and microbial growth, preferably avoiding antibiotics. Non-healing wounds that reach a critical state of colonization, with a bacterial bioburden, generally defined as $\geq 10^5$ c.f.u. g^{-1} tissue, have greatly increased tissue damage [24].

We have previously documented the anti-inflammatory activity of CPZO, which decreases the levels of inflammatory cytokines [9]. Moreover, our clinical observations that infected ulcers become clear after local CPZO application led us to hypothesize that it could possess bactericidal activity. Therefore, we examined the effects of CPZO on the growth and viability of bacteria frequently complicating chronic wounds, such as *S. aureus* and *P. aeruginosa* [25]. In the initial phase of our study we observed bactericidal activity of CPZO against *S. aureus* grown in suspension, but not against *E. coli* and *P. aeruginosa* even after 14 days in contact with CPZO. As the genetic features of these bacteria allow them to form mature biofilms on inert surfaces, such as plastic or polystyrene (Table 1), which more closely resemble actual infections, we next examined the ability of CPZO to inhibit biofilm formation by clinical strains of *S. aureus*. Although this dilution-based test is the gold standard to determine bacterial susceptibility to various compounds, this assay is not the most accurate to examine the bactericidal activity of CPZO due to its adhesive nature.

Therefore, in order to reproduce *in vitro* the conditions of chronic wounds (formation of mature biofilms) and hence to test the activity of CPZO under real life-resembling conditions, mature biofilms of *S. aureus* and *P. aeruginosa* were subjected to direct contact with 400 mg of CPZO and examined in an *in vitro* model of wound colonization. By these means, after 72 h in the presence of CPZO, the *S. aureus* biofilm was reduced by 100% (~8 logs) and the *P. aeruginosa* one was reduced by 99.99% (~6 logs). In a previous work, CPZO led to a reduction of ~5 logs at 24 h and complete anti-biofilm activity at 48 h post-incubation, similar results to those reported by Kiamco *et al.* [26].

Considering the possible mechanisms of action of CPZO, maltodextrin is a hyperosmotic agent with known anti-biofilm activity [27] that also promotes the proliferation of fibroblasts and granular and epithelial tissue to improve the healing process [28, 29]. On the other hand, ZnO nanoparticles cause a 20–60% biofilm reduction of Gram-positive bacteria [30]. ZnO interacts with the cell membrane, leading to its disruption by oxidative stress (OS) and increased permeability with osmotic cell death [31]. The reduced action against Gram-negative bacteria could be due to lower cell wall permeability that decreases intake by the cell [32]. The accumulation of Zn ions around the bacterial surface leads to cell death due to membrane damage [33–35]. Taken together, these data suggest that ZnO is lytic to bacterial cells. It would appear that the combination of a hyperosmotic antimicrobial agent with an ionic antimicrobial agent in CPZO increases the effectiveness of both compounds.

Another hyperosmotic agent that is often used to treat wounds is honey, which can cause a ~5 log reduction in bacterial load [29]. Similarly, 6M NaCl reduces *Enterococcus faecalis* and *P. aeruginosa* biofilms by ~6 log after 72 h exposure [36]. Silver-impregnated pads are another alternative to reduce *S. aureus* biofilm by 0–4 log. Unfortunately, silver ions are rapidly inactivated after release into the medium and are known to cause bacterial resistance [29]. Antibiotics in combination with hyperosmotic agents also have proven anti-biofilm activity. Thus, Falghoush *et al.* [27] found a ~5.5 log reduction with maltodextrin plus tobramycin and a ~5 log reduction with sucrose plus tobramycin against *Acinetobacter baumanii*. On the other hand, Kiamco *et al.* [26], obtained a 2 log reduction of *S. aureus* biofilm with a combination of maltodextrin and vancomycin. As already mentioned, it is now preferred that treatments are free of antibiotics to reduce the risk of resistance. The definite anti-biofilm activity of CPZO adds to its known anti-inflammatory and pro-healing properties [8–15].

Because CPZO it is not an antibiotic, it is unlikely that it will eventually induce resistance. As mentioned above, the bacteriological findings reported here confirm our clinical impressions over several years of use of CPZO to treat CVUs [9, 11], which follows the observation that after several days of CPZO application, wounds no longer have heavy secretions with improvement of the surrounding cellulitis [37]. As an additional advantage, it must be mentioned that after extensive clinical experience with chronic wounds, CPZO does not appear to have any toxic or unpleasant side effects.

Conclusions

Because its anti-inflammatory properties are capable of promoting the healing of complicated chronic ulcers and its antimicrobial action prevents and controls microbial colonization, we propose that CPZO should be considered as a first-line therapeutic option for CVUs and other chronic ulcers.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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