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Weak organic acid synergy towards the prevention of catheter blockages

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Running title: Organic acids for catheter blockage prevention

Summary

Background

Up to 50% of all long-term catheterised individuals experience recurrent episodes of urinary catheter infections and blockages, leading to urine retention, pyelonephritis and septicaemia if the catheter is left *in situ*. We have previously reported the synergistic activity of weak organic acid (WOA) combinations against nosocomial uropathogens.

Aim

To investigate the efficacy of selected WOAs, citric acid and propionic acid, alone and in combination, on prevention of crystalline biofilm formation and catheter blockages.

Methods

Static crystallisation assays and dynamic *in vitro* bladder model assays, with scanning electron microscopy, were performed for determination of bacterial viability, urinary pH and time to catheter blockage.

Findings

The rate of encrustation around the catheter eyeholes was reduced in the presence of the citric acid/propionic acid combination, extending the time to blockage three-fold.

Conclusion

Synergistic WOA combinations identified herein represent promising alternatives to antibiotics to combat the global healthcare burden of catheter-associated urinary tract infections and related blockages.

Keywords: Catheter blockage; synergy; weak organic acid; crystalline biofilm; uropathogen

Introduction

Infection of indwelling urinary catheters by urease-producing pathogens, such as *Proteus mirabilis*, leads to the formation of crystalline biofilms, device encrustation and subsequent blockage of the catheter lumen [1]. Without timely catheter replacement and/or treatment, the resulting retention of urine in the bladder can lead to pyelonephritis and septicaemia. Alternatively, crystalline deposits on the catheter surface can cause trauma to the bladder mucosa and urethra upon removal of the catheter [2]. Indeed, recurrent episodes of catheter infections and blockages are reported in up to 50% of all long term-catheterised individuals, with annual costs of managing urinary catheter infections in Western Europe estimated at £1 billion [3].

Urease-induced hydrolysis of urea in the urine results in the release of ammonia. This leads to elevation of urinary pH, with subsequent precipitation of magnesium and calcium phosphates to crystals of struvite and carbonate apatite [4]. Aggregation of these crystals results in the formation of urinary stones, while accumulation within the biofilm matrix leads to crystalline biofilms which can block the flow of urine through the catheter lumen [1, 5]. Released ammonia can damage the urothelial glycosaminoglycan layer, rendering the urothelium unprotected against bacterial infection, while also inducing direct damage and inflammation of uroepithelial cells [6, 7].

The urease produced by *P. mirabilis* has been demonstrated to hydrolyse urea six to ten times faster than the enzyme produced by other species such as *Morganella morganii*, *Proteus vulgaris* and *Providencia rettgeri* [6]. Indeed, *P. mirabilis* has been isolated from up to 40% of biofilm communities present on catheters retrieved from long-term catheterised patients and *Proteus* species have been isolated from 70% of infectious urinary stones [4, 5, 8]. In conjunction with urease-production, the ability of *P. mirabilis* to swarm over catheter surfaces has been hypothesised to facilitate the formation of extensive crystalline biofilms [9, 10].

Currently investigated approaches for managing catheter encrustation and infection include the use of bladder irrigations with saline and antiseptics, such as chlorhexidine and mandelic acid [11, 12]. Saline has, however, shown little efficacy in preventing catheter blockages and, furthermore, uropathogen resistance to chlorhexidine has been reported [11, 13, 14]. Washouts have also been demonstrated to cause damage and irritation to the bladder mucosa [15]. Alternatively, other strategies have focused on inhibition of the enzyme urease. Many identified urease inhibitors, including quinones, phosphoramidates and acetohydroxamic acid,

are, however, associated with issues of toxicity and stability [6, 9]. For example, despite the promising efficacy shown by acetohydroxamic acid in the reduction of catheter encrustation, the accompanying side-effects render this drug poorly tolerated by patients [9, 16]. Inflation of the catheter retention balloon with antimicrobials including triclosan and mandelic acid, which then diffuse into the urine, has also been proposed as a strategy to control catheter encrustation [2, 17, 18]. Concerns have, however, been raised over the generation of triclosan resistance as a result of overexploitation of this agent [19].

All urinary catheters remain susceptible to encrustation therefore efficacious strategies to prevent catheter blockages are urgently needed [5]. We have previously reported the promising antibacterial activity of synergistic combinations of weak organic acids (WOAs), including citric acid, malic acid, propionic acid, mandelic acid, lactic acid, benzoic acid, pyruvic acid and hippuric acid, against uropathogens. In this work we investigate the efficacy of citric acid and propionic acid, alone and in combination, in comparison to the known urease inhibitor, acetohydroxamic acid, against *P. mirabilis*-induced catheter encrustation and blockage.

Materials

Citric acid, propionic acid, acetohydroxamic acid, L-histidine ($\geq 98.5\%$), L-cysteine ($\geq 97\%$), reduced glutathione ($\geq 98\%$), magnesium chloride hexahydrate, sodium chloride, sodium sulfate, trisodium citrate, disodium oxalate, potassium dihydrogen phosphate, potassium chloride, ammonium chloride, gelatine, tryptone soya broth, urea, creatinine and calcium chloride were purchased from Sigma Aldrich (Poole, Dorset, UK). *P. mirabilis* ATCC 51286 was purchased from LGC Standards (Middlesex, UK) and maintained on cryotubes in 15% glycerol at -80°C (VWR Chemical). Phosphate-buffered saline (PBS), low swarm agar (LSW), Mueller-Hinton broth (MHB) and quarter-strength Ringer's solution (QSRS) were obtained from Oxoid Ltd (Hampshire, UK). All-silicone 14 Ch male Foley catheters and drainage bags were purchased from Bard Ltd. (West Sussex, UK).

Methods

Preparation of artificial urine

Artificial urine (AU) used in the *in vitro* bladder model assay was prepared as two solutions which were mixed before use, as described by Nzakizwanayo *et al.* [5]. Components of solution

1 (3.25 g magnesium chloride hexahydrate, 23 g sodium chloride, 11.5 g sodium sulfate, 3.25 g trisodium citrate, 0.1 g disodium oxalate, 14 g potassium dihydrogen phosphate, 8 g potassium chloride, 5 g ammonium chloride, 25 g gelatine and 5 g tryptone soya broth (TSB)) were dissolved in 850 mL dH₂O. The pH was adjusted to ~pH 5.7 by the dropwise addition of NaOH or HCl. dH₂O was then added to bring to a final volume of 1 L before autoclaving five times. Components of solution 2 (2.45 g calcium chloride and 125 g urea) were dissolved in 400 mL dH₂O before sterilising by filtration through 0.45 µm pore size filters. Finally, solutions 1 and 2 were added to 3.6 L of sterile dH₂O.

The AU employed in the static crystallisation studies was prepared as described above but with a higher concentration of calcium chloride (0.65 g/L) and TSB (10 g/L), and replacement of gelatine with creatinine (1.1 g/L).

Preparation of acid stock solutions

Stock solutions were prepared in AU at the following concentrations: citric acid (96 mg mL⁻¹), propionic acid (11 mg mL⁻¹) and acetohydroxamic acid (1 mg mL⁻¹). The solutions were sterilised by syringe filtration with a 0.20 µm filter and stored at 8°C.

Neutraliser preparation

Universal neutraliser of L-histidine, L-cysteine and reduced glutathione was prepared to neutralise the effects of residual acids in the microbiological tests. A solution of 1.0 g L-histidine, 1.0 g L-cysteine and 2.0 g reduced glutathione was made up to 20 mL in dH₂O and then sterilised by syringe filtration with a 0.20 µm filter. 500 µL of the universal neutraliser solution was then added to each 20 mL of the medium used to give respective concentrations of 0.125% w/v L-histidine, 0.125% w/v L-cysteine and 0.25% w/v reduced glutathione [20].

Static crystallisation assay

Overnight broth cultures of *P. mirabilis* were diluted in AU to a bacterial density of 1 x 10⁵ cfu mL⁻¹. Citric acid and propionic acid, alone and in combination, and the urease inhibitor, acetohydroxamic acid, were added to provide final concentrations of 0.75 mg mL⁻¹, 0.35 mg mL⁻¹ and 1.00 mg mL⁻¹, respectively. WOAs were used at concentrations representing 0.5 x MIC values. 100 µL of the test solutions and control, with no WOA or acetohydroxamic acid, were added to wells of a flat bottomed 96-well plate. The plate was then incubated at 37°C in an orbital incubator at 100 rpm. UV absorbance readings at 600 nm and measurements of urine

pH were performed at 0, 1, 2, 3, 4, 6 and 24 h. In parallel, viable counts of the inoculum were determined at each time interval by removing aliquots (600 μ L) from McCartney bottles containing control and test solutions (20 mL), serially diluting in neutraliser-supplemented QSRS and plating onto low-swarm agar. SEM images of crystals formed in the AU were recorded at 24 h. The assay was performed on three independent occasions [4].

***In vitro* bladder model**

The bladder model was set up as described by Macleod and Stickler, and a schematic is shown in Figure 1 [8]. A double-walled glass chamber, representing the bladder, was maintained at 37°C by a water jacket. All-silicone 14 Ch male Foley catheters were inserted into each bladder and retention balloons were filled with 10 mL sterile water. Catheters were attached to drainage bags to form a closed system. The turbidity of *P. mirabilis* cultures grown to the logarithmic phase was adjusted in AU to obtain a final inoculum density of 1×10^9 cfu mL⁻¹. 10 mL of the bacterial suspension was added to the bladder and allowed to establish for 1 h before flow was initiated. AU, with and without citric acid (0.75 mg mL⁻¹) and propionic acid (0.35 mg mL⁻¹), concentrations representing 0.5 x MIC values, was supplied to the bladders at a constant flow rate of 0.75 mL min⁻¹. The models were operated until the control catheters blocked. The process was then repeated with the models operated until blockage of the test catheters. Urinary pH was measured and viable bacterial cells in residual urine in the bladders were enumerated when flow of AU was initiated, when the control catheters blocked and when each test catheter blocked [5, 21].

Catheters were removed from the models upon blockage of the control and test catheters. Sections (1 cm in length) immediately below the eye-holes were cut from each catheter, as shown in Figure 2, and the lumens imaged with SEM after mounting onto aluminium holders with carbon tape. The SEM was operated at 5 – 15 kV [2]. Duplicates were performed on three independent occasions.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 8.0 for Mac (GraphPad Software Inc., San Diego, USA). Statistical differences in bacterial viability, pH and absorbance of AU, with and without the presence of WOAs and acetohydroxamic acid, in the static crystallisation assay were analysed using a two-way analysis of variance (ANOVA). Statistical differences in the time for catheter blockage, with and without the presence of WOAs, were determined using

a one-way ANOVA, whereas statistical differences in bacterial viability and pH of the residual urine in the bladder when flow of AU was initiated, when the control catheters blocked and when the test catheters blocked were determined using a two-way ANOVA. In all cases $p < 0.05$ was taken to denote significance. Where a statistically significant difference was identified, Tukey's multiple comparison post-hoc test was carried out.

Results and discussion

Static crystallisation assay

The effect of WOAs on the crystallisation of magnesium and calcium phosphates in *P. mirabilis*-infected AU was investigated, firstly, by static crystallisation assays. The bacterial density, pH and optical density of AU were determined after various periods of incubation and are displayed in Figures 3 (a) and (b), respectively.

As shown in Figure 3 (a) the WOAs demonstrated no bactericidal activity against *P. mirabilis* due to the employment of these agents at sub-MIC concentrations. In addition, Morris and Stickler have previously reported the absence of a bactericidal effect of acetohydroxamic acid against *P. mirabilis* at concentrations up to 1 mg mL^{-1} [22]. With regards to pH, after 24 h incubation there were no significant differences between the pH of the control AU with no WOAs and the AU containing citric acid or propionic acid alone (Figure 3 (b)), with pH values increasing from ~pH 6 to ~pH 9.4 due to the urease-induced hydrolysis of urea to ammonia. In contrast, the pH values of the AU containing the citric acid/propionic acid combination and the AU containing acetohydroxamic acid were significantly lower than the control after 24 h incubation, with respective values of pH 6.2, pH 6.3 and pH 9.4. The significantly lower pH of the acetohydroxamic acid-containing AU was expected since acetohydroxamic acid is reported to cause complete non-reversible, non-competitive inhibition of urease, thereby preventing urea hydrolysis, ammonia production and resultant pH elevation [23].

The controlled urinary pH in the presence of the WOA combination and/or acetohydroxamic acid had important implications with regards to crystal precipitation. As shown in Figure 3 (b), absorbance values for AU containing the citric acid/propionic acid combination and the acetohydroxamic acid-containing AU were significantly lower than the corresponding values for control AU and AU containing the individual WOAs after 24 h incubation. Carbonate apatite and struvite crystals have previously been reported to precipitate at pH values ≥ 6.8 and ≥ 7.2 , respectively [23, 24], whereas in the presence of the WOA combination or

acetohydroxamic acid the urinary pH remained below these values. Prywer *et al.* have previously reported longer induction times for struvite, with a corresponding reduction in the number of crystals formed, in the presence of curcumin due to inhibition of urease [4].

These optical density findings were supported by the SEM images shown in Figure 4.

X-shaped and coffin-like crystals of struvite were observed in *P. mirabilis*-infected AU in the absence of the WOAs, as shown in Figure 4 (a). A similar extent of crystallisation was observed in AU with the presence of the individual WOAs, whereas a significant reduction in coffin-like crystals was observed in the acetohydroxamic acid-containing AU. Moreover, crystallisation was completely prevented in the presence of the WOA combination. The presence of the WOA combination maintained the AU in the static model undersaturated with respect to struvite formation by lowering urinary pH [4]. These findings have important implications with regards to the promising potential clinical utility of pH-lowering agents for the prevention of catheter encrustation.

***In vitro* bladder models**

The time for catheters to become blocked by *P. mirabilis* crystalline biofilms in *in vitro* bladder models supplied with infected AU, with and without the presence of WOAs, is displayed in Figure 5.

Models were inoculated with a bacterial density of 1×10^9 cfu mL⁻¹ to simulate a high level of infection [5]. Catheters supplied with *P. mirabilis*-infected AU were blocked by crystalline biofilms within $43.9 \text{ h} \pm 4.18$. These findings confirm those of Stickler *et al.* who reported blockage of all-silicone catheters in *P. mirabilis*-infected models within 40 h [2]. Catheters supplied with propionic acid-containing AU were susceptible to blockage within a similar timeframe ($44.2 \text{ h} \pm 0.93$). In contrast, in the presence of citric acid, the time to catheter blockage was extended two-fold ($75.1 \text{ h} \pm 11.1$). Furthermore, the catheters supplied with the citric acid/propionic acid combination drained freely for over six days, with a significantly extended time to blockage of $152 \text{ h} \pm 23.5$.

Inflation of catheter retention balloons with triclosan solutions has previously been reported to inhibit the urease-induced elevation of urinary pH in the presence of *P. mirabilis*, and the corresponding encrustation of catheters [2, 17, 19]. The pH and number of viable cells within

the residual urine in the bladders when the flow of AU was initiated, when the control catheters blocked and when each test catheter blocked is shown in Figures 5 (b) and (c). The pH of AU devoid of WOAs significantly increased from pH 8.27 to pH 8.89, while the density of *P. mirabilis* remained $\sim 10^9$ cfu mL⁻¹ by the time of catheter blockage (43.9 h). In the case of the WOA-containing AU, the urinary pH was significantly lower than the pH of AU devoid of WOAs upon initiation of urine flow. By the time of the control catheter blockage, however, the pH of the propionic acid- and the citric acid/propionic acid-containing AU had increased to values of pH 8.86 and 9.05, respectively, and only the citric-acid containing urine (pH 8.13) displayed a significantly lower pH than the urine devoid of WOAs.

Calcium and magnesium phosphates crystallise at pH values above their nucleation pH (pH ≥ 6.8 and ≥ 7.2 , respectively) [23, 24]. The urinary pH of patients who experience recurrent episodes of catheter encrustation and blockage has largely been reported to exceed these values [2, 3, 25]. In contrast, we herein reveal the absence of crystallisation at pH values approaching pH 9. Similarly, Nzakizwanayo *et al.* reported significant reductions in catheter encrustation, with a dose-dependent increase in the time for catheter blockage, upon treatment with thioridazine in the absence of any effect on elevation of urinary pH [5]. The mechanism behind suppression of crystalline biofilm formation was attributed to the thioridazine-induced inhibition of efflux activity.

With regards to bacterial viability, significantly lower cell densities were reported in the WOA-containing AU (4.5×10^7 cfu mL⁻¹, 2.2×10^7 cfu mL⁻¹ and 5.9×10^7 cfu mL⁻¹ for citric acid-, propionic acid and citric acid/propionic acid-containing AU, respectively) than the AU devoid of WOAs (1.1×10^9 cfu mL⁻¹) at the time of control catheter blockage. This reduced cell density suggests impairment of bacterial growth as a potential mechanism contributing to the delay in blockage. While this was not anticipated due to use of the WOAs at concentrations 0.5 x MIC, the MIC values were determined in standard MHB and not in AU with the dynamic conditions of the bladder models. Similarly, Nzakizwanayo *et al.* reported reduced numbers of *P. mirabilis* in the presence of thioridazine and fluoxetine in bladder models when used at concentrations 0.5 x and lower than their MICs [5].

Figure 6 displays images of catheter eyeholes following removal from the bladder models at the time of control catheter blockage and blockage of each of the test catheters.

The images in Figure 6 show complete blockage of the eye-holes with crystalline deposits at the time of respective catheter blockages. In contrast, at the time of the control catheter blockage, the catheters supplied with the citric acid- and citric acid/propionic acid-containing AU appeared free of encrustation. This was anticipated due to the significantly extended time to catheter blockage reported for the catheters supplied with citric acid alone and the acid combination.

Sections (1 cm in length) were cut from the catheters following removal from the bladder models at the time of control catheter blockage and blockage of each test catheter, and the lumens were examined by SEM, as shown in Figure 7.

In addition to the eyeholes, the micrographs in Figure 7 provide visual confirmation that the lumens of catheters removed from models supplied with the citric acid- and citric acid/propionic acid-containing AU were free of crystalline material at the time of control catheter blockage. In contrast, extensive accumulation of crystalline material was visible throughout the lumen of the blocked control catheter. Accumulation of crystalline material was observed within the lumen of the catheter supplied with the propionic acid-containing AU at the time of control catheter blockage due to the non-significant differences between the time to blockage of these two catheters (43.9 h and 44.2 h for control and propionic acid test catheters, respectively).

These findings demonstrate the efficacy of citric acid and the citric acid/propionic acid combination in maintaining patency of catheter eyeholes and lumens, and thereby allowing urine drainage for extended periods. Clinical application of WOAs, ideally through localised delivery at the catheter surface, therefore holds promise with regards to the prevention of bladder and urethral trauma upon removal of encrusted catheters and, importantly, in preventing life-threatening episodes of pyelonephritis and septicaemia resulting from urinary retention [2].

Conclusion

Further research is needed to provide greater understanding of the mechanisms behind the WOA-mediated prevention of crystallisation and to develop approaches to facilitate the employment of WOAs in controlling catheter blockages, ideally through localised delivery in the catheterised urinary tract. Nevertheless, based on the promising findings herein, namely the

significantly extended time to catheter blockage in the presence of the WOAs, there is considerable scope for use of WOA combinations to maintain lumen patency and help combat this global health priority.

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Conflict of interest

None declared.

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Figures

a, i

iii

b, i

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(a,i)

(ii)

(iii)

(iv)

(b,i)

(ii)

(iii)

Figure legends

Figure 1. Schematic of the *in vitro* bladder model set-up (modified from Nzakizwanayo *et al.*) [21].

Figure 2. Image showing catheter section immediately below the eye-hole used for SEM analysis.

Figure 3. (a) Viability of *P. mirabilis*, and (b) optical density at 600 nm and pH of *P. mirabilis*-infected AU in the presence and absence of WOAs (citric acid (0.75 mg mL^{-1}) and propionic acid (0.35 mg mL^{-1})), and acetohydroxamic acid (1 mg mL^{-1}) over a 24 h incubation period at 37°C . pH values are displayed by the symbols with no connecting lines. Concentrations of WOAs represent $0.5 \times \text{MIC}$ values. Each assay was repeated on three independent occasions and representative curves are displayed. Error bars represent positive standard deviations of the mean values.

Figure 4. SEM images of struvite crystals grown in *P. mirabilis*-infected AU in the absence (a) and presence of citric acid (0.75 mg mL^{-1}) (b), propionic acid (0.35 mg mL^{-1}) (c), citric acid/propionic acid ($0.75 \text{ mg mL}^{-1}/0.35 \text{ mg mL}^{-1}$) (d), and acetohydroxamic acid (1 mg mL^{-1}) (e) after 24 h incubation at 37°C . Images were acquired at 500x magnification and scale bars represent $200 \mu\text{m}$.

Figure 5. (a) The time taken for catheter blockage when supplied with *P. mirabilis*-infected AU with and without the presence of citric acid (0.75 mg mL^{-1}) and propionic acid (0.35 mg mL^{-1}), alone and in combination. (b) pH and (c) bacterial density of residual urine in the bladders when flow of AU was initiated, when the control catheters blocked and when each test catheter blocked. Error bars represent S.D.'s of the mean values ($N = 6$).

Figure 6. Images of catheter eyeholes following removal from bladder models at (a) the time of control blockage ($43.9 \text{ h} \pm 4.18$) when supplied with AU (i) devoid of WOAs, and containing (ii) citric acid, (iii) propionic acid and (iv) citric acid/propionic acid combination, and (b) at the time of test blockage when supplied with AU containing (i) citric acid ($75.1 \text{ h} \pm 11.2$), (ii) propionic acid ($44.2 \text{ h} \pm 0.93$) and (iii) citric acid/propionic acid combination ($152 \text{ h} \pm 23.5$). Representative images are shown ($N = 6$).

Figure 7. SEM images of catheter cross-sections directly below the eyeholes. (a) Catheters removed from bladder models at the time of control blockage ($43.9 \text{ h} \pm 4.18$) when supplied with AU (i) devoid of WOAs, and containing (ii) citric acid, (iii) propionic acid and (iv) citric acid/propionic acid combination, and (b) catheters removed from bladder models at the time of test blockage when supplied with AU containing (i) citric acid ($75.1 \text{ h} \pm 11.2$), (ii) propionic acid (44.2 ± 0.93) and (iii) citric acid/propionic acid combination (152 ± 23.5). Representative images are shown ($N = 6$).