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Basic Research

Chlorhexidine Antiseptic Irrigation Eradicates *Staphylococcus epidermidis* From Biofilm: An In Vitro Study

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Abstract

Background Antiseptic and antibacterial solutions used for intraoperative irrigation are intended to kill bacteria and thereby decrease the incidence of surgical site infections. It is unknown if the concentrations and exposure times of irrigation solutions commonly used for prophylaxis in clean cases (povidone-iodine 0.35% for 3 minutes) are effective against bacteria in biofilm that are present in implant infections. Currently, povidone-iodine (0.35%), chlorhexidine (0.05%), sodium hypochlorite (0.125%), and triple antibacterial solution are all being used off-label for wound irrigation after surgical débridement for orthopaedic infections.

Questions/purposes Do commonly used antibacterials and antiseptics kill bacteria in established biofilm at clinically relevant concentrations and exposure times?

Methods *Staphylococcus epidermidis* (ATCC#35984) biofilms were exposed to chlorhexidine (0.025%, 0.05%, and 0.1%), povidone-iodine (0.35%, 1.0%, 3.5%, and 10%), sodium hypochlorite (0.125%, 0.25%, and 0.5%,), and triple antibacterial solution (bacitracin 50,000 U/L, gentamicin 80 mg/L, and polymyxin 500,000 U/L) for 1, 5, and 10 minutes in triplicate. Surviving bacteria were detected by 21-day subculture. Failure to eradicate all bacteria in any of the three replicates was considered to be "not effective" for that respective solution, concentration, and exposure time.

Results Chlorhexidine 0.05% and 0.1% at all three exposure times, povidone-iodine 10% at all three exposure times, and povidone-iodine 3.5% at 10 minutes only were effective at eradicating *S epidermidis* from biofilm. All concentrations and all exposure times of sodium hypochlorite and triple antibacterial solution were not effective.

Conclusions Chlorhexidine is capable of eradicating *S epidermidis* from biofilm in vitro in clinically relevant concentrations and exposure times. Povidone-iodine at commonly used concentrations and exposure times, sodium hypochlorite, and triple antibacterial solutions are not.

Clinical Relevance This in vitro study suggests that chlorhexidine may be a more effective irrigation solution for *S epidermidis* in biofilm than other commonly used

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solutions, such as povidone-iodine, Dakin's solution, and triple antibiotic solution. Clinical outcomes should be studied to determine the most effective antiseptic agent, concentration, and exposure time when intraoperative irrigation is used in the presence of biofilm.

Introduction

Irrigation is used during orthopaedic procedures to keep tissues moist, remove tissue debris and bacterial contaminants from the wound, and, in the case of surgical débridement for infection, remove bacteria and fragments of biofilm with the clinical goal of eradicating established biofilm-based infections. For clean surgery cases, with or without implants, antibacterial drugs are commonly added to the irrigation solution in an attempt to kill bacterial contaminants that are not physically removed from the wound by irrigation. Bacitracin, an aminoglycoside, and polymyxin often are added in combination for broad-spectrum activity. Adding antiseptics as opposed to antibacterial drugs to intraoperative irrigating solutions to reduce contaminants has gained attention, most commonly povidone-iodine 0.35% for 3 minutes before closing [3]. Decreased risk of surgical site infections (SSI) has been reported after primary THAs and TKAs and spine procedures with the use of a povidone-iodine irrigation solution, however these cases do not have established biofilm present [3, 7, 8]. Based on in vitro data, chlorhexidine may be superior to povidone-iodine for bacterial eradication from biofilms in select orthopaedic applications [24]. Chlorhexidine is an antiseptic widely used for skin decolonization and as therapeutic irrigation in dental applications. Chlorhexidine is chondrotoxic and its use is contraindicated in the setting of native cartilage [10, 28]. A third antiseptic that has been used anecdotally to treat biofilm on implant surfaces is chlorine in the form of sodium hypochlorite, known as Dakin's solution; however, data supporting the use of sodium hypochlorite in the management of biofilms are lacking.

When irrigation is used after surgical débridement for infection, the bacteria in the wound include sessile phenotypes in fragments of biofilm or areas of retained biofilm, which are inherently less responsive to antiseptics than planktonic microorganisms [15]. Bacterial persister cells in biofilm are resistant to host defenses and therefore must be eradicated pharmacologically or surgically removed, whereas planktonic persister cells can be eradicated by the host [15]. Although the minimum biofilm eradication concentration (MBEC) is a measure of the lowest level of antibiotics needed for therapeutic effect, antiseptics are used at the highest concentration that is tolerated by tissues. We propose that clinically relevant concentrations of antiseptic solutions can be determined "effective" or "not effective" for eradication of bacteria from biofilm using methodology similar to an MBEC assay which determines the breakpoint concentration of antibacterials that achieve total eradication of all living bacteria in biofilm [6].

Povidone-iodine (0.35%), chlorhexidine (0.05%), and sodium hypochlorite (0.125%) are all being used off-label for wound irrigation after surgical débridement for orthopaedic infections without comparative bacterial kill data to choose among them. We therefore asked the question: Do commonly used antibacterials and antiseptics kill bacteria in established biofilm at clinically relevant concentrations and exposure times?

Materials and Methods

This in vitro study was designed to determine if chlorhexidine, povidone-iodine, sodium hypochlorite, or triple antibacterial (bacitracin, gentamicin, and polymyxin) is capable of eradicating all viable bacteria from biofilms exposed to clinically relevant concentrations for clinically relevant exposure times. The exposure times studied bracket the 3- to 5-minute time reported in several studies, which spans the times used by us and colleagues [3, 7, 8]. We feel exposure times greater than 5 minutes to be unrealistic for clinical use on a consistent basis and therefore not clinically relevant. We considered concentrations acceptable for direct exposure to tissue to be clinically relevant: chlorhexidine 0.05% or less, povidone-iodine 0.35% % or less, and sodium hypochlorite 0.125% or less [2, 3, 7, 16, 19, 21, 23, 25]. The concentrations studied bracket the commercially available concentrations (chlorhexidine 0.05%, sodium hypochlorite 0.125%) for each agent that are available for clinical use (povidone-iodine 0.35%), or used by other surgeons [3, 4, 7, 8].

Mature *Staphylococcus epidermidis* (ATCC 35984) biofilms were exposed to each concentration of the four irrigation solutions for three exposure times (1, 5, 10 minutes) in triplicate for a total of (3 + 4 + 3 + 1) x 3 x 3 = 108 specimens. Normal saline with no active antimicrobial agent was used as a negative control. Wells with no biofilm were used as a positive control on each plate. After exposure to an irrigation solution, remaining viable bacteria were detected on 21-day subcultures [6].

Biofilms were grown by propagating a single colony of *S epidermidis* (ATCC 35984) for 18 hours at 37° C in 5 mL of glucose-enriched tryptic soy broth (TSB; 1 wt% glucose by adding 7.34 mg glucose per mL of TSB; Becton Dickinson, Franklin Lakes, NJ, USA). The *S epidermidis* suspension was diluted 1:100 with the TSB with glucose and incubated in 96-well plates (Sarstedt, Newton, MA, USA) for 7 days to form mature biofilms [12]. The growth

media was removed and the biofilm was gently rinsed with sterile deionized water to remove residual media and planktonic bacteria before exposure to an irrigation solution. Chlorhexidine solutions were prepared by diluting chlorhexidine gluconate 4% solution (Scrub Care® Exidine 4% solution; CareFusion Leawood, Shawnee Mission, KS, USA) with deionized water to concentrations of 0.025%, 0.05%, and 0.1%. Povidone-iodine solutions were prepared by diluting povidone-iodine 10% solution (Scrub Care® Povidone Iodine Topical Solution Paint; CareFusion Leawood) with deionized water to concentrations of 0.35%, 1%, 3.5%, and 10%. Sodium hypochlorite solutions were prepared by diluting bleach (Clorox®; The Clorox Company, Oakland, CA, USA) with deionized water to concentrations of 0.125%, 0.25%, and 0.5%. Triple antibacterial solution was prepared in the hospital pharmacy to concentrations of bacitracin 50,000 IU/L, gentamicin 80 mg/L, and polymyxin 500,000 IU/L.

Exposure to irrigation solutions was accomplished by replacing the growth media with one of the concentrations of each irrigation solution or a control for 1, 5, and 10 minutes in triplicate.

After exposure, the irrigation solutions were removed and the biofilms were gently rinsed with deionized water, then 200 μL of TSB with glucose was placed in each well and the biofilm was physically scraped from the bottom surface with a micropipette (Eppendorf Reference®; Eppendorf, Hauppauge, NY, USA). The biofilm suspension was transferred to test tubes, vortexed on setting 10 for 30 seconds (Mini Vortexer; VWR, Radnor, PA, USA), sonicated at 40 kHz for 1 minute (Aquasonic Model 75D; VWR) to disrupt biofilm structure, vortexed on setting 10 for an additional 30 seconds, then incubated at 37° C for 21 days. Visual turbidity caused by growth of surviving biofilm organisms was documented as "not effective" to eradicate all viable bacteria (Fig. 1) [6, 29]. Growth was assessed daily for each well up to Day 21.

Failure to eradicate all bacteria in any of the three replicates was considered to be "not effective" for that respective solution, concentration, and exposure time. The subculture data were considered to be nominal (effective or not effective), so the replicates were not averaged [6]. The lowest effective concentration and exposure time for each irrigation solution were tabulated.

Results

The minimum effective concentration and exposure time combinations were 0.05% chlorhexidine for 1 minute, 3.5% povidone-iodine for 10 minutes, and 10% povidone-iodine for 1 minute (Table 1). All exposure times of lower concentrations of chlorhexidine and povidone-iodine and all concentrations and all exposure times of sodium hypochlorite and triple antibacterial solution were not effective. All positive controls were effective and all negative controls were not effective (Table 2).

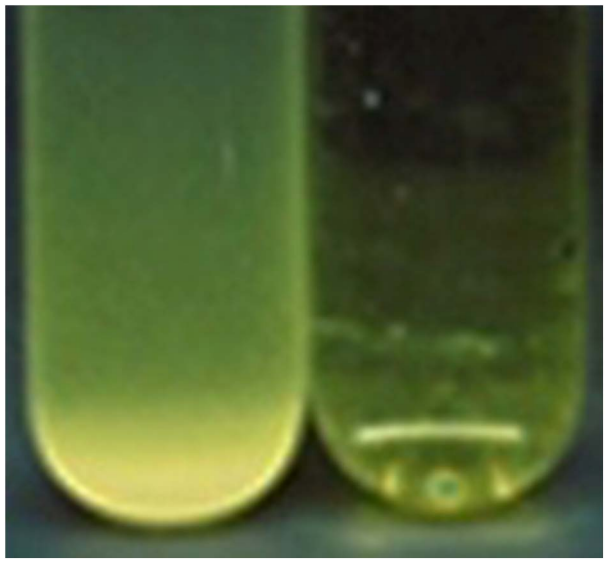

Fig. 1 Visual turbidity of a subculture from the biofilm after exposure to an irrigation solution as seen in the tube on the left was considered persistence of viable bacteria and not effective to eradicate all viable bacteria. The tube on the right is clear with no visible turbidity at 21 days, which was considered negative for growth or effective at eradication of all viable bacteria from the biofilm.

Discussion

Although antibacterial and antiseptic solutions are being used intraoperatively to reduce the risk of colonization in primary surgery and after débridement for biofilm-based orthopaedic infections, there are no comparative microbiologic data of which we are aware for their effect on sessile bacteria, and clinical outcomes data are limited to their use in case series of noninfected clean procedures [3, 7, 8]. We therefore asked: Do currently used antiseptic and antibacterial solutions eradicate bacteria from established biofilm at clinically relevant concentrations and exposure times? We found that 0.05% chlorhexidine was effective at killing biofilm-based *S epidermidis* with exposure time as short as 1 minute, but sodium hypochlorite, triple antibacterial, and normal saline were not effective at all concentrations and times tested. Although povidone-iodine was capable of killing sessile *S epidermidis,* it took either a 30-fold increase in concentration at clinically relevant exposure times (10% povidone-iodine for 1 minute) or 10-fold increase at triple the exposure time (3.5% povidone-iodine for 10 minutes). A 10-minute exposure time is not realistic

Table 1. Minimum concentration (wt%) of irrigation solution that was effective at eradication of *Staphylococcus epidermidis* from biofilm.

	Time (minutes)		
Solution	**1 minute**	**5 minutes**	**10 minutes**
Chlorhexidine (%)	0.05	0.05	0.05
Povidone-iodine (%)	10	10	3.5
Sodium hypochlorite (%)	none	none	none
Triple antibiotic	none	none	none

for clinical use and 10% povidone-iodine has been shown to be toxic in vivo [25]. Although 0.35% povidone-iodine may decrease surgical site infections with no adverse effects attributable to povidone-iodine when used as an irrigation solution during primary joint replacement and spine procedures [3, 7, 8, 13], our data suggest that it may not be effective when used during the treatment of an established periprosthetic joint infection in which a biofilm exists.

There are limitations to our study. First, we used in vitro biofilm and therefore the findings may not apply to in vivo biofilms. Because we have found that biofilms are generally more susceptible to antimicrobials in vivo than in vitro, we believe the data do not overestimate effectiveness of the antiseptic agents use in irrigation during surgeries for established biofilm-based infection. Second, we studied only a single organism. ATCC 35984 was chosen as a relevant microorganism because *S epidermidis* is a common orthopaedic pathogen, responsible for approximately 19% to 31% of hip and knee periprosthetic joint infections, and this strain is known to be a robust former of biofilm [12, 18, 20]. Our data may not be representative of antiseptic effectiveness for other orthopaedic pathogens. Similar to our data, Schwecter et al. [24] and Smith et al. [26] reported chlorhexidine is superior to povidone-iodine on methicillin-resistant *S aureus* biofilm, but required a minimum concentration of 2%, which is higher than the effective concentration of 0.05% we found. The difference may be microorganism-specific. Further study will be required to determine the chlorhexidine concentration required against other orthopaedic pathogens. Third, we did not use serum for the povidone-iodine solution. Iodine absorption by serum protein decreases the effectiveness of povidone-iodine in vivo. While we found that povidone-iodine required higher concentrations and longer exposure times than are clinically relevant, our data may overestimate the effectiveness of povidone-iodine, strengthening our finding that povidone-iodine is not effective. Fourth, we did not study local tissue toxicity. The concentrations we studied included concentrations above those commonly used clinically; however, the aim of this investigation is to identify the concentrations needed to kill sessile *S epidermidis,* not determine what concentration is safe. Ten percent povidone-iodine is intended for external

Table 2. Bacterial eradication by irrigation agent

	Concentration (%)	Exposure time (minutes)		
Solution		**1**	**5**	**10**
	0.025	+	+	+
Chlorhexidine	0.05	−	−	−
	0.1	−	−	−
	0.35	+	+	+
	1	+	+	+
Povidone-iodine (%)	3.5	+	+	−
	10	−	−	−
	0.125	+	+	+
Sodium hypochlorite (%)	0.25	+	+	+
	0.5	+	+	+
Triple antibiotic		+	+	+
− control		+	+	+
+ control		−	−	−

+= growth within 21 days; − = no growth at 21 days.

use only. Povidone-iodine and chlorhexidine solutions are known to irritate mucous membranes and may cause adverse local tissue effects at concentrations above those currently being used clinically for irrigation. Chlorhexidine has been shown to be cytotoxic and its use is cautioned [1, 5, 9, 11, 14, 27]. However, some in vitro studies have used much higher concentrations and/or exposure times than are used clinically [9, 11, 14, 17]. Despite that some in vitro studies have shown chlorhexidine to be cytotoxic, multiple in vivo studies have shown that there is not an increase in wound-healing complications or other adverse effects, other than chondrotoxicity, compared with controls [19, 21-23]. We interpreted our data based on concentrations considered acceptable for local irrigation. Finally, we did not confirm the bacteria that grew on 21-day subcultures were *S epidermidis*. Our positive control groups provide assurance that the risk of growth from contamination in the 21-day subcultures is low, as there was no growth in any of the positive controls. Therefore, we believe it is very unlikely that the growth on subcultures were not *S epidermidis*.

Our protocol was designed to represent currently used concentrations and exposure times as reported by others. Sanchez et al. [23] reported improved wound healing in wounds irrigated daily with 0.005% and 0.05% chlorhexidine in a dog model compared with wounds irrigated daily with normal saline. Best et al. [2] reported that 1-minute exposure of 0.05% chlorhexidine was not toxic to normal human cartilage in vitro. However, the same exposure was toxic to osteoarthritic cartilage. Reading et al. [21] reported similar results in a rat model, concluding that 0.05% chlorhexidine could be used on normal cartilage if rinsed away after 1 minute of exposure time. Using a guinea pig model, Platt and Bucknall [19] reported no increase in adverse tissue reactions and superior antibacterial activity of 0.05% chlorhexidine when compared with saline, 10% povidone-iodine, and 0.1% benzalkonium chloride. Severyns et al. [25] reported 0.05% chlorhexidine to be much less toxic than 10% povidone-iodine when used for irrigation just before performing femoral vessel repair in a rat model. Saatman et al. [22], in a guinea pig model, irrigated surgical wounds and abrasions daily with 4% and 0.5% chlorhexidine for up to 21 days. There was no difference in the histologic appearance of incision and abrasions when compared with saline controls. However, case reports of the accidental use of dilute chlorhexidine during arthroscopic procedures in the knee have shown extensive chondrolysis [10, 28]. However, since chondrolysis is not a concern during THA and TKA (provided that the patella is resurfaced in the latter), some of us (MJS, CSE) have started to use dilute chlorhexidine for irrigation or lavage during those procedures; however, to our knowledge, no results using this approach have been reported. The potential benefits of using oxidizing antiseptic irrigation solutions during total joint arthroplasty or during the treatment of an established infection should be balanced with the potential local toxic effects. In vitro studies suggest that 0.05% chlorhexidine chondrotoxicity is time-dependent. As noted, at least two studies [2, 21] suggest that the use of a 005% chlorhexidine solution for very brief exposures (1 minute) do not appear to be directly chondrotoxic to healthy cartilage; if this intervention is going to be evaluated clinically, perhaps it should first be studied in the setting of prosthetic joint infection where chondrotoxicity is not an issue. Any such studies still should seek to minimize toxicity to local tissues by keeping exposures short, and using copious irrigation with a nontoxic substance (eg, normal saline) after irrigation with chlorhexidine to minimize residual chlorhexidine left in the wound.

Our study differs from work by Schwechter et al. [24] in that we used total eradication on 21-day subcultures to determine effectiveness rather than a 3-log reduction in colony-forming units, which does not eradicate persister cells that can propagate infection. Our protocol is similar to the MBEC assay used by Castaneda et al. [6], and positive controls uniformly produced turbid cultures. We believe all viable persister cells were reliably detected.

Chlorhexidine was effective in vitro at killing *S epidermidis* in biofilm at a clinically available concentration (0.05%) with an exposure time of 1 minute, whereas povidone-iodine required concentrations that are too high or require prolonged exposure times; sodium hypochlorite and antibacterials were ineffective. Our in vitro data need confirmation with clinical outcomes studies and suggest that antiseptic agent, concentration, and exposure time are important determinants when studying outcomes for intraoperative irrigation in the presence of biofilm.

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