Efficacy of combination of chlorhexidine and protamine sulphate against device-associated pathogens

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Objectives: The objectives of this study were to examine: (i) the potential in vitro synergy of combining protamine sulphate (PS) with chlorhexidine (CHX); (ii) the in vitro spectrum and durability of antimicrobial activity of CHX + PS-coated catheters; and (iii) the in vivo efficacy of CHX + PS-coated catheters in comparison with silver-hydrogel-coated and uncoated catheters.

Methods: The potential synergistic antimicrobial and antibiofilm activities of CHX and PS were investigated in vitro by the MIC and biofilm assays. The spectrum and durability of antimicrobial activity of CHX + PS-coated catheters were studied in vitro by using a serial plate transfer method. The in vivo efficacy of CHX + PS-coated catheters was assessed in a rabbit model against Escherichia coli.

Results: In vitro studies showed that the combination of CHX + PS has a synergistic inhibitory effect on E. coli and provides a significant synergistic antibiofilm and antimicrobial activity against E. coli, Pseudomonas aeruginosa and Staphylococcus epidermidis. Furthermore, catheters coated with CHX + PS provided a broad-spectrum and enduring in vitro antimicrobial activity over a 10 day period. The in vivo efficacy study demonstrated that subcutaneously implanted CHX + PS-coated catheters in rabbits were significantly less likely to become colonized (2/28 = 7%) than either silver-hydrogel-coated (25/28 = 89%; P < 0.001) or uncoated catheters (18/28 = 64%; P < 0.001) by E. coli.

Conclusions: The synergistic, broad-spectrum and durable in vitro activity of the CHX + PS combination and the robust in vivo efficacy of catheters coated with this unique composition encourage clinical evaluation of this innovative approach.

Keywords: device-related infections, antimicrobial activity, Escherichia coli

Introduction

Medical devices constitute an increasingly essential component of modern healthcare. Infection is generally the most common serious complication of these devices. The escalating use of long-established and new medical devices has further magnified the problem of device-associated infections, particularly in patients at inherently high risk of infection.1 Device-associated infections are generally cumbersome to manage, have great impact on the quality of life, result in excessive prolongation of hospital stays and are expensive to treat.2–5 The serious medical complications, problematic management and economic sequelae of device-related infections have prompted a keen interest in exploring innovative preventive approaches.6

Bacterial colonization of the medical devices is a prelude to device-related infection. Antimicrobial modification of the surface of medical devices has the potential of resisting bacterial adherence to the device and, possibly, preventing device-related infection. Although the lack of in vitro efficacy usually indicates the absence of in vivo efficacy, not all antimicrobial-coated surfaces that repel bacterial adherence in vitro can prevent device colonization and device-related infection in vivo. This variable in vivo efficacy could be attributed, at least in part, to the type of antimicrobial agents used for surface treatment of the device. We elected to explore the efficacy of the unique combination of chlorhexidine plus protamine sulphate (CHX + PS) because: (i) CHX possesses broad-spectrum antimicrobial activity7 that is partly attributed to disruption of the cellular plasma membrane,8

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and the cationic polypeptide PS increases the permeability of cell membrane, thereby facilitating the transport of antimicrobial compounds to the cytoplasm;\textsuperscript{9,10} and (ii) both CHX\textsuperscript{11} and PS\textsuperscript{12} have been separately reported to be active \textit{in vivo} against biofilm-associated infections.

The objectives of this study were to examine: (i) the potential \textit{in vitro} synergy of combining CHX and PS; (ii) the \textit{in vitro} antimicrobial activity and durability of CHX + PS-coated catheters; and (iii) the \textit{in vivo} anti-infective efficacy of CHX + PS-coated catheters in comparison with silver-hydrogel-coated and uncoated catheters. Since urinary catheters are the most commonly utilized medical devices and are associated with a relatively high rate of colonization and infection,\textsuperscript{13,14} we used bladder catheters in this study.

Materials and methods

Chemicals, microorganisms and culture conditions

All used chemicals (including media ingredients) were of analytical grade and purchased from Sigma-Aldrich (St Louis, MO, USA) or BD Diagnostic Systems (Sparks, MD, USA). We tested eight device-associated clinical isolates of \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, \textit{Proteus mirabilis}, \textit{Pseudomonas aeruginosa}, \textit{Staphylococcus aureus}, \textit{Enterococcus faecalis}, vancomycin-resistant \textit{Enterococcus faecium} (VRE) and \textit{Candida albicans}, plus one American Type Culture Collection (ATCC) isolate of \textit{Staphylococcus epidermidis}. All strains were maintained at \(-80\)^\circ\text{C} in 15\% glycerol and recovered onto Luria–Bertani (LB) agar or tryptic soy agar (TSA). The assay for zone of inhibition and microbiological assessment of catheter segments explanted from animals were performed using Mueller–Hinton agar and microtiter plates (Corning Inc., New York, NY, USA). The 100-fold concentrated aqueous stock solutions of CHX and PS were prepared by sterile distilled water. The wells without antimicrobial agents served as control. After incubation for 24 h, the medium containing planktonic cells in each well was removed and the biofilm was rinsed with PBS. A 2 mL of PBS to each well was sonicated for 1 min and the disaggregated biofilm was mixed well with the pipette tip, as previously reported.\textsuperscript{16} The 1 mL suspension from each well was then diluted 10-fold and 100 \(\mu\text{L}\) aliquots from each dilution were inoculated onto TSA plates. The plates were incubated at 37\(^\circ\text{C}\) for 24 h and colonies were enumerated.

Catheters

We tested three groups of 14 French urinary catheters: (i) sterile uncoated silicone catheters (Rusch, Inc., GA, USA); (ii) silicone catheters (Rusch, Inc.) that we coated with CHX + PS by dipping in a solution that contained CHX (400 mg/mL) and PS (100 mg/mL)\textsuperscript{17} and then gas sterilized with ethylene oxide; and (iii) sterile silver-hydrogel-coated catheters (Bardex I.C.\textsuperscript{6} Foley catheter, C.R. Bard, Inc., Covington, GA, USA), the most commonly utilized surface-modified urinary catheter in North America.

In \textit{vivo} spectrum and durability of antimicrobial activity of coated catheters

The \textit{in vitro} spectrum and durability of CHX + PS-coated catheters in comparison with control uncoated catheters were assessed by using a modified Kirby–Bauer technique.\textsuperscript{18} One centimetre long segments of uncoated and CHX + PS-coated silicone catheters (4 mm external diameter) were pressed onto the centre of the agar plate that had been freshly inoculated with one of the six tested organisms. After incubating the agar plate at 37\(^\circ\text{C}\) for 24 h, the overall zone of inhibition was determined by measuring the diameter of the clear zone of inhibition perpendicular to the long axis of the catheter segment. The actual zones of inhibition were recorded by subtracting the external diameter of the catheter (4 mm) from each measurement. The catheter segments were transferred daily to fresh lawns on Mueller–Hinton agar. The diameter of the zone of inhibition (in mm) is expressed as the mean value from three tested samples.

In the clinical setting, coated urinary catheters would be in constant contact with urine, thereby allowing for detachment of the antimicrobial coating agents. To simulate this process, the CHX + PS-coated and uncoated control catheter segments were placed in separate 500 mL flasks and soaked in 100 mL of sterile artificial urine medium.\textsuperscript{19} The flasks were incubated at 37\(^\circ\text{C}\) with 100 rpm shaking. Half of the artificial urine medium was replaced by fresh medium every 2 days, as described previously.\textsuperscript{20} Catheter segments
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were removed from the flask after 1, 3, 5 and 7 days to perform adhesion assay against \( E. \ coli \) and \( S. \ epidermidis \).\(^{21} \) The catheter segments were rinsed in 1 mL PBS and placed in 10 mL BHI broth. The tubes were inoculated with 100 \( \mu \)L of bacterial suspension (10\(^7\) cfu/mL) and incubated in a water bath for 3 h at 37 C with gentle shaking. After incubation, the catheter segments were washed three times in sterile normal saline and transferred to 1 mL of sterile normal saline. Adherent bacteria were removed by sonication for 30 s, followed by vortexing for 1 min. Bacterial cells were serially diluted in saline and then plated onto LB agar plates. Plates were incubated at 37 C for 24 h and colonies were counted.

In vivo efficacy of coated catheters in rabbit model of \( E. \ coli \) infection

The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at Baylor College of Medicine. A previously described rabbit model of Gram-negative bacterial infection of subcutaneously inserted devices was used.\(^{21} \) Female New-Zealand White, specific pathogen-free rabbits (body weight 2–3 kg) were anaesthetized by intramuscular injection (0.5 mL/kg body weight) of a mixture of ketamine (70 mg/kg body weight) and acepromazine (2 mg/kg body weight). To simulate the practice of administering peri-operative antibiotic prophylaxis in human patients, each animal received immediately after induction of anaesthesia an intramuscular injection of vancomycin (20 mg/kg), which is active against Gram-positive organisms but not \( E. \ coli \). The backs of rabbits were shaved, then prepared and draped in a sterile fashion. Six 2 cm long catheter segments, including two CHX + PS-coated, two silver-hydrogel-coated and two uncoated, were subcutaneously inserted 4 cm lateral to the spine and away from each other. A total of 84 catheter segments were placed in 14 rabbits. Based on the findings of a pilot trial that was initially performed to determine the optimal bacterial inoculum required for colonization of most catheter segments, a 50 \( \mu \)L aliquot of \( 10^5 \) cfu/mL for a clinical strain of \( E. \ coli \) was inoculated onto the surface of inserted catheter segments. After suturing the wounds and achieving sternal recumbence, the rabbits were monitored daily for signs of local infection, sepsis or major distress.

Bacteriological assessment

Rabbits were sacrificed 1 week after surgery. Blood samples were collected from rabbits prior to euthanasia and inoculated onto trypticase sheep agar plates containing 10% sheep blood. To prevent possible contamination of the catheter segment during explantation by organisms at and around the surgical site, an incision was made 2 cm medial to the surgical wound to remove the catheter segment utilizing an electrosurgical cutter. All retrieved catheter segments were quantitatively cultured by using the sonication technique, with a detectability limit of 20 cfu.\(^{21} \) After explanting the catheter segments, qualitative swab cultures of the site adjacent to the catheter segments were obtained. Bacterial growth was assessed after incubating the inoculated agar plates at 37 C for 48 h.

The two primary outcomes of the animal study were catheter colonization and catheter-related infection. Catheter colonization was defined as isolation of the inoculated strain of \( E. \ coli \) from sonication cultures of explanted catheter segment. The diagnosis of catheter-related infection was made in the presence of \( E. \ coli \) colonization of explanted catheter segments plus growth of \( E. \ coli \) from swab cultures of subcutaneous tissue adjacent to the catheter segment. A secondary outcome of the mean bacterial cfu retrieved from the explanted catheter segments was also compared among the three study groups.

Statistical analysis

The rates of catheter colonization and catheter-related infection were compared between the different groups by using a two-tailed Fisher’s exact test (STATA, version 8; Stata-Corp., College Station, TX, USA). The mean bacterial counts retrieved from the explanted catheter segments were compared among the three groups by using the two-tailed Student’s t-test with unequal variance. A \( P \) value of <0.05 indicated significant differences.

Results

Antimicrobial activity of CHX and PS

The MICs of CHX, PS and the CHX + PS combination for bacterial and fungal organisms are shown in Table 1. The MICs of CHX ranged from 0.195 (for \( S. \ epidermidis \)) to 12.5 mg/L (for \( P. \ aeruginosa \)) and were generally lower than the MICs of PS which ranged from 3.25 (for \( S. \ epidermidis \)) to >200 mg/L (for \( K. \ pneumoniae \) and \( P. \ mirabilis \)). The FICI for CHX + PS combination varied from 0.5 to 1.25 with an appreciable synergistic inhibitory effect on \( E. \ coli \).

Antibiofilm activity of CHX and PS

As Figure 1 shows, the combination of CHX + PS significantly inhibited biofilm formation due to \( E. \ coli \), \( P. \ aeruginosa \) and \( S. \ epidermidis \) as compared with either no antimicrobial agent or individual antimicrobial agents (\( P < 0.05 \)), thereby indicating possible synergistic effects between CHX and PS. The biofilm formed by \( P. \ aeruginosa \) was almost completely inhibited when CHX and PS were used together at a concentration of 25 mg/L each. Although synergy between CHX and PS was not observed when assessing biofilm formation by \( K. \ pneumoniae \), \( E. \ faecalis \) and \( C. \ albicans \), the CHX + PS combination inhibited more than 99% biofilm formation by each of these three organisms (0.0001%, 0.01% and 0.001%, respectively).

In vitro spectrum and durability of antimicrobial activity of CHX + PS-coated catheters

The CHX + PS-coated catheter segments displayed zones of inhibition against all six tested organisms, with enduring antimicrobial activity after 10 days of serial plate transfer (Figure 2). No zone of inhibition was detected around the control uncoated catheter segments. Furthermore, antimicrobial coating of catheters prevented \( \geq 80\% \) of \( E. \ coli \) and \( S. \ epidermidis \) colonization after soaking the catheter in sterile artificial urine medium for 1, 3, 5 and 7 days (Figure 3).

In vivo efficacy of CHX + PS-coated catheters

All 14 rabbits tolerated surgery well and exhibited no evidence of sepsis or failure to thrive. Blood cultures from all 14 sacrificed rabbits were sterile. Two out of 28 (7%) CHX + PS-coated catheters, 25/28 (89%) silver-hydrogel-coated catheters and 18/28 (64%) uncoated catheters became colonized with \( E. \ coli \).
The CHX + PS-coated catheters were significantly less likely to be colonized than either silver-hydrogel-coated catheters \( (P < 0.001) \) or uncoated catheters \( (P < 0.001) \), and there was no significant difference \( (P = 0.055) \) in the rate of colonization of silver-hydrogel-coated versus uncoated catheters. Furthermore, 1/28 (4%) CHX + PS-coated catheters, 12/28 (43%) silver-hydrogel-coated catheters and 14/28 (50%) uncoated catheters resulted in infection due to \( E. \) coli. The CHX + PS-coated catheters were significantly less likely to cause infection than either silver-hydrogel-coated catheters \( (P = 0.001) \) or uncoated catheters \( (P < 0.001) \), and there was no significant difference \( (P = 0.79) \) in the incidence of infection caused by silver-hydrogel-coated versus uncoated catheters.

The mean numbers of bacterial colony counts retrieved from catheter segments were 4.6 \( \times 10^6 \) cfu in the CHX + PS-coated catheter group, 2.5 \( \times 10^6 \) cfu in the silver-hydrogel group and 8.3 \( \times 10^6 \) cfu in the uncoated group. The mean number of bacterial colony counts retrieved from uncoated catheter segments was significantly higher than those originating from CHX + PS-coated \( (P = 0.028) \) or silver-hydrogel-coated catheters \( (P = 0.023) \). There were, however, no significant differences in the mean number of bacterial colony counts retrieved from CHX + PS-coated catheters versus silver-hydrogel-coated catheters \( (P = 0.23) \).

**Discussion**

We assessed the potential antimicrobial enhancing effect of PS on the antimicrobial activity of CHX against planktonic catheter-associated organisms and found either synergy or no interaction between CHX and PS. The CHX + PS combination was notably synergistic against \( E. \) coli, a very common uropathogen, and was not antagonistic against any of the other tested pathogens.

Because of the relatively slow bacterial growth within the biofilm, inhibition of antimicrobial activity by biofilm substances and poor penetration of some antimicrobials into the biofilm, the MICs for biofilm-embedded organisms can be up to 100–1000-fold higher than those for planktonic counterparts. The results of this study reaffirm the patterns of resistance to antimicrobial agents by biofilm-embedded organisms. For instance, a CHX concentration of 12.5 mg/L CHX, which is 64-fold higher than its MIC (0.195 mg/L) for planktonic \( S. \) epidermidis, was 1000-fold higher than its MIC (0.195 mg/L) for planktonic \( S. \) epidermidis organisms, failed to completely inhibit the growth of biofilm-embedded \( S. \) epidermidis. Although one option to counter this clinical quandary would be to use antibiotics at supratherapeutic concentrations as is the case with antibiotic lock solutions, the use of high concentrations of CHX poses safety concerns in the clinical setting. Therefore, in this study, we evaluated the impact of combining PS with CHX in an attempt to enhance the antimicrobial activity of CHX. The CHX + PS combination was significantly more active against \( E. \) coli, \( P. \) aeruginosa and \( S. \) epidermidis biofilms than either agent alone. These results support previous reports describing the synergistic antimicrobial and antibiofilm impact of combining PS with either antibiotics (rifampicin, ciprofloxacin or vancomycin) or non-antibiotic compounds (\( N,N-[1,2 \) phenylene] dimaleimide, EDTA or ovotransferrin) both in vitro and in vivo. Furthermore, PS has been reported to bind firmly to surfaces such as stainless steels and to inhibit surface colonization by \( Staphylococcus \) spp.33
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CHX + PS-coated devices are intended to inhibit bacterial presence both on the device surface and within the biofilm layer surrounding the implanted devices. The biofilm layer contains a variety of host-derived adhesins to which different organisms variably adhere. For instance, *S. aureus* adheres tightly to fibronectin, fibrinogen, collagen and, to a lesser extent, laminin, whereas *S. epidermidis* adheres only to fibronectin but not to the other host-derived adhesins. 34,35 Gram-negative bacilli, enterococci and *Candida* account for about two-thirds, one-quarter and one-tenth, respectively, of catheter-associated urinary tract infections, whereas staphylococci, Gram-negative bacilli and *Candida* account for almost three-quarters, one-fifth and one-tenth, respectively, of infections associated with central venous catheters. 1,36 The results of this study indicate that devices coated with the combination of CHX and PS provide in vitro durability against a variety of Gram-negative bacilli (*E. coli* and *K. pneumoniae*) and Gram-positive cocci (*E. faecalis*, *S. epidermidis* and *S. aureus*) as well as *C. albicans*. In addition, coated catheters exhibited significantly less colonization (*P*, 0.05) between viable cells adherent to CHX + PS-coated catheter and uncoated control catheter.

Lower numbers of *E. coli* organisms adhered in vivo to both the silver-hydrogel-coated and the CHX + PS-coated catheters as compared with uncoated catheters. Even more importantly, this study demonstrated that devices whose surfaces were modified with CHX + PS, but not with silver-hydrogel, reduced in vivo the incidence of device colonization and device-related infection by *E. coli*. This superior in vivo efficacy of CHX + PS-coated versus silver-hydrogel-coated catheters against infection by *E. coli* may be attributed to three factors: (i) production of appreciable zones of inhibition by the former but not the
latter catheters (because silver molecules are relatively tightly bound to the surface of the catheter) taking into consideration that the production of no or small zone of inhibition in vitro by other types of antimicrobial coatings can predict the lack of anti-infective efficacy in vivo;37 (ii) the relatively long in vitro durability of antimicrobial activity of CHX + PS-coated catheters against catheter-associated pathogens, in comparison with the previously reported findings on silver-hydrogel-coated catheters;38 and (iii) the in vitro synergistic antibiofilm activity of the CHX + PS combination that could be attributed to the ability of PS to increase the permeability of microbial cell membrane and dilate the ion channels, thereby facilitating the transport of other antimicrobial compounds to the cytoplasm.9

In summary, this study indicated that the CHX + PS combination may provide a strong and synergistic antimicrobial and antibiofilm activity in vitro and, more importantly, demonstrated a robust in vivo protection against E. coli that is afforded by incorporating this unique antimicrobial composition onto devices. It would be prudent to further evaluate the potential of using the CHX + PS composition as an anti-infective coating to prevent infections associated with medical devices, particularly those that are primarily infected by Gram-negative bacteria as is the case with urological and intra-abdominal devices. As with other types of antimicrobial-coated devices, it is essential to judiciously limit the use of such anti-infective approaches to patients who either have an unacceptably high risk of infection or in whom infection can lead to serious medical consequences and/or economic sequelae.

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Transparency declarations

The right to the coating method that was used to incorporate the combination of CHX and PS onto the surfaces of devices was assigned by Dr Darouiche and Mr Mansouri to their employer Baylor College of Medicine. Dr Darouiche has served as a consultant for Kane Biotech Inc.

References


