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ORIGINAL ARTICLE

In vitro microbicidal, anti-biofilm and cytotoxic effects of different commercial antiseptics

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Abstract

Topical antiseptics are widely used for wound treatment, with the goal of disrupting biofilm capacity. We analysed the effectiveness of a variety of antiseptics to inhibit various stages of biofilm formation and to remove biofilms in vitro as well as the agents' cytotoxic effects on fibroblasts. We found that the chlorine-releasing agents exhibited immediate anti-biofilm effects in the short term, with lesser cytotoxicity than agents prepared from more stable compounds, such as biguanide or modified diallyl disulfide-oxide, which, conversely, have better long-term effectiveness. Among the examined organisms, Gram-positive bacteria and *Candida albicans* were the most sensitive to the antiseptics, whereas *Pseudomonas aeruginosa* and *Acinetobacter baumannii* were relatively resistant to them. Formulations whose mechanisms of action involve the release of chemically active chlorine were more effective when administered in solution than the gel form, likely because of the stability of the active ingredients during or after preparation of the formula. Interestingly, hypochlorous acid and some superoxidation solutions were effective in preventing biofilm formation within a short time period and showed virtually no toxicity. Our study indicates that most antiseptics remain effective long enough to prevent biofilm formation; thus, even brief application of an antiseptic agent during initial wound treatment can lead to better wound management outcomes.

Introduction

Loss of skin integrity because of a lesion carries a risk of infection (1). Wounds always present some microbial contamination, the degree of which is controlled by the host and contaminating flora (2–5). Under conditions of uncontrolled biota growth or a favourable biological environment, microorganisms can reach unlimited numbers such that infection sets in (6). Moreover, a favourable environment creates a biologically stable form because of the various microorganisms that are present. These microorganisms, together with host detritus, exudates and numerous additional factors, sustain a stable colonisation in the wound, in most cases as a biofilm (5,7).

A biofilm is a community of microorganisms that grow and organise in an extracellular matrix (ECM) of proteins, DNA and polysaccharides derived from microbial metabolism (8). Biofilm formation is a complex process with three stages: adhesion, aggregation and maturation. In the first stage, during the first 4 hours of growth, microorganisms adhere to inert or live surfaces by electrostatic forces. Once adhesion has occurred, aggregation begins after 6–12 hours of inoculation. Microorganisms synthesise polysaccharides and other elements that comprise the ECM. Finally, after 12 hours, the microorganisms form the characteristic structure of a mature biofilm (9).

Key Messages

- effectiveness of an antiseptic depends on its ability to control, or much better, eliminate microorganisms in planktonic or biofilm stages, simultaneously with the lowest eukaryotic cell damage

- biochemically, antiseptic stable substances are highly toxic for microorganisms as well as for eukaryotic cells because of their permanence in the environment
- unstable molecules, such as halogens, can control early microorganism growth, with very limited eukaryotic damage
- nevertheless, biofilm impairment or its elimination becomes a higher challenge, where antiseptic treatment frequency and toxicity must be considered

A biofilm offers physical (10) and metabolic barriers (11) that make microorganisms in biofilms much more resistant to antimicrobials compared with microorganisms that grow independently or as plankton. Bacteriostatic, bactericidal and fungicidal strategies that are effective under simple conditions (i.e. planktonic cultures (12)) do not have the desired effect under routine clinical conditions (13). Despite the great variety of commercially available antiseptics and antibiotics (14), management of infected wounds still largely depends on the knowledge level of the treating clinician (2,3), the type and sensitivity of pathogens in the wound, the availability of substances or medications and the compliance of patients and caretakers.

The objective of any treatment for an infected wound is localised or systemic control of microbial colonisation to prevent or eradicate infection (15). Topically applied antiseptics are increasingly being used because they are effective almost immediately. Antiseptics can present various mechanisms of action, such as protein coagulation and precipitation, alteration of cell wall or membrane permeability and specific or generalised toxicity to the bacterial enzymatic systems. Through these mechanisms, the antiseptics kill (12,15–17) or inhibit the growth of saprophytic and pathogenic bacteria. However, antiseptics are also cytotoxic, which can be harmful to the granulation tissue (18,19). In this study, we analysed the bacteriotoxicity of different commercially available antiseptics (20) at different stages of biofilm formation as well as their cytotoxicity against human fibroblasts (21). Our goal was to understand which chemical groups and compounds are biologically safe and effective for microbial control.

Materials and methods

We tested the abilities of different commercial antiseptics to inhibit biofilm formation (microbial aggregation, adhesion and maturation) and to eliminate biofilm. We also tested their cytotoxicity against human fibroblasts, considering the abundance and key role of these cells in the healing process.

Microorganisms and cells

For our anti-biofilm tests, we used *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, *Enterococcus faecalis* and *Candida albicans*. All strains were obtained from isolates of clinical origin, identified using the VITEK® 2 system and characterised phenotypically as biofilm producers by crystal violet staining (data not shown). Cytotoxicity tests in eukaryotic cells were conducted with fibroblasts obtained from a healthy 50-year-old female patient who underwent abdominoplasty and consented to the use of the cells for research purposes.

Antiseptic and solution preparation

We tested the following antiseptic formulas: 0·057% sodium hypochlorite (NaClO; Anasept®, Anacapa Technologies, California), 0·008% modified diallyl disulfide-oxide (DADS-M; ACCUA Aseptic®, Cellpharma, Mexico City, Mexico), a superoxidation solution (SOS) containing 0·0008% NaClO, 0·0032% hypochlorous acid (HClO), 3% sodium lithium magnesium fluorosilicate (Microdacyn®, Morepharma, Mexico City, Mexico), 0·033% HClO (Vashe®, SteadMed Medical, Fort Worth, TX), 0·1% polyaminopropyl biguanide (biguanide; Prontosan®, B. Braun, Melsungen, Germany) and an electrolysed SOS of neutral pH with 0·002% active chlorine (Estericide®, Esteripharma, Mexico City, Mexico). All antiseptics were in liquid, pharmaceutical and gel forms, except for Vashe®, which is only available as a solution.

Before performing the microbial and fibroblast cytotoxicity tests, we prepared 1%, 5% and 10% solutions of each antiseptic (w/v for gels and v/v for liquids). As the positive control for antisepsis, microbial cultures were incubated in the presence of 10% phenol (Sigma-Aldrich, St. Louis, MO). All microbial tests were conducted in quintuplicate. All cell (eukaryotic) tests were carried out in quadruplicate. For all data, we used the average of two independent experiments. The standard deviation never exceeded 15% of each value.

In vitro inhibition of single-species biofilm formation

To measure the inhibition of biofilm formation, we used the method described by Dosler *et al.* (16) Briefly, each strain was statically cultured in brain–heart infusion broth (BHI) for 24 hours at 37°C. We prepared suspensions of each strain equivalent to the MacFarland 0.5 standard [$1{\cdot}5 \times 10^8$ colony-forming units (CFU)] in BHI-glucose (22). We distributed 100 µl of the suspensions in each well of a sterile 96-well flat-bottomed plate, together with 100 µl of each antiseptic at various concentrations. We incubated the plates at 37°C for different times to test the inhibition of each stage of biofilm formation: 2 and 4 hours for microbial adhesion, 6 and 12 hours for microbial aggregation and 24 and 48 hours for biofilm maturation. To establish the different stages for biofilm formation, we previously performed kinetics of growth for every microorganism (23) (data not shown). After the corresponding incubation time, we removed the medium from the plates and washed each well three times with sterile saline solution. Plates were dried for 2 hours at room temperature. We stained the biofilms or their residues for 20 minutes with 200 µl of 0·1% (w/v) crystal violet solution. We removed the stain, washed away the excess with saline solution and dried the plate for 2 hours. Finally, we solubilised the stain with 200 µl of 95% ethanol for 30 minutes. We measured the optical density (OD) of the solution at 540 nm on a spectrophotometer (xMArk™, Bio-Rad Laboratories, Inc., California). The OD obtained from a culture in the absence of

any antiseptic (negative control) was considered 100% adhesion, aggregation or maturation.

In vitro elimination of single-species biofilms

To measure biofilm elimination, the same procedure was performed as described for inhibition, except that we first incubated the microbial cultures statically in BHI for 24 hours at 37°C. Once the biofilm was formed, we gently removed the medium. We washed the wells three times with sterile saline solution to remove free bacteria and added 100 µl BHI-glucose and 100 µl of each antiseptic at various concentrations. We stained the biofilms or residues with crystal violet and measured the OD by spectrophotometry. We designated the OD of a culture in the absence of any antiseptic (negative control) as 100% biofilm.

Cytotoxicity study

We seeded $6{\cdot}25 \times 10^4$ cells/cm^2 of confluent cultures of fourth-passage human fibroblasts on a 96-well culture plate (2×10^4 cells/well). We cultivated cells in 200 µl of D-MEM, 10% foetal bovine serum and 2 mM glutamine for 24 hours in a humid environment at 37°C and 5% CO_2. We removed the supernatant and added 200 µl of culture medium containing 1%, 5% or 10% antiseptic (liquid/gel). We incubated the cultures for 6, 12, 24 and 48 hours and then assessed cell viability using the test medium of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction (24). We added 10 µl of a 5 µg/ml MTT solution to each well and incubated the cultures for 3 hours. We removed the supernatant and washed each well with saline solution at room temperature. We dissolved formazan precipitate with 200 µl dimethyl sulfoxide/isopropanol and measured the solution by colorimetry at 570 nm with an iMARK™ Microplate Absorbance Reader (Bio-Rad Laboratories, Inc.) We considered the OD of a culture in the absence of any antiseptic (negative control) to represent 100% viability.

Data analysis

In analysing the anti-biofilm and cytotoxicity effects, any antiseptic that produced at least a 50% reduction in the biofilm (ED50) or fibroblast population (LD50) compared with the negative control was considered to be effective or cytotoxic, respectively (25,26).

Results

Specificity and effectiveness of antiseptics depend on formulation and treatment time

Although the availability of antiseptics for wound care has grown considerably in the last 10 years, clinical staff continue to choose antiseptics based on company recommendations, limited testing or current availability. In this study, we evaluated different antiseptics used for wound cleaning and antisepsis, and they were classified by the chemical group of the active or primary ingredient. We tested these antiseptics using clinically isolated microorganisms that are often resistant to various antibiotics to observe the effects in a setting closer to the hospital reality (data not shown) (7). We created an effectiveness table based on the ED50 at different intervals and concentrations of each active ingredient to facilitate the appropriate choice of antiseptic formulation (Table 1).

Test results for biofilm inhibition at 2 hours showed that almost all of the antiseptics had a major effect, particularly for Gram-positive bacteria and *C. albicans,* but did not completely block the surface adhesion of microorganisms. Biguanide showed a general effect. Other antiseptics (e.g., NaClO) completely inhibited adhesion of *E. faecalis* and *C. albicans*, while allowing growth of Gram-negative bacteria. *P. aeruginosa* was only inhibited by biguanide and at high concentrations of DADS-M (Figure 1A). After 4 hours of treatment, the effect remained for Gram-positive bacteria, but there was clear recovery of most of the Gram-negative microbial colonies treated with some forms of NaClO and HClO. Inhibition of aggregation was only achieved for Gram-positive, *S. maltophilia* and *C. albicans* microorganisms and was primarily achieved by treatment with biguanide, DADS-M, SOS (Estericide) and HClO (Figure 1B).

After microbial adhesion, the next step in biofilm formation is cell aggregation, when the primary colonies are formed. In our tests, after 6 hours of antiseptic treatment, only biguanide effectively inhibited microbial aggregation. HClO only permitted aggregation of *P. aeruginosa* while inhibiting the other microorganisms. The remaining antiseptic formulae had limited effect, inhibiting aggregation of Gram-positive bacteria, *C. albicans* and *S. maltophilia* (Figure 1C). After 12 hours of treatment, aggregation was inhibited for some microbial cultures only. In particular, HClO, which was highly effective at 6 hours, began to lose its effectiveness for *K. neumoniae, S. maltophilia* and *C. albicans* after 12 hours. DADS-M and biguanide at high concentrations were effective at inhibiting aggregation at 12 hours (Figure 1D).

The last phase in biofilm formation is maturation. In the mature biofilm, the microbial cells are embedded in an ECM that they themselves have produced. Some cells exist in a quiescent state, which can make pharmaceutical control more difficult (7,27). Certain microorganisms that were greatly inhibited before biofilm maturation (e.g. *S. aureus* and *E. faecalis*) showed limited biofilm formation capability after 24 hours of antiseptic treatment. Most Gram-negative microorganisms were not inhibited by the test antiseptics, except for biguanide (Figure 1E). We observed the same trend (i.e. only Gram-positive bacteria were controlled) after 48 hours of treatment (Figure 1F). At 24 and 48 hours of treatment, non-biguanide antiseptics permitted biofilm formation for *C. albicans*, which was controlled only by NaClO stabilised in saline solution. Not all of the antiseptics had an effect that was directly proportional to the concentration of the active ingredient. This result is perhaps because microorganisms are able to modify their external structures in response to the environment. This 'sensing' effect has been observed with ethanol. Concentrations of ethanol greater than that required for the bactericidal effect (70%) only achieve bacteriostatic effects (28).

Table 1 Effects of various antiseptics on biofilm formation and human fibroblast cytotoxicity

	Liquids																			
	Inhibition of adhesion (6 hours)					Inhibition of aggregation (12 hours)					Inhibition of maturation (24 hours)					Elimination of biofilm (48 hours)				
Strains	NaClO	HClO	DADS-M	SOS	Be.	NaClO	HClO	DADS-M	SOS	Be.	NaClO	HClO	DADS-M	SOS	Be.	NaClO	HClO	DADS-M	SOS	Be.
P. aeruginosa	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−
A. baumannii	−	+	+	+	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−
K. pneumoniae	+	+	−	+	+	−	+	+	+	+	−	−	−	−	+	−	−	−	−	−
S. maltophilia	+	+	+	+	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	+
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. faecalis	+	+	+	+	+	+	+	−	−	+	−	+	+	+	+	+	+	+	+	+
C. albicans	+	+	+	+	+	+	+	+	+	+	−	−	−	−	+	−	−	−	−	+
	Citotoxicity																			
Fibroblasts	+	−	+	−	+	−	+	+	−	+	+	+	−	−	+	−	+	+	−	+

	Gel															
	Inhibition of adhesion (6 hours)				Inhibition of aggregation (12 hours)				Inhibition of maturation (24 hours)				Elimination of biofilm (48 hours)			
Strains	NaClO	DADS-M	SOS	Be.	NaClO	DADS-M	SOS	Be.	NaClO	DADS-M	SOS	Be.	NaClO	DADS-M	SOS	Be.
P. aeruginosa	−	+	+	−	−	+	+	+	−	−	−	+	−	−	−	+
A. baumannii	−	−	−	+	−	−	−	−	−	−	−	+	−	−	−	−
K. pneumoniae	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−	−
S. maltophilia	+	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
S. aureus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
E. faecalis	+	−	−	+	+	+	+	+	−	+	+	+	−	+	+	+
C. albicans	+	+	+	+	+	+	+	+	+	−	−	+	+	+	+	+
	Citotoxicity															
Fibroblasts	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+

+, antiseptic or cytotoxic effect based on ED50 or LD50 determination, respectively; −, no evident toxicity; NaClO, sodium hypochlorite; HClO, hypochlorous acid; DADS-M, modified diallyl disulfide-oxide; SOS, superoxidation solution; Be, biguanide.

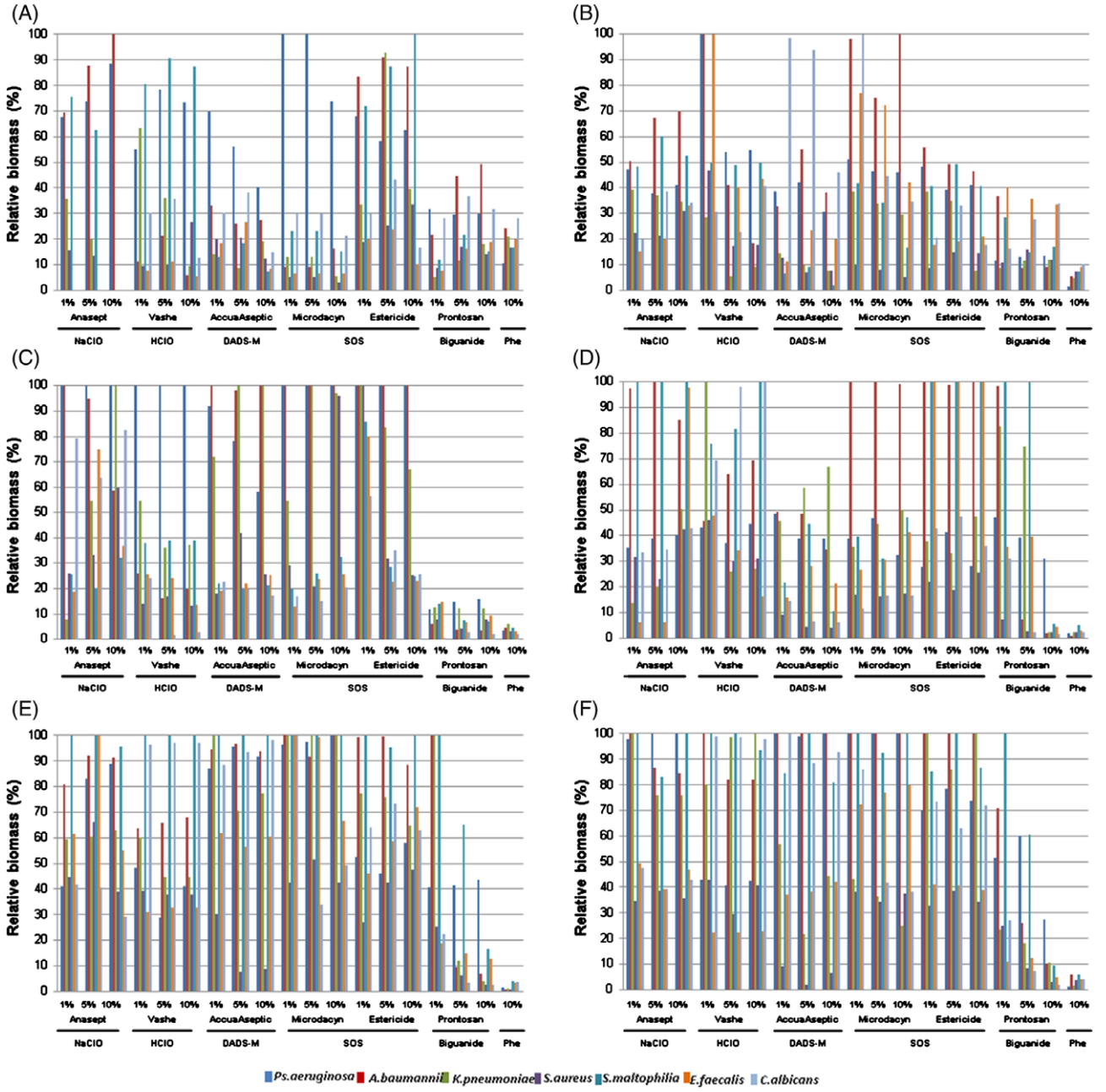

Figure 1 In vitro assessment of the inhibition of biofilm formation by liquid antiseptic formulations. Inhibition of microbial adhesion at 2 hours (A) and 4 hours (B), microbial aggregation at 6 hours (C) and 12 hours (D) and biofilm maturation at 24 hours (E) and 48 hours (F). NaClO, sodium hypochlorite; HClO, hypochlorous acid; DADS-M, modified diallyl disulfide-oxide; SOS, superoxidation solution; Phe, phenol (positive control).

Commercial wound care antiseptics are generally available as liquids or gels. It is thought that the semisolid gel form is not easily removed by exudation and may remain on wounds for a prolonged period of time (29). In some cases, antiseptic gel can help retain moisture in the lesion (29,30). However, we found that biofilm development was greater in cultures that were treated by antiseptics in gel form, with the exception of biguanide (Figure 2). This result may have occurred because it is difficult to stabilise and maintain gases, such as NaClO, in the pharmaceutical form or because of the limited bioavailability of the active ingredient for either NaClO or DADS-M.

To be effective, an antiseptic must be capable of biofilm eradication, which is a more complex effect than interfering in biofilm formation. To eliminate mature biofilm, it is not sufficient to apply microbiocides. Removal strategies for the ecosystem are required, such as the breakdown or removal of the ECM containing the microorganisms (19,31). Wound care antiseptic suppliers frequently claimed that their formulae have this capability. Therefore, we tested the effectiveness of several antiseptics in eliminating mature biofilm. In general, antiseptics were only able to eliminate biofilms partially for Gram-positive bacteria and *C. albicans* (Figure 3). Effects were evident early,

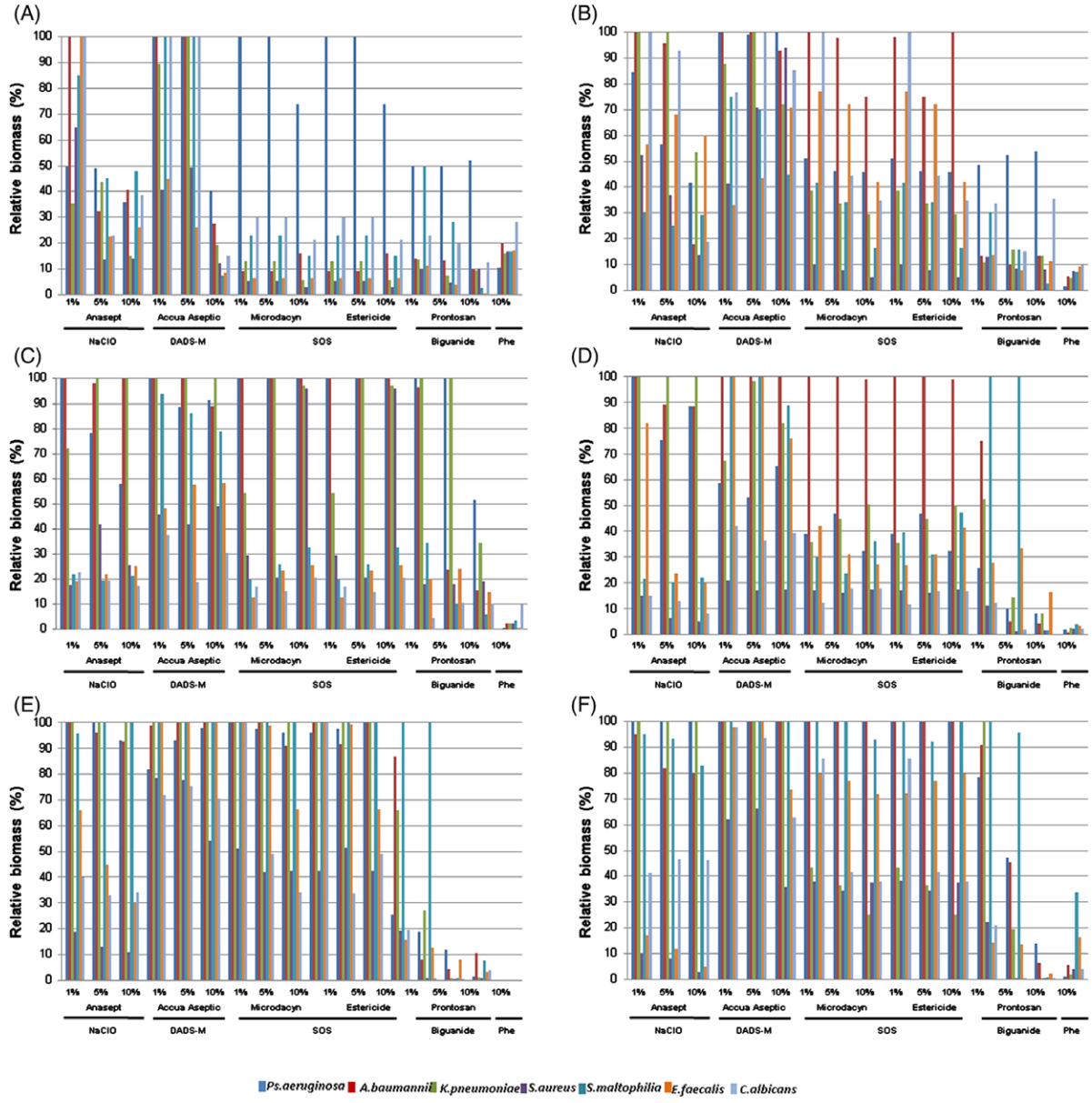

Figure 2 In vitro assessment of the inhibition of biofilm formation by gel antiseptic formulations. Inhibition of microbial adhesion at 2 hours (A) and 4 hours (B), microbial aggregation at 6 hours (C) and 12 hours (C) and biofilm maturation at 24 hours (D) and 48 hours (E). NaClO, sodium hypochlorite; DADS-M, modified diallyl disulfide-oxide; SOS, superoxidation solution; Phe, phenol (positive control).

at 6 hours of treatment, especially for formulations based on biguanide and DADS-M. This finding indicates that active chlorine generators (NaClO, HClO and SOS) do not have sufficient activity to eliminate biofilms. As we found in the biofilm formation inhibition tests, gel antiseptic formulations were not as effective as liquids for biofilm elimination (Figure 3). This result is interesting because the industry maintains that wound care gels can penetrate biofilms better than liquids (32).

Cytotoxicity to human fibroblasts depends on antiseptic formulation and treatment time

Wounds are a complex environment formed by the wounded organ or tissue, humoral and cellular elements and contaminating microorganisms. The effectiveness of local antiseptics depends not only on their ability to control the microbial flora and biofilm formation but also on their cytotoxicity (19). We evaluated the cytotoxicity of various antiseptics (Table 1) against eukaryotic cells (human skin fibroblasts).

Cell activity, evidenced by mitochondrial MTT reduction, showed that gel formulations were highly cytotoxic, showing LD50 at the concentrations and times tested (Figure 4). Fibroblast toxicity varied depending on the active ingredient, mainly for DADS-M and biguanide. This cytotoxic effect prevented recovery of the cell population even after 48 hours of treatment, when the active ingredient activity should decrease (Figure 5). After 6 hours of treatment, only with the HClO solution was

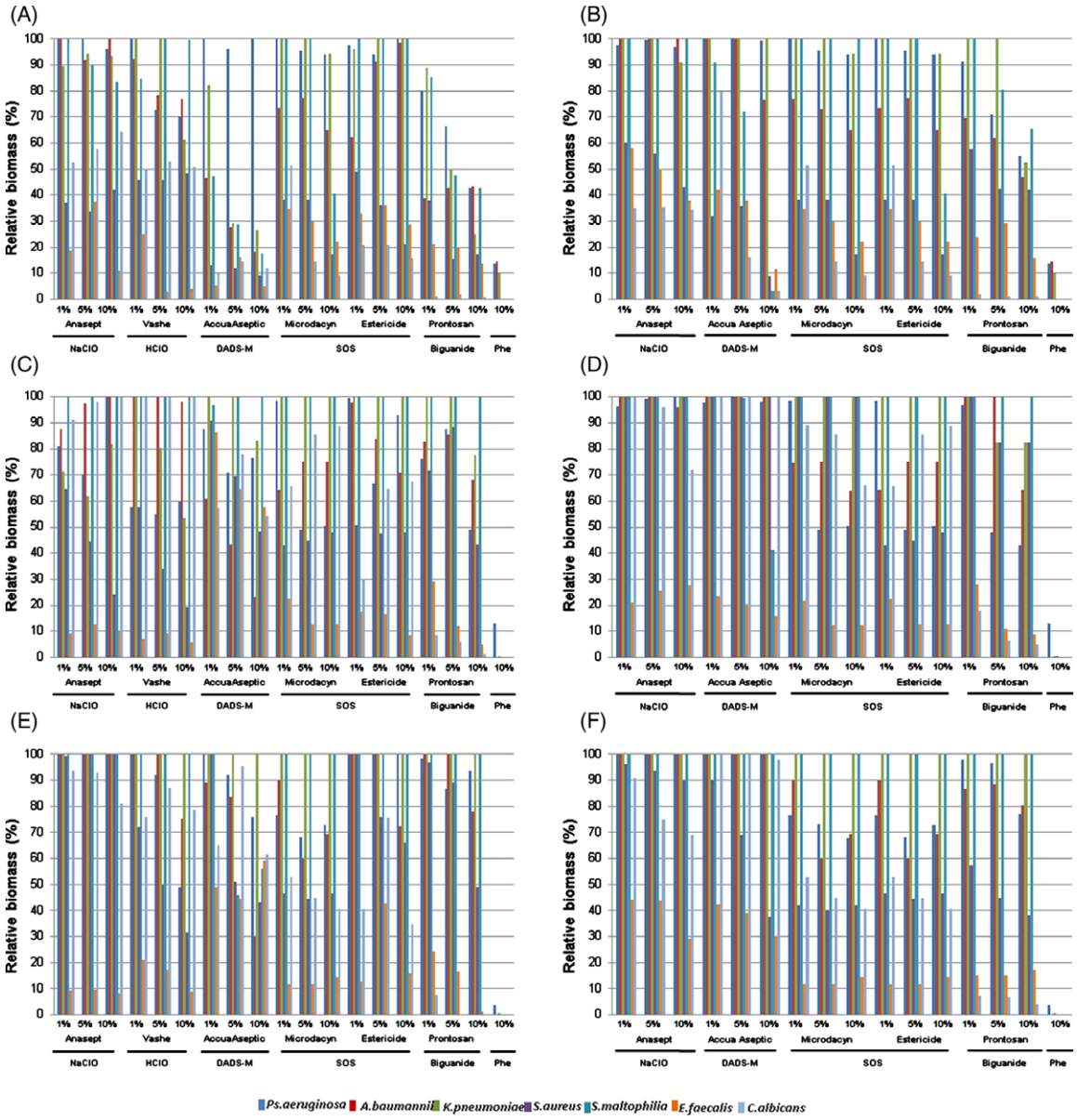

Figure 3 Biofilm elimination. Effects of liquid (A, C and E) and gel (B, D and F) antiseptic formulations on tested microorganisms at 6 hours (A and B), 12 hours (C and D) and 24 hours (E and F). NaClO, sodium hypochlorite; HClO, hypochlorous acid; DADS-M, modified diallyl disulfide-oxide; SOS, superoxidation solution; Phe, phenol (positive control).

the fibroblast population maintained at more than 50% viability (Figure 5A). This effect remained at 48 hours (Figure 5 B–D). The NaClO and SOS formulations showed a recovery trend of the fibroblast population after 24 hours of treatment. However, by 48 hours, a clear toxic effect could be seen (Figure 5C and D, respectively). In this experiment, the cytotoxic effects from treatment with various antiseptics had a direct relationship with the tested population.

Discussion

The success of the healing process depends on the completion and integrity of each of its phases. An adequate wound bed is essential to successful healing. Routine treatment must involve wound tissue removal, control of infection, moisture balance and migration of epithelial cells, known as the TIME concept (33). Although these events are interrelated, infection is the factor that most often upsets the balance. Thus, bacterial control is a priority for a wounded patient.

In our study, we evaluated the capabilities of various antiseptic formulations to inhibit biofilm development. Although active chlorine-liberating formulations, such as NaClO, HClO and some SOSs, had bactericidal activities above 99·99% (34), this effect occurs only for the in vitro treatment of planktonic forms. In contrast, the actual medical scenario requires the control of biofilm formation (35). Although nearly all of the tested antiseptics prevented the early formation of single-species biofilms, their effectiveness decreased with time

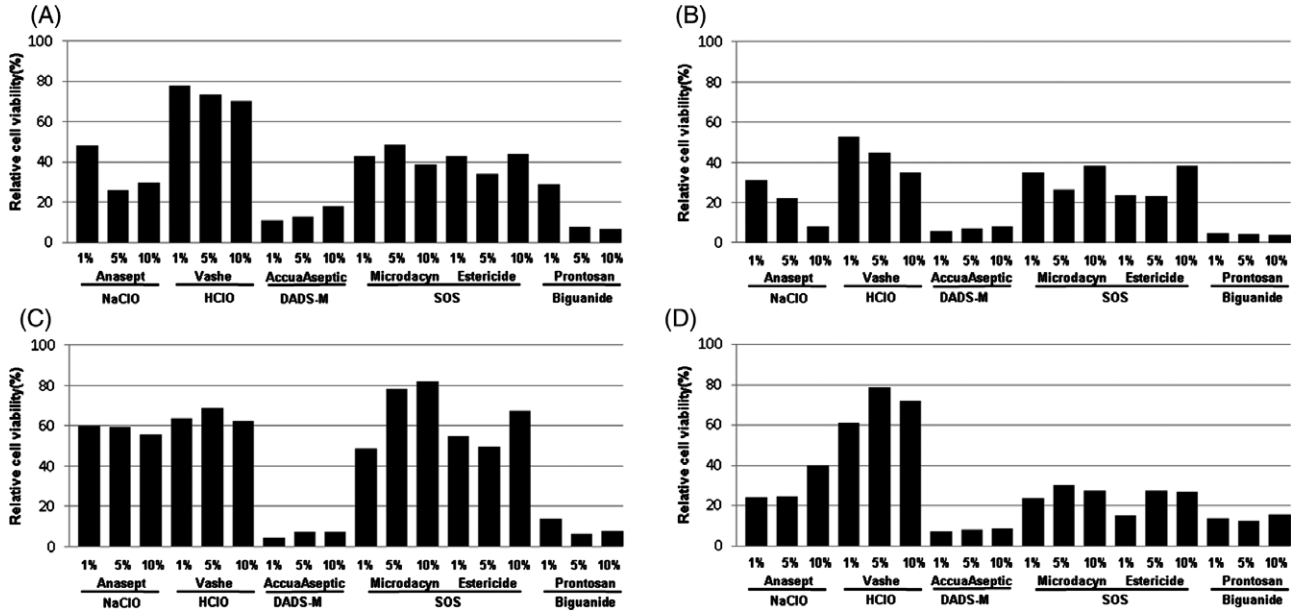

Figure 4 Cytotoxicity of various liquid antiseptic formulations against human fibroblasts at 6 hours (A), 12 hours (B), 24 hours (C) and 48 hours (D) compared with control (culture medium). NaClO, sodium hypochlorite; HClO, hypochlorous acid; DADS-M, modified diallyl disulfide-oxide; SOS, superoxidation solution.

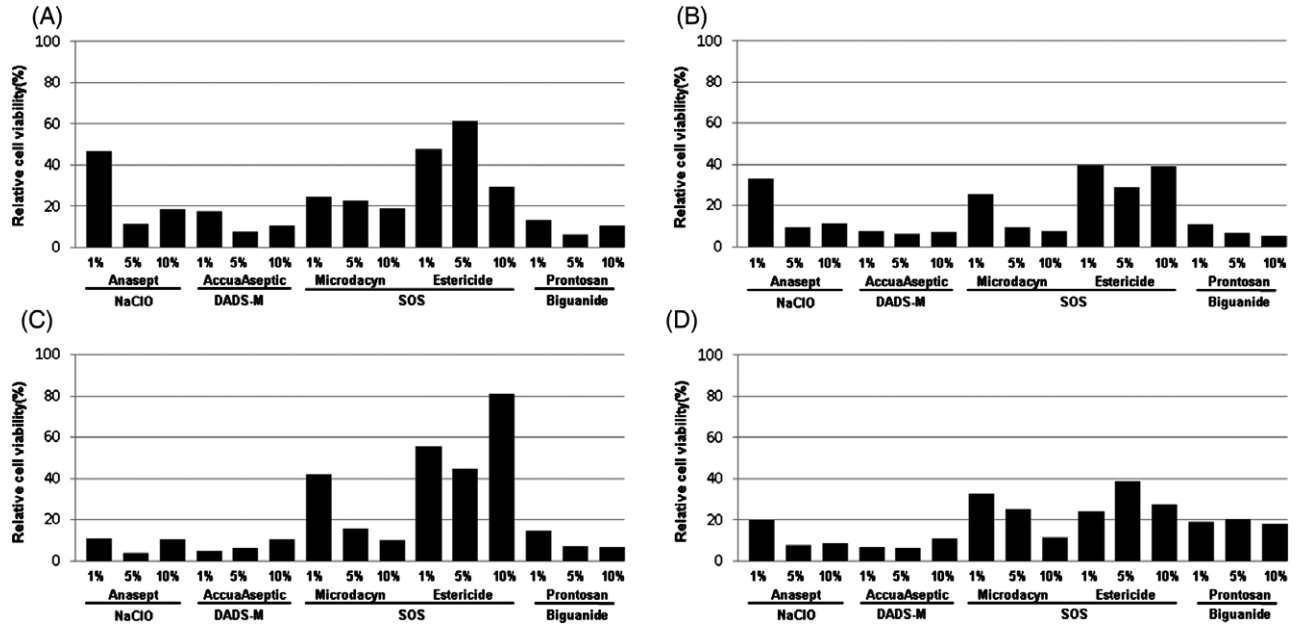

Figure 5 Cytotoxicity of various gel antiseptic formulations against human fibroblasts at 6 hours (A), 12 hours (B), 24 hours (C) and 48 hours (D) compared with control (culture medium). NaClO, sodium hypochlorite; DADS-M, modified diallyl disulfide-oxide; SOS, superoxidation solution.

as the active ingredients, especially gases dissolved in aqueous media, changed into harmless molecules (36). In particular, it has been demonstrated that HClO can reduce the biofilm microbial population as well as polysaccharides and proteins in the biomass. It means that HClO could avoid biofilm formation and/or disrupt the biofilm itself (37).

Antiseptics with stable active ingredients, such as DADS-M (38) and biguanide (39), whose bacteriotoxicity has been reported as among the most stable and sustained, also had limited effects, particularly against Gram-negative bacteria. In addition to the ability of antiseptics to prevent biofilm formation, we believe that it was important to understand their anti-biofilm effects. In a complete, already structured habitat, it is not sufficient for an antiseptic to kill active phase microorganisms; they must also be able to eliminate the microorganism–ECM complex. Moreover, the quiescent state of microorganisms in the biofilm core is a challenge. Although it may be feasible to eliminate surface species,

encapsulated cells can be inaccessible to antimicrobial systems (40). As expected, in this situation, anti-biofilm activity was very restricted. Most of the tested formulations were only effective in the first 6 hours and only on Gram-positive microorganisms and *C. albicans*. Afterwards, there was clear recovery associated to the residual mass from the original biofilm, leaving the possibility that microorganisms could reform a new structured colony. For that reason, debridement as well as repeated antiseptic treatments are needed to eradicate in vivo the whole colony (41). With the method we have followed, it is impossible to know whether living cells are still present in the residual mass; further microbial viability experiments are needed to know it (i.e. MTT bacterial assays).

Interestingly, the same antiseptics in aqueous gel that form more quickly lost their capacity to interfere in biofilm formation and elimination. This finding contradicts expectations that a semisolid can remain longer on the wound surface (42,43). The choice of the optimal vehicle(s) used during gel formulation is a critical issue for wound care. Not only must they provide moisture to the injury, but also gel formulation must maintain drug stability during shelf time as well as an appropriate release during their administration. Several works have demonstrated that during hydrogel formulation, different excipients have variations in drug release (44,45). With regards to chemical stability, SOSs and chlorine can be exhausted into hydrogel because their high reactivity (46); this idea is supported by our results because chlorine containing and SOSs were less effective against biofilm formation and elimination than DADS-M or biguanide. Otherwise, gel formulation's cytotoxicity could be attributed solely to changes in the culture medium isotonicity or to additives required to prepare hydrogels as the corresponding antiseptic solutions showed less damage to cultures than gels.

Wound bed preparation involves the formation of granulation tissue, and fibroblasts play a key role in this process. As an antiseptic must be able to protect the eukaryotic environment while controlling infection in a wound, data from animal and clinical studies have shown inhibition of wound repair, starting as far back as Fleming A. assays (47) and more recently Kramer (48). NaClO has particularly been thoroughly evaluated, and some of its main deleterious effects on wound healing involve blood flow cessation as well as local tissue toxicity (18,49).

To know the potential cytotoxicity of antiseptic formulations against fibroblasts cultures, we assessed cell viability by the MTT assay. In general, the effectiveness of the antiseptics was associated with cytotoxicity, except for HClO that showed an early response to biofilm formation without being excessively cytotoxic to fibroblasts. Although the active chlorine generators all had microbiocidal activity, there were differences in how the active chlorine was released and took effect, given that eukaryotic cells were not equally susceptible to the various antiseptics.

Regarding HClO, our results are similar to those reported by Sakarya *et al.* 2014 (50), where the biomass derived from the biofilms prepared from *S. aureus, P. aeruginosa* and *C. albicans* (all of them ATCC strains, while ours were clinical isolates) diminished after HClO treatment in a dose-dependent pattern. In that work, Sakarya's group also evidenced that total planktonic microorganism kill at 1/64, 1/8 and 1/4 dilutions, (1·6, 12·5 and 25%, respectively). In our study, we only assessed the biomass formed by the planktonic microorganism's growth in enriched medium, in such a manner that results, after 2 hours of antiseptic treatment, could be similar to those reported by Sakarya *et al.* Other important issues are that Sakarya's group used 75 times less microorganisms than us, and we followed the method by the Clinical and Laboratory Standards Institute (22). Besides, they prepared their experiments in PBS that is only an isotonic solvent, and in consequence, microorganisms are 'growing' under stress and they are more susceptible. Meanwhile in this work, we diluted microorganisms in an enriched culture medium, where environment partially mimics clinical condition, where exudate is rich in nutrients. Even in these conditions, we demonstrated microbicidal properties after HClO treatment, with the less cytotoxicity among the assessed antiseptics.

In conclusion, microbiocidal activity is directly related to cytotoxicity in most antiseptic formulations. Treating clinicians must use the right criteria for choosing the appropriate antiseptic treatment scheme. In this study, we have presented our results graphically, offering a practical option to the wound care clinician for evaluating commercially available antiseptics based on risk/benefit of application. Inevitably, a complete anti-biofilm effect is only possible with repeated debridement and antiseptic treatments, which we did not test in our system because of the model. This aspect is a limitation of the in vitro models. For that reason, randomised controlled trials must be performed, where antiseptics tested in this work can be assessed with the approach of creating clinical guidelines. In addition, biofilms in wounds include various microorganisms that coexist and synergise (51).

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