Efficacy of concurrent application of chlorhexidine gluconate and povidone iodine against six nosocomial pathogens

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Background: Chlorhexidine gluconate (CHG) and povidone iodine (PI) are rarely used concurrently despite a lack of evidence regarding functional incompatibility of these agents. Methods: CHG and PI, alone and combined, were evaluated against Staphylococcus aureus (methicillin-susceptible S. aureus [MSSA]) and methicillin-resistant S. aureus [MRSA]), Staphylococcus epidermidis (MRSE), Acinetobacter baumannii, Pseudomonas aeruginosa, and Escherichia coli using checkerboard microbroth dilution techniques. Minimum bactericidal concentration (MBC) was the concentration (percent wt/vol) that reduced bacterial burden ≥5-log10 colony-forming units/mL at 2 hours when compared with bacterial densities in growth controls. Fractional bactericidal concentration indexes (FBCIs) were calculated to determine CHG and PI compatibility. Additionally, tissue plugs from freshly excised porcine vaginal mucosa were infected with S. aureus (MSSA), treated for 2 hours with CHG 3%, PI 5%, or CHG 3% and PI 5% combined and then viable bacteria on the tissue plugs enumerated. Results: In broth, CHG demonstrated dose-dependent bactericidal activity, whereas PI activity was all-or-none. All isolates studied were similarly susceptible to CHG (MBCs: 0.0078% ± 0.0019%, 0.0069% ± 0.0026%, 0.0024% ± 0.0005%, 0.0024% ± 0.0005%, 0.0059% ± 0.00%, and 0.0029% ± 0.00%, respectively). The MBCs of PI were identical (0.625%) for all isolates. Overall, FBCI calculations showed indifference. Treatment of MSSA-infected porcine tissue for 2 hours demonstrated that the CHG-PI combination was superior to either antiseptic alone. Conclusion: FBCIs, determined in broth culture, indicate that combining CHG and PI had no negative impact on antisepsis. Moreover, data from an ex vivo porcine mucosal infection model suggest a potential benefit when combining the 2 antiseptic agents. Key Words: Chlorhexidine gluconate; povidone iodine; antiseptic; bacteria; fractional bactericidal concentration index.

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Nosocomial infection prevention involves a multi-pronged approach: infection control, antibiotic prophylaxis, and antisepsis.1 The emergence of multidrug-resistant organisms underscores the importance of using antiseptic agents as a strategy for infection prevention.2 In general, antiseptics have a broader spectrum of activity than antibiotics. Antiseptics often have multiple targets, reducing the likelihood that bacteria will develop resistance, whereas antibiotics tend to have specific intracellular targets. Also, resistance to antiseptics may be overcome by increasing the concentration or time of exposure.3 Chlorhexidine gluconate (CHG) and polyvinylpyrrolidone iodine (povidone iodine; PI) are 2 of the most commonly used antiseptic agents for antimicrobial skin preparation prior to surgery and central venous catheter (CVC) insertion.4 Many studies have compared the individual antiseptic activity of these 2 compounds, but few studies have evaluated the activity of the compounds in combination. In addition, studies investigating the combined effects of CHG and PI have applied the antiseptics sequentially. Langgartner et al concluded that skin disinfection with a propanol/CHG solution followed by PI was superior to either regimen alone in preventing CVC colonization, whereas Guzel et al found that cleaning the skin with 15% CHG followed by 10% PI was safe and effective for skin antisepsis prior to neurosurgical intervention.5,6 Our study examined the antimicrobial effects of an aqueous solution containing both CHG and PI. Therefore, the bacteria were exposed to the 2 compounds simultaneously.

CHG is a polycationic biguanide with broad-spectrum antimicrobial activity. CHG damages the...
outer bacterial surface layers, thereby promoting its own uptake and subsequently attacking the cytoplasmic or inner membrane of the organism.\textsuperscript{3, 7} At low concentrations, CHG reduces membrane fluidity, osmoregulation, and metabolic capacity.\textsuperscript{8} At higher concentrations, such as those in commercially available preparations, the membrane becomes liquid crystalline and loses its integrity.\textsuperscript{8} CHG has broad-spectrum activity against bacteria and yeast. It has low antiviral activity and is not sporicidal but can prevent spore activity against bacteria and yeast. It has low antiviral activity and is not sporicidal but can prevent spore development.\textsuperscript{5} In addition to its direct effect on microbes, CHG can bind to the outermost layer of the epidermis and to mucous membranes, which provides a lingering or persistent antimicrobial effect.\textsuperscript{9, 10}

PI is a complex of iodide and a solubilizing carrier, polyvinylpyrrolidone, which acts as a reservoir of “free” iodine (the active component). The iodine is slowly released and delivered to the bacterial cell surface where it penetrates the cell membrane and inactivates key cytosolic proteins, fatty acids, and nucleotides.\textsuperscript{11} Slow release of iodine from the PI complex in solution minimizes iodine toxicity towards mammalian cells. Iodine has broad-spectrum antibacterial activity, as well as activity against fungi, protozoa, viruses, and some bacterial spores.\textsuperscript{12}

CHG and PI have different cellular targets and different mechanisms of action. These differences may prove beneficial when using these 2 antiseptics in combination. CHG damages the outer membrane, which, in turn, would augment access to the intracellular targets necessary for the bactericidal action of PI. Moreover, PI’s activity is more immediate, whereas CHG’s activity occurs later and lingers,\textsuperscript{9, 13} implying that these 2 compounds may work cooperatively. In clinical practice, the use of CHG and PI in combination is commonly avoided despite a lack of evidence regarding functional incompatibility of these agents. The aim of this study was to determine whether the combined activity of CHG and PI against clinically relevant pathogens is inferior to the activity of either agent alone.

MATERIALS AND METHODS

Bacteria

Clinical isolates of methicillin-sensitive and methicillin-resistant \textit{Staphylococcus aureus} (MSSA and MRSA, respectively), methicillin-resistant \textit{S. aureus epidermidis} (MRSE), multidrug-resistant \textit{Acinetobacter baumannii} (resistant to cefazolin, cefuroxime, and ceftriaxone), \textit{Pseudomonas aeruginosa}, and a strain of \textit{Escherichia coli} (resistant to cefotaxime) were grown in Todd Hewitt broth at 37°C overnight. For the checkerboard microdilution assays, the overnight cultures were adjusted to an OD\textsubscript{595} (optical density) of \(\sim1.0\) or bacterial density of \(\sim10^7\) colony-forming units (CFU)/mL in cation-adjusted Mueller Hinton broth. The number of bacteria in the culture was verified using standard saline dilution and plating techniques. The adjusted bacterial suspensions were used to inoculate the 96-well microtiter plates resulting in inoculums of \(\sim10^5\) CFU/mL.

MSSA (MN8) was chosen as the model organism in the studies utilizing the ex vivo porcine model of infection. A 1-mL aliquot of an overnight culture grown in Todd Hewitt broth at 37°C was centrifuged to pellet the cells and washed in RPMI 1640 media (no supplements). The washed cell pellet was resuspended in RPMI 1640 media to an OD\textsubscript{595} of \(\sim0.5\), which resulted in \(\sim1 \times 10^6\) CFU in the 2-\(\mu\)L volume used to infect the tissue plugs.

Antiseptics

Aqueous CHG 3% wt/vol was prepared from a 20% wt/vol stock (Sigma-Aldrich Corporation, St. Louis, MO) in water and further diluted before addition to microtiter plates. CHG was tested over a concentration range of 0.0018% to 0.375%. The concentration (percent wt/vol) of CHG in commercial products ranges from 0.12% in oral rinses to 4% in topical skin cleansers.

Polyvinylpyrrolidone iodine (PI) 10% wt/vol (Sigma-Aldrich Corporation) was prepared from powder, in water. PI was tested over a concentration range of 0.078% to 2.5%. The concentration of PI in commercial preparations is usually 10%, but ophthalmic preparation solutions contain only 5%.

Higher concentrations were required for antiseptic activity in the porcine ex vivo mucosal experiments due to the interaction of CHG and PI with mucosal proteins. The concentrations of CHG and PI alone were 3% and 5%, respectively. After combining a 6% solution of CHG with an equal volume of 10% PI, the final concentrations of CHG and PI in the mixture were the same as the individual concentrations: 3% and 5%, respectively.

Microdilution assay

The checkerboard method was used to determine the minimum bactericidal concentration (MBC) of each antiseptic agent, both alone and in combination. We defined the MBC as the concentration that reduced the bacterial burden by \(\geq5\)-log\textsubscript{10} CFU/mL when compared with the bacterial burden in the growth control wells. This was an adaptation of the French standard T72300, which defines an antiseptic as bactericidal if the challenge inoculum is reduced by 5 log\textsubscript{10} following exposure for 1 minute.\textsuperscript{14} We selected a 2-hour end point to assess sustained bacterial killing based on the approximate time elapsed from preoperative skin
preparation to conclusion of routine surgical procedures.\textsuperscript{15,16} CHG and PI were 2-fold serially diluted across and down microtiter plates containing cation-adjusted Mueller Hinton broth. The bacterial suspension that was prepared from the overnight culture was used to inoculate the microtiter plates. The desired inoculum was $\sim 1 \times 10^6 \text{CFU/mL}$. The plates were incubated at $37^\circ\text{C}$ for 2 hours, and then an antiseptic neutralizing solution containing Triton X-100, Tween-80, lecithin, and sodium thiosulfate was added to each well. After neutralization, the number of viable bacteria in each well was determined by saline dilution and plating techniques using a WASP2 spiral plater (Microbiology International, Frederick, MD). After overnight incubation at $37^\circ\text{C}$, the colonies on the plates were counted using an automated colony counter (ProtoCOL; Microbiology International).

**Fractional bactericidal concentration index**

Fractional bactericidal concentration indexes (FBCIs) were calculated to determine the compatibility of CHG and PI when used in combination, as have been described previously.\textsuperscript{17,18} The concentration of PI in the presence of CHG that produced a $\geq 5\log_{10}$ decrease in bacterial density was divided by the concentration of PI that produced the same effect when used alone. The same calculation was performed for CHG. The sum of these 2 fractions is the FBCI:

$$\frac{\text{MBC}_{\text{PI in combination}}}{\text{MBC}_{\text{PI alone}}} + \frac{\text{MBC}_{\text{CHG in combination}}}{\text{MBC}_{\text{CHG alone}}}$$

The results were categorized as follows: FBCI < 0.5 ($S = $ synergistic); 0.5 $\geq$ FBCI < 1.0 ($PS = $ partially synergistic); FBCI = 1.0 ($A = $ additive); 1.0 $>$ FBCI $\leq$ 4.0 ($I = $ indifferent); and FBCI > 4.0 ($AN = $ antagonistic). The FBCI was determined for each organism a minimum of 3 times.

**Ex vivo porcine vaginal mucosa culture**

An ex vivo porcine vaginal mucosal infection model was utilized to determine the efficacy of CHG and PI alone and in combination in the presence of biologic tissues and proteins. Specimens of normal porcine vaginal mucosa were excised from animals at slaughter and transported to the laboratory in RPMI 1640 media supplemented with 10% fetal calf serum, penicillin (50 IU/mL), streptomycin (50 mg/mL), and amphotericin B (2.5 $\mu$g/mL). Tissue was utilized within 3 hours of excision. Tissue plugs of uniform size were obtained from the porcine vagina using a 5-mm biopsy punch. Excess muscle tissue was trimmed away with a scalpel. Tissue plugs were washed in fetal calf serum-supplemented antibiotic-free RPMI 1640 media 3 times. The plugs were then placed mucosal side up on a 0.4-$\mu$m cell culture insert in 6-well plates containing fresh serum and antibiotic-free RPMI 1640. Bacterial suspension (2 $\mu$L) containing approximately $1 \times 10^6 \text{CFU}$ of the MSSA isolate was pipetted onto the outer mucosal surface of each tissue plug. Inoculated tissue plugs were incubated at $37^\circ\text{C}$ in 7% CO$_2$ for 2 hours prior to treatment with antiseptics to allow the bacteria to recover. Uninoculated tissue plugs were included as controls. After 2 hours incubation of infected or control tissue plugs, 10 $\mu$L of each antiseptic agent alone or a mixture of both was applied to the mucosal surface using a pipet. Tissue plugs were returned to the incubator ($37^\circ\text{C}, 7\%\text{ CO}_2$) for a 2-hour treatment period. After incubation, tissue plugs were transferred to 5-mL centrifuge tubes containing 250 $\mu$L of the same solution that was used to neutralize the activity of CHG and PI in the broth studies (Triton X-100, Tween-80, lecithin, and sodium thiosulfate). Each plug was homogenized for 30 seconds using an Omni International tissue homogenizer (Marietta, GA) at the highest setting to release bacteria into solution. Tissue homogenates that were either neat or serially diluted in phosphate-buffered saline were applied to sheep blood agar plates using a spiral plater (Microbiology International). The following day,
colonies were enumerated using an automated colony counter (ProtoCOL; Microbiology International).

RESULTS

This study determined the antiseptic activity of CHG and PI alone and when applied simultaneously against clinically relevant nosocomial pathogens: MSSA, MRSA, MRSE, A baumannii, P aeruginosa, and E coli. In general, low concentrations of CHG (<0.00295%, MRSA; <0.000365, A baumannii) were determined to have no effect on CFU/mL over the 2-hour time frame of the microbroth experiments. These results implied that CHG was bacteriostatic at low concentrations (Fig 1A), which was consistent with previous reports.7 At higher concentrations (>0.008%), CHG was bactericidal (≥5-log10 decrease in CFU/mL) for all organisms tested. Conversely, the activity of PI was all-or-none (Fig 1B). Once the bactericidal concentration threshold (0.625%) was reached, there was a ≥5-log10 decrease in the number of viable bacteria for all studied organisms.

All study organisms were similarly susceptible to CHG. MBCs of CHG alone were 0.0078% ± 0.0019%, 0.0069% ± 0.0026%, 0.0024% ± 0.0005%, 0.0024% ± 0.0005%, 0.0059% ± 0.0%, and 0.0029% ± 0% for MSSA, MRSA, MRSE, A baumannii, P aeruginosa, and E coli, respectively (Table 1). The MBC of PI alone was identical (0.625%) in 3 or more independent experiments and across the different species tested (Table 1). As reported previously, CHG was a great deal more potent than PI.13,19,20

Bactericidal activities of CHG and PI combined against MRSA, a representative gram-positive organism, and A baumannii, a representative gram-negative organism, are illustrated in Fig 2. Synergy was determined if the kill-curve generated using data from the 2-agent combinations shifted to the left of the curve that was generated using single agent data, ie, the concentration of the single agent (depicted on the x-axis) that was necessary to produce a ≥5-log10 decrease in the number of viable bacteria was significantly reduced when that agent was combined with sub-MBC concentrations of the second agent. For the MRSA isolate, when sub-MBCs of PI (<0.625%) were combined with sub-MBC of CHG (<0.0069%), the CHG curve was not affected, ie, no shift (Fig 2A). When sub-MBCs of CHG were combined with a sub-MBC of PI, the bacterial load was reduced, but the concentration of PI required to produce ≥5-log10 decrease in the number of viable bacteria remained at 0.625% (Fig 2B). The data for A baumannii showed a slight shift to the left when sub-MBCs of PI (<0.625%) were combined with CHG (<0.0024%). However, this shift was not great enough to suggest additivity or synergism (Fig 2C). When sub-MBCs of CHG were combined with PI at a sub-MBC, the number of bacteria recovered decreased. The higher concentrations of CHG (0.002930% and 0.005860%) were above the MBC of CHG alone (0.0024%) and, predictably, produced a ≥5-log10 decrease in the number of viable bacteria (Fig 2D).

Table 1 lists the FBCIs for each of the bacteria, which were calculated to determine the overall effect of combining CHG and PI, ie, antagonistic, synergistic, additive, or indifferent. Overall, using the microbroth dilution assay, the combination of CHG and PI had no effect on antisepsis; FBCIs = indifferent for all organisms (1.0 > FBCI ≤ 4.0). In 3 independent experiments, the average FBCIs for MSSA, MRSA, MRSE, A baumannii, P aeruginosa, and E coli were 2.00, 2.33, 1.85, 1.49, 1.66, and 2.83, respectively. The average FBCI for E coli was the highest at 2.83 but still within the range of indifference for 2 out of 3 experiments.

Finally, treatment of MSSA-infected porcine mucosal tissue with either 5% PI or 5% CHG individually for 2 hours resulted in numerically lower bacterial counts compared with untreated control (Fig 3). When PI and CHG were combined in solution before applying to the infected tissue, the resulting decrease in bacterial burden (>3-log10) was significantly different from control tissues.

<p>| Table 1. MBCs and FBCIs of PI and CHG alone and in combination against 6 nosocomial pathogens* |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Organism</th>
<th>MBC of PI alone</th>
<th>MBC of PI combined</th>
<th>MBC of CHG alone</th>
<th>MBC of CHG combined</th>
<th>FBCI</th>
</tr>
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<td>0.625</td>
<td>0.0069</td>
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<td>0.0024</td>
<td>0.0023</td>
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<tr>
<td>Acinetobacter baumannii</td>
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<td>0.519</td>
<td>0.0024</td>
<td>0.0017</td>
<td>1.49</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
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<td>0.519</td>
<td>0.0059</td>
<td>0.0049</td>
<td>1.66</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>0.625</td>
<td>0.625</td>
<td>0.0029</td>
<td>0.0053</td>
<td>2.83</td>
</tr>
</tbody>
</table>

MBC, minimum bactericidal concentration defined as the concentration that reduced the bacterial burden by >"greater than or equal to" 5-log10 CFU/mL when compared with the bacterial burden of the growth control.

*Average MBCs of PI and CHG alone, average MBCs of PI when combined with CHG, average MBCs of sub-MBC concentrations of CHG when combined with sub-MBC concentrations of PI, and the calculated FBCIs of the combination.

FBCI = (MBC PI in combination)/(MBC PI alone) + (MBC CHG in combination)/(MBC CHG alone); FBCI < 0.5 (S = synergistic); 0.5 ≤ FBCI < 1 (PS = partially synergistic); FBCI = 1 (A = additive); 1.0 > FBCI ≤ 4.0 (I = indifferent); and FBCI > 4.0 (AN = antagonistic).
DISCUSSION

The aim of this study was to evaluate the potential interaction between CHG and PI and the effect the combination had on antimicrobial activity using checkerboard microdilution assays and infected porcine mucosal tissue explants. The efficacy of these antiseptics used individually is well established. However, to our knowledge, this is the first report evaluating the efficacy of these 2 antiseptic agents used concurrently. PI has rapid bactericidal activity, but activity is diminished shortly after contact with organic matter present in skin.21,22 CHG is not as fast acting but exhibits sustained antimicrobial activity and is not readily neutralized by organic matter.7 We surmised that these 2 agents would compliment one another by creating an initial and rapid kill of resident flora followed by a sustained antibacterial effect, which could prove quite beneficial in the clinical setting.

Our strategy for determining the efficacy of PI and CHG when used concurrently against nosocomial pathogens differed from previously published studies. First, we chose a well-established in vitro checkerboard microbroth dilution method but determined the efficacy at 2 hours instead of the 18 to 24 hours used commonly when determining minimum inhibitory concentrations (MICs) and MBCs in other studies.9,23,24 We chose a 2-hour end point to more closely approximate in-use conditions when antiseptics are employed to decontaminate skin prior to surgery and to evaluate the prolonged effect during surgery.15,16 Second, we defined the MBC as the concentration (percent wt/vol) that reduced the bacterial burden by $5\log_{10}$ CFU/mL when compared with the bacterial burden in the growth control wells when typically MBC is defined as 99.9% or 3-log$_{10}$ kill of the original inoculum.9,23,24 FBCI was calculated using the MBC data obtained using broth microdilution checkerboard techniques. FBCI, also referred to as the interaction index or $\Sigma$FBC, mathematically expresses the interaction between 2 antimicrobial agents and is an accepted method for determining synergism.25 Finally, we chose to validate the in vitro findings using an ex vivo full-thickness mucosal model because in vitro broth techniques have been shown to overestimate the antibacterial efficacy of biocides.5,21,22

The results from the checkerboard microdilution assays performed using the 6 nosocomial organisms indicate that there is no functional incompatibility when combining CHG and PI. In fact, at sub-MBC concentrations, CHG may enhance the antiseptic activity of PI alone. Furthermore, our ex vivo full-thickness mucosal model of infection suggests that there may be a benefit to combining the 2 antiseptic agents against MSSA. It is well-known that CHG and PI are useful skin antiseptics, but simultaneous application of these 2 agents for use in clinical situations is rare despite a lack of evidence to

(P < .05, analysis of variance followed by Bonferroni post-test for multiple comparisons, n = 3).
support incompatibility. Recently, there have been 2 reports in the literature in which sequential use of CHG and PI was superior to use of either agent alone. Langgartner et al demonstrated that bacterial colonization of CVCs was significantly reduced when skin was disinfected with a propanol/CHG preparation for 1 minute followed by PI disinfection for 1 minute prior to CVC insertion. The authors conclude that CHG and PI may have synergistic disinfectant activity. In another study, skin flora was evaluated after cleaning the skin with CHG and PI for preparation prior to neurosurgery. Guzel et al showed that a small amount of bacteria persisted after the CHG preparation but that, when CHG was followed by PI application, the cultures showed no growth. Together, our data and these recent reports suggest that there may be clinical benefits to using a combination of these agents to prepare skin prior to surgery.

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References