Use of adhesion counts to help predict symptomatic infection and the ability of fluoroquinolones to penetrate bacterial biofilms on the bladder cells of spinal cord injured patients

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There were three objectives to the present study: (1) compare the bladder infection rate and extent of biofilm formation for seven untreated spinal cord injured (SCI) patients and seven given prophylactic co-trimoxazole, (2) identify a level of bacterial adhesion to bladder cells which could be used to help predict symptomatic infection, and (3) determine from *in vivo* and *in vitro* studies whether fluoroquinolones were effective at penetrating bacterial biofilms. The results showed that the infection rate had not changed with the introduction of prophylaxis. However, the uropathogenic population had altered subsequent to the introduction of prophylaxis with *E. coli* being replaced by *E. faecalis* as the most common cause of infection. In 63% of the specimens from asymptomatic patients, the bacterial counts per cell were < 20, while 81% of specimens from patients with at least one sign and one symptom of urinary tract infection (UTI) had > 20 adherent bacteria per bladder cell. Therefore, it is proposed that counts of > 20 bacteria adherent to sediment transitional epithelial bladder cells may be predictive of symptomatic UTI. Clinical data showed that fluoroquinolone therapy reduced the adhesion counts to < 20 per cell in 63% of cases, while trimethoprim-sulfamethoxazole only did so in 44%. Further *in vitro* testing showed that ciprofloxacin (0.1, 0.5 and 1.0 µg/ml) partially or completely eradicated adherent biofilms from 92% of spinal cord injured patients' bladder cells, while ofloxacin did so in 71% cases and norfloxacin in 56%. These findings have important implications for the detection and treatment of bacteriuria in spinal cord injured patients.

Keywords: bladder infections; bacterial adhesion; fluoroquinolone treatment; spinal cord injury patients.

Introduction

Symptomatic urinary tract infection (UTI) still remains a potentially life-threatening disease in spinal cord injured (SCI) patients and those with neurogenic bladder disturbances.¹ The issue of whether or not to treat UTI and asymptomatic bacteriuria (ABU) in these patients has recently been raised² and recommendation made that asymptomatic bacteriuria should not be treated.

However, this recommendation was made without the knowledge that adherent bacterial biofilms exist on bladder cells and devices whether or not antibiotics are administered^{3,4,5} and the presence of these biofilms correlates with a significant loss in bladder cell viability.⁴ The use of prophylaxis, primarily with cotrimoxazole, is considered an effective method to prevent full blown bladder infection in otherwise healthy premenopausal women.⁶ However, its role as a prophylactic agent in SCI patients is less clear.

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The present study was undertaken to address three questions: (1) Does prophylactic management with co-trimoxazole lower bladder infection rates and eradicate adherent biofilms in SCI patients? (2) Does the number of adherent bacteria indicate whether or not a symptomatic infection will arise? (3) Are fluoroquinolones effective at penetrating and eradicating adherent biofilms? The latter question arises from *in vitro* and *in vivo* observations that of the many antimicrobial agents used to prevent and treat biofilm related infections, only ciprofloxacin, a potent fluoroquinolone, has shown good activity to date^{5,7,8} (Preston CAK *et al*, unpublished).

Materials and methods

Patients

Fourteen SCI patients, aged 20–66 years (three females, 11 males), undergoing a hospital rehabilitation program provided written consent to participate in the study, approved by Parkwood Hospital and the University of Western Ontario Health Review Board. In all cases, bladder management comprised intermittent catheterization.

Each patient provided urine samples for 8 weeks. The urine specimens were cultured using standard microbiology laboratory techniques, and sediment transitional cells were Gram stained and the number of adherent organisms enumerated on 50 cells. In addition, cells from each sample were sonicated to remove adherent bacteria, and these organisms were then inoculated onto blood and brain heart infusion agar plates to determine the numbers of viable bacteria which had been attached to the cells.

The patients were monitored for signs and symptoms of UTI, namely spasticity, fever, cloudy and foul odour urine. Seven patients did not receive prophylactic therapy and seven were given daily co-trimoxazole (160 mg trimethoprim, 800 mg sulfamethoxazole). Symptomatic UTI was actively treated with an agent to which the infecting bacteria was sensitive: namely, ciprofloxacin (500 mg b.i.d.), norfloxacin (400 mg b.i.d.), amoxicillin

(500 mg q8h), ampicillin (250 mg q.i.d.), co-trimoxazole (160 mg trimethoprim, 800 mg sulfamethoxazole b.i.d.), gentamicin (100 mg q8h) or nitrofurantoin (50 mg q.i.d.).

In vitro incubation of epithelial cells with antibiotics

In order to determine if fluoroquinolones were able to kill adherent bacteria on the patients' epithelial cells, three concentrations (0.1, 0.5, 1.0 µg/ml) of ciprofloxacin, ofloxacin and norfloxacin were incubated with 10⁵ cells in 1 ml buffer for 24 hours at 37°C. The cells were then sonicated to remove adherent bacteria, and these were then washed and quantitated by culture on agar. The percentage reduction compared to untreated controls was calculated.

Scanning electron microscopy

A previous study³ had illustrated the presence of biofilms on bladder cells of SCI patients. In the latest study, several samples were processed for scanning electron microscopy to confirm these findings. A photomicrograph is included in Figure 1, not only for this purpose but also to illustrate the

Figure 1 Scanning electron micrograph showing a microcolony of enterococci adherent to a transitional bladder cell of a spinal cord injured patient. The photograph appears slightly out of focus, but in fact this is due to the deposition of a conditioning film onto the surface of the cell and encasing the microorganisms.

clumping of the organisms and the appearance of a conditioning film over the biofilm.

Results

The seven patients who had no antibiotic prophylaxis had an infection rate per sample of 68%, which was not statistically higher than the 54% infection rate for the seven patients given prophylactic co-trimoxazole. However, as shown in Table I, the uropathogens isolated were quite different in each group, with *E. coli* being replaced by *E. faecalis* as the dominant pathogen upon use of co-trimoxazole.

An examination of the numbers of bacteria adherent per bladder cell showed a mean of $44 \pm \text{SD}49$ in 27 samples from the untreated patients, which was not statistically different from the mean of 39 ± 42 per cell from 26 samples from patients given co-trimoxazole prophylactically.

Sixteen specimens were able to be processed by sonication. There was no correlation between the number of adherent bacteria counted on cells by light microscopy and those found by sonication and quantitative culture (Fig 2). However, 11 of 16 (69%) samples with mean adhesion counts < 20 per cell had no bacteria recovered after sonication, whereas viable bacteria were recovered from all samples with means > 20 per cell.

A combined analysis of bacterial adhesion counts from patients without signs or symptoms of UTI showed that in 19 of 30 (63%)

Figure 2 This graph presents the correlation between the number of bacteria visualized by Gram stain and light microscopy to be adherent to bladder cells from SCI patients, and the number of actual viable bacteria recovered from each sample of 10^5 cells. A constant of 0.5 has been added to the count data.

cell specimens, the mean count was < 20 per cell. In 26 of 32 specimens (81%) where patients had at least one sign and one symptom of UTI, the adhesion counts were > 20 per cell. Cloudy and foul odour urine per se would not be indicative of symptomatic infection and thus another symptom had to be recorded, such as spasticity, plus confirmation by culture of $> 10^5$ organisms per ml urine.

A further examination of bladder cells from patients actively treated with ciprofloxacin and norfloxacin showed a mean of < 20 bacteria per cell in five of eight samples (63%), compared to 14 of 32 (44%) when the patients were given co-trimoxazole.

All the isolated organisms tested for antibiotic penetration *in vitro* were sensitive to the fluoroquinolones, except one strain of *E. faecalis*. *In vitro* incubation of cells with adherent bacteria and the three fluoroquinolones showed that all three agents were equally effective at killing the adherent organisms (Table II). The increased eradication of bacteria at higher concentrations was not linear. The eradication of the adherent bacteria was partial or complete in 92% of the ciprofloxacin treated specimens, 71% of the ofloxacin (one sample was lost) and 56% of the norfloxacin specimens.

Table I Recovery of uropathogens in numbers $> 10^5$ per ml urine from spinal cord injured patients untreated or given prophylactic co-trimoxazole

	% Recovery	
	No treatment	Co-trimoxazole
<i>E. coli</i>	35	0
<i>P. aeruginosa</i>	18	13
<i>K. pneumoniae</i>	18	0
<i>S. epidermidis</i>	18	0
<i>E. faecalis</i>	11	80
Gram positive cocci	0	7

Table II *In vitro* effect of fluoroquinolones on specimens of viable bacterial biofilms adherent to bladder cells from SCI patients, compared to untreated controls

Antibiotic	Concentration of antibiotic used <i>in vitro</i>	Mean % reduction in bacterial viability	Biofilm eradication shown as no. of samples		
			Complete	Partial	None
Ciprofloxacin	0.1 µg/ml	89	1	10	1
	0.5 µg/ml	91	2	8	2
	1.0 µg/ml	93	3	9	0
Ofloxacin	0.1 µg/ml	88	0	10	2
	0.5 µg/ml	88	1	5	5 ^a
	1.0 µg/ml	90	2	7	3
Norfloxacin	0.1 µg/ml	86	0	7	5
	0.5 µg/ml	86	0	5	7
	1.0 µg/ml	89	0	8	4

^aOne sample was lost.

Discussion

The findings of the present study verified the presence of viable, and often dense, bacterial biofilms adherent to the transitional epithelial cells of SCI patients. The biofilms were often seen by microscopy to be embedded within a conditioning film (Fig 1). Although only 14 patients were examined, there was no evidence to indicate that the UTI rate had decreased with the use of prophylactic co-trimoxazole. A study of 81 patients is now underway to determine more conclusively whether prophylactic therapy altered the infection rate and to what extent it impacted upon uropathogen strain, patient wellbeing and cost. In the present study, there was strong evidence to indicate that *E. faecalis* had replaced *E. coli* as the major cause of UTI following the introduction of prophylaxis. A previous Japanese study of patients with complicated UTI (underlying neurogenic bladder dysfunction and cancer) has shown a high incidence of enterococcal infection and drug resistance to many antimicrobial agents, including co-trimoxazole (57%).⁹ Of the few cases treated with ciprofloxacin, norfloxacin, enoxacin and ofloxacin, good eradication rates were found with few cases of recurrence. This compares favourably with the findings of the present study where *in vitro* fluoroquinolone concentrations were 100–1000-fold lower than those present in the bladder of patients treated with these

antibiotics.

One of the major problems in the management of SCI patients is predicting when the asymptomatic bacteriuria will go on to cause symptomatic infection and morbidity. The data obtained in the present study indicated that signs and symptoms of UTI were associated with mean bacterial adhesion counts of over 20 organisms per cell. Clearly, these data alone are not sufficient to influence clinical decision making and larger numbers of SCI patients and other patient groups must be tested to confirm the observation. The key will be to produce a diagnostic assay which is sufficiently sensitive and specific to put these *in vitro* findings into practice. By providing a physician with information on the nature and extent of bladder cell colonization, it should be feasible to better decide if antibiotic therapy is required.

An examination of the bacterial adhesion counts showed that there was some correlation between the ability of more adhesive (and perhaps more virulent) uropathogens to cause symptomatic UTI, in agreement with *in vitro* results for *E. coli*.¹⁰ In cases where there was no correlation between adhesion per cell and numbers of bacteria recovered from sonicated cells (Fig 2), there could be several explanations. (1) A number of the adherent bacteria may have been killed by antibiotics, but still have remained attached. (2) The light microscopy

technique may have underestimated the numbers of adherent bacteria, especially cocci which, as Figure 1 shows, can be present in small microcolonies.

The potential of fluoroquinolones, especially ciprofloxacin, to penetrate adherent biofilms appears to have some merit, based upon *in vitro* studies.^{7,8} In the present study, ciprofloxacin and norfloxacin therapy reduced the adhesion counts to < 20 per cell in 63% of cases and co-trimoxazole only did so in 44%. Admittedly, this difference could be due to relative activity of the antibiotics rather than ability to penetrate biofilms. However, further *in vitro* studies confirmed the potency of three fluoroquinolones at killing adherent bacteria on the epithelial cells, and showed that low doses had a partial effect. These findings do not necessarily mean that fluoroquinolones should be used prophylactically or as the drug of choice to treat UTI in SCI patients. Certainly, there are arguments for using less expensive products such as co-trimoxazole, which at our institution costs around 10 cents per tablet compared to having to use active therapy to clear an infection, for example using 400 mg norfloxacin q12 h at \$2 per tablet or 500 mg q12 h ciprofloxacin at \$2.60 per tablet. However, unless an

antibiotic is able to penetrate and/or eradicate bacterial biofilms, its effectiveness is questionable (except that it may prevent spread of planktonic *E. Coli* to the kidney) and its usage could result in the emergence of more problematic pathogens.

Clinical trials will be required to verify that ciprofloxacin and other fluoroquinolones can truly eradicate biofilms and provide a clinically significant endpoint. Further studies would also be useful with shorter courses (which have been effective in otherwise healthy adults)¹¹ or even sub-minimum inhibitory concentrations of ciprofloxacin, to reduce adhesion and temporarily prevent recurrence of infection, without giving rise to a significant increase in the number of drug resistant uropathogens.

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