Eradication of microorganisms embedded in biofilm by an ethanol-based catheter lock solution

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Abstract

Background. Interdialytic locking of catheters with antimicrobial agents is frequently used for preventing catheter-related infections, often associated with biofilm formation. We determined the bactericidal effect of 60% ethanol (ETOH) versus a 46.7% trisodium citrate (TSC) solution on biofilm embedded in silicone catheters.

Methods. Four- and 24-h biofilms of Staphylococcus aureus, S. epidermidis, Pseudomonas aeruginosa, Klebsiella pneumoniae and Candida albicans established in a microfermentor were exposed to ETOH and TSC for up to 24 h and the number of remaining viable microorganisms was determined.

Results. ETOH 60% was significantly more effective than 46.7% TSC in rapidly eradicating sessile cells from all microorganisms tested. A 20-min ETOH 60% treatment completely eradicated the Gram-negative bacilli and C. albicans biofilms, which initially contained up to 10^8 and 10^5 cells, respectively. Gram-positive cocci biofilms only showed a significant 2.6–4.3 log reduction in the initial viable counts after 20 min of ETOH 60% treatment, with eradication occurring after 30 min. Confocal laser scanning microscopy observation of ETOH-treated biofilm showed sparse cells with respiratory activity. TSC 46.7% eradicated none of the tested microorganisms. In contrast, ETOH 60% totally eradicated planktonic cells, whereas TSC had significant bactericidal activity against K. pneumoniae, P. aeruginosa and C. albicans after 20 min, 1 and 24 h, respectively, but none on the Staphylococcus species.

Conclusions. This in vitro study demonstrates the superior antimicrobial activity of ETOH 60% in contrast to TSC 46.7% in eradicating biofilm formed on a silicon catheter. Hence, ethanol-based solution shows promise as a catheter lock solution.

Keywords: biofilm; catheter; infection; lock solution

Introduction

Although native arteriovenous fistula is the recommended vascular access for haemodialysis provision [1], haemodialysis catheters are being increasingly used [2] because of fistula failure, late recognition of end-stage renal disease, difficulty in achieving placement and shortage of resource. The use of catheters for dialysis provision in end-stage renal disease patients is associated with an increase in life-threatening infectious complications, which are major sources of morbidity, mortality and overcosts [3]. In these patients, the incidence of catheter-related bacteraemia
varies widely, ranging from 2 to 6 per 1000 catheter days [3].

Bacterial growth on the inner surface of the catheter with biofilm formation is frequent and may occur within days of catheter placement [4,5]. Catheter-related bacteraemia often arises from this biofilm [4–7] most frequently associated with coagulase-negative staphylococci and *Staphylococcus aureus*, but other important pathogens such as Gram-negative bacilli and *Candida albicans* can also be involved [8]. Systemic antibiotics frequently fail to treat biofilm-associated infections since sessile bacteria within the biofilms are usually less susceptible to killing by antimicrobial agents than their rapidly growing planktonic counterpart cells [9,10]. The inhibition of biofilm formation represents an attractive strategy for preventing catheter-associated infections. Filling catheter lumens with very high concentrations of antibiotics for extended periods (antibiotic locks) achieves supraphysiologic antibiotic concentrations that overcome the relative antimicrobial resistance of sessile bacteria. A variety of antibiotics have been used as prophylactic antimicrobial dialysis catheter lock solutions, including gentamycin, cefazolin plus gentamicin, minocyclin or cefotaxime, and were effective in preventing catheter-related bacteraemia [11–15]. However, there are concerns, because of the potential development of resistant organisms and the risk for systemic toxicity due to antibiotic solution leak from the catheter lumen into the circulation [16]. Non-toxic antibiotic agents are, therefore, greatly needed. Several studies indicate that 4% citrate plus 1.35% taurolidine [17] or 30% citrate [18] may be effective in preventing catheter-related bacteraemia. However, an in vitro study shows that taurolidine fails to eradicate *Pseudomonas aeruginosa* or *Candida* sp. biofilm [19], and studies on the efficacy of 30% citrate to eradicate sessile bacteria are scarce.

Concentrated ethanol (ETOH) might be a promising lock solution because of its antimicrobial activity against a broad range of planktonic bacteria and fungi, its low cost and its universal availability. There is no evidence of acquired resistance to concentrated ETOH despite its extensive and longstanding use as an antiseptic. Nor are there any studies showing hypersensitivity related to ETOH administration. Preliminary reports suggest that catheter locking with concentrated ETOH can be performed without severe side effects [20–23]. However, there are only few data on the ability of concentrated ETOH to eliminate sessile bacteria [24,25]. The aim of this study was to determine the time killing curve of 60% ETOH and 46.7% citrate on biofilm of five clinically relevant microorganisms.

**Materials and methods**

**Microbial strains and culture conditions**

*Staphylococcus epidermidis* CIP 68.21, *S. aureus* CIP 65.25 (methicillin resistant), *P. aeruginosa* ATCC 27853, and *Klebsiella pneumoniae* LM21 and *C. albicans* SC5314 were selected for the study. Bacterial strains were grown on the Luria Bertani medium (M63B1) and the fungal species in the 0.67% yeast nitrogen base (YNB, Difco) supplemented with 0.4% glucose. The organisms were maintained at −70 °C in their respective medium with 15% glycerol, and on each occasion the biofilm was established from the original stock.

**Silicone catheter biofilm formation**

Biofilm formation on a silicone catheter was performed in 60-ml aerated microfermentors as described by Ghigo [26] and at www.pasteur.fr/recherche/unites/Ggh/matmet.html. Sterile silicone catheter segments (each 1 cm long) (DualCath®, Medcomp, Harleyville, PA, USA, and Hemotech, Ramonville, France) were fixed onto the internal removable glass slide of the microfermentors. Strains from the frozen stocks were cultured in the M63B1-0.4% Glu or YNB-0.4% Glu medium overnight. An inoculum of 10⁷ bacilli, 10⁷ cocci or 10⁷ *C. albicans* cells was used to inoculate microfermentors containing the silicone segments. Continuous flow of 100 ml/h of either M63B1-0.4% Glu medium (bacterial strains) or YNB-0.4% Glu (yeast) and constant aeration with sterile press air (0.3 bar) were used to obtain continuous flow-through culture conditions. Our experimental model received a high input of fresh medium to avoid significant planktonic growth. After 4 and 24 h of incubation, the catheter segments were removed from the incubator and separated from the device. The biofilms formed on the catheter segments were resuspended in 5 ml M63B1 minimal or YNB medium by sonication and vortexing. Serial dilutions of the resulting suspensions were performed and plated onto appropriate agar plates to determine the number of viable cells [Colonel Forming Unit (CFU)] after an overnight incubation at 37 °C. The bacteria count was expressed as a decimal logarithm (log10); the limit of detection in our experimental conditions was 1.6 log10 (40 CFU) per catheter segment.

**Treatment protocol**

The antibiotic activities of both ETOH 60% (v/v) and trisodium citrate (TSC) were determined using 4- and 24-h biofilms. After incubation, the catheter segments harbouring biofilm were removed. Each segment was carefully rinsed in 1 ml of saline, and then placed in a tube containing 1 ml of the following lock solutions: (i) ETOH 60% (prepared with 95% ETOH diluted in a 0.9% sodium chloride solution), (ii) 46.7% TSC (CitraLock(tm), Hemotech, Ramonville, France) and (iii) saline (control). For every organism, the experiments were repeated in triplicate or quadruplicate, and during each treatment assay, catheter segments were exposed to the different solutions for up to 24 h at 37 °C. To determine the effects of decreasing concentrations of ETOH over time, 24-h *S. epidermidis* biofilm was treated with ETOH 60% for 10 min, followed by ETOH 40% and 20%, respectively for an additional 10 min each. Subsequently, the catheter segments were removed, rinsed once with saline and the number of adherent viable microorganisms was determined as described above. In addition, the biofilm biomass was determined before any treatment for each strain in triplicate. The catheter segments were exposed to treatments for 20, 30, 60, 240 and 1440 min. Longer periods of ETOH treatment were performed with 24-h *S. epidermidis* biofilm, including 48 and 72 h length time. The effect of the treatment solutions was also tested with planktonic cells; 10⁷ cells from overnight culture of the microorganisms in appropriate media were incubated for the same lengths of time with the different treatment solutions. After incubation, the cells were diluted in saline and the number of CFU was determined using the same procedure.

**Statistical analysis**

The data are expressed as means ± SD decimal logarithm (log10) of CFU. The bacterial reduction factor (RF) was calculated as the difference between the log10 value before treatment and the posttreatment log10 value. RF was considered as eradication if posttreatment viable bacterial count was below the limit of detection. Differences in RF between the treatment groups were compared using the Mann–Whitney *U*-test. A *P*-value < 0.05 was considered significant.

**Fluorescent staining and Microscopic observations**

To assess the cell viability, respiratory activity was determined by staining the bacteria within biofilm with 0.5 mg/ml of CTC (Polysciences, Inc., Warrington, PA, USA). DAPI (10 µg/ml; Polysciences, Inc., Warrington, PA, USA) was used for enumeration of the total cells [27]. The untreated or ETOH-treated biofilm coupons were incubated with 0.5 ml of CTC and DAPI staining solutions at 37 °C in the dark, for 1 h and 15 min, respectively. The biofilm samples were then imaged with a LSM510 Meta microscope (Carl Zeiss MicroImaging, Inc., le Pecq, France) with 40XNA Plan-Neofluar objective. All imaging was performed at room temperature. Figures were processed using LSM Image Browser software and Photoshop 7 (Adobe).
Fig. 1. Number of viable cells (CFU) per catheter segment before and after treatment for 20 min, 30 min, 1 h, 4 h and 24 h with ETOH 60%, TSC 46.7% and saline (control) of S. epidermidis (A), S. aureus (B), K. pneumoniae (C), P. aeruginosa (D) and C. albicans (E) 4- and 24-h biofilms and planktonic cells. The results are expressed as means ± standard deviations (indicated as error bars) of at least three independent assays. The detection limit was 40 CFU/catheter segment and is represented by a dashed lane. ∗Significant difference (P < 0.05) between lock solution and control (saline); ‡Significant difference between ETOH 60% and both TSC 46.7% and control.

Results

Before any treatment, the counts of organisms (log 10 CFU) within 4-h biofilms were 4 for S. aureus, 5 for S. epidermidis, K. pneumoniae and C. albicans, and 7 for P. aeruginosa. Regarding the 24-h biofilm, the initial bacteria counts were 5 for C. albicans, 6 for S. aureus and S. epidermidis and 8 for K. pneumoniae and P. aeruginosa. The counts of
each viable test organism in biofilms after up to 24 h of exposure to the test solutions are shown in Figure 1(A–E). After 20-min exposure, treatment with ETOH 60% induced a significant reduction in 4 and 24-h biofilms by comparison with saline and TSC, whatever the microorganisms tested. The eradication of microorganisms was observed after 20-min ETOH 60% exposure in 4 and 24-h P. aeruginosa, K. pneumoniae, C. albicans biofilms and 4-h S. epidermidis biofilm, whereas 30-min ETOH 60% exposure was required to eradicate 4 and 24-h S. aureus biofilms and 24-h S. epidermidis biofilm. Using decreasing ETOH concentrations over the same period of time (60% followed by 40% and 20% for 10 min each) with 24-h S. epidermidis biofilm also induced total eradication of viable bacteria. In addition, longer periods of ETOH 60% exposure (48 and 72 h) of 24-h S. epidermidis biofilm did not impair the eradication process. Figure 2 shows the CLSM CTC-DAPI stained images of untreated and 30-min ETOH-treated S. epidermidis 24-h-old biofilms. The untreated biofilm showed mostly red and blue stained cells with a typical architecture, indicating that the majority of the cells within the biofilm were actively respiring (Figure 2). In contrast, a few red cells were visible in the ETOH-treated biofilm, which indicates that ETOH drastically reduced cell respiration activity after 30 min of contact (Figure 2).

Whatever the planktonic microorganisms tested, ETOH 60% achieved eradication after 20-min exposure. TSC treatment never achieved eradication in sessile microorganisms and in planktonic microorganisms only for P. aeruginosa after 24-h exposure.

Discussion

The bactericidal effect of ETOH on planktonic microorganisms is well documented and is rapidly achieved, but a few studies have reported the germicidal effect of concentrated ETOH on biofilm. Using monospecies biofilms formed in a kinetic microfermentor, we showed that a 60% ETOH solution rapidly eradicates sessile microorganisms adherent to silicon catheter fragments. In our study, performed with biofilms incubated for 4 and 24 h, 20-min treatment with a 60% ETOH solution completely eradicated biofilms formed with C. albicans and the Gram-negative bacilli, K. pneumoniae and P. aeruginosa. Although a significant decrease in the counts of 4- and 24-h S. aureus biofilms and 24-h S. epidermidis was observed after 20 min with the same treatment, it took more than 30 min for them to be entirely eradicated. ETOH is known to eliminate bacterial cells by denaturing proteins and causing membrane leakage. In our experiment, CLSM images of 30-min ETOH-treated biofilm of S. epidermidis revealed that the inability to form colonies on an agar medium was associated with the loss of respiratory activity by most cells (Figure 2). When reincubated in broth for 24 h, there was, however, remultiplication of the staphylococcal organisms embedded in the 30-min ETOH-treated biofilm and not with biofilm incubated for longer periods of time, probably because a few cells were not fully inactivated by ETOH within the biofilm. The relative ETOH resistance of staphylococcal biofilm could be related to a limited diffusion of the solution within the biofilm owing to the matrix composition and, therefore, to exposure of bacterial cells within the biofilm to lower concentrations of ETOH. Furthermore, our experiments were performed with high initial inocula, between 5 and 8 log10 per cm of catheters, probably larger than the bacterial counts yielded by a quantitative tip culture of catheter in clinical practice [28]. Nevertheless, whether the bactericidal effect observed with a 60% ETOH solution is sufficient in vivo to eradicate most of the adherent microorganism remains speculative.

In an in vitro assay using a static multiwell incubation model, Chambers et al. showed that after 16 h of adhesion, Candida, Gram-positive cocci and Gram-negative bacilli were killed by 1 h of exposure to 70% ETOH, whereas longer periods of treatment were required to eradicate 40- and 72-h biofilms of Candida and/or Gram-negative bacilli [29]. A 60-min exposure to a 25% ETOH solution was shown to suppress but not to eradicate mature biofilms of S. aureus and C. parapsilosis [24]. Similar results were obtained by Sherertz et al. with ETOH solutions ranging from 10% to 100% that produced at least 3 log units of killing of S. aureus on a silicone catheter after 2 and 4 h of treatment [30]. However, it is difficult to draw a firm conclusion from the various studies because of the diversity
in strains, lock solutions and experimental models used for biofilm growth.

To prevent catheter thrombosis, anticoagulant agents are often used in the lock solutions and some of them have shown specific activity against microbial agents, particularly concentrated citrate [3]. Several studies showed the efficacy of citrate lock solutions in preventing dialysis catheter infections. The ability of citrate to prevent experimental biofilm formation and, therefore, the germicidal effect of citrate is debatable [31,32]. Sodium citrate is a potent permeabilizer of the cell wall of Gram-negative bacteria [33] and probably acts by impairing cellular integrity. In our study, none of the biofilms tested, whatever the strain or the maturity of the biofilm, was eradicated by TSC, even though significant decreases in the number of biofilm viable cells were observed, mostly with *K. pneumoniae*, *P. aeruginosa* and *C. albicans*. The bactericidal effect of TSC on sessile Gram-positive bacteria is less pronounced after 1-h exposure for *S. aureus* and 24-h exposure for *S. epidermidis*. TSC did not reduce significantly the bacterial count of the planktonic cells even after 24-h exposure, contrary to what was obtained by Weijmer et al. in a previous study [34] using TSC 30%, but performed with lower microbial inocula.

Surprisingly, we observed a higher growth of planktonic *S. epidermidis* after 24-h TSC exposure on comparison with saline. Such a result could be related to previous findings showing that low TSC concentrations strongly stimulate staphylococci growth [31,32].

The eradication of experimental biofilms on catheters with concentrated ETOH suggests that it could be an attractive lock solution to prevent catheter infections. Its *in vivo* efficacy remains to be determined, since this study was conducted *in vitro* with monospecies biofilms devoid of host-derived proteins, which normally cover indwelling devices and may compromise the antibacterial activity of the lock solutions. Clinical trials are in progress to assess the safety and efficacy of ETOH as a catheter lock solution.

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Conflict of interest statement. Hemotech provided the silicone catheters.

References

The Pan-Thames EPS study: treatment and outcomes of encapsulating peritoneal sclerosis

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Abstract

Background. Encapsulating peritoneal sclerosis (EPS) is a disease process that can occur as a complication of peritoneal dialysis (PD). The aim of this study was to make a general assessment of the clinical features, diagnosis, management and outcome of PD-related EPS cases from London and South-East England.

Methods. Questionnaires were sent to 11 PD units in March 2007; cases were identified retrospectively. Outcome data on surviving patients were collected in March 2008.

Results. A total of 111 patients were identified; the mean time on PD was 82 months (range 8–247). Mortality increased with length of time on PD, being 42% at <3 years (n = 12), 32% at 3–4 years (n = 19), 61% at 5–6 years (n = 31), 54% at 7–8 years (n = 24), 75% at 9–10 years (n = 8) and 59% at >10 years (n = 17). Twelve patients had no previous peritonitis episodes, 28 had one previous episode, 30 had two previous episodes and 33 had three or more previous episodes. Of the patients with PD details available, 41/63 were high (>0.81) transporters and 44/71 had ultrafiltration <1 l/24 h, but 7/63 were low average transporters (0.5–<0.65) and 27/71 had ultrafiltration >1 l/24 h and a few had significant residual renal function. Sixty-five (59%) patients had their PD discontinued prior to diagnosis (51 HD; 14 transplanted). CT scans were performed on 91 patients and laparotomy on 47 patients. Drug treatment consisted of tamoxifen, immunosuppression or both. The median survival was 15 months in patients treated with tamoxifen (n = 17), 12 months in patients treated with immunosuppression (n = 24) and 21 months in patients who received both (n = 13), against 13 months (n = 46) in patients who received no specific treatment. Adhesionolysis was performed in 5 patients, and 39 patients were given parenteral nutrition. The overall mortality was 53% with a median survival of 14 months and a median time to death of 7 months.

Conclusion. This is one of the largest cohorts of patients with EPS in the literature. Long-term survival occurred in over 50%, regardless of the various treatments strategies undertaken by the centres.

Keywords: encapsulating peritoneal sclerosis; peritoneal dialysis; multicentre retrospective study; treatment

Introduction

Encapsulating peritoneal sclerosis (EPS) is an uncommon but serious complication of long-term peritoneal dialysis...