Intravesical Nitric Oxide Delivery for Prevention of Catheter-Associated Urinary Tract Infections

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The use of indwelling urinary catheters is a major risk factor for urinary tract infection; and despite the availability of numerous preventive regimens, this condition is still extremely common. In earlier studies we have demonstrated the inhibitory effects of nitrite and ascorbic acid on bacterial growth in urine. When combined, these compounds generate antibacterial reactive nitrogen species, including the gas nitric oxide. We have now tested in a laboratory model of the urinary bladder whether filling of the catheter retention balloon with nitrite and ascorbic acid would generate measurable amounts of NO outside the membrane and whether this would affect bacterial growth in the surrounding urine. Two strains of Escherichia coli, one strain isolated from a patient (U1106024) and one reference strain (ATCC 25922), were tested. Nitric oxide gas was generated in the silicone balloon and readily diffused into the urine. When control catheters with ascorbic acid but without nitrite were used, bacterial counts increased from 9.0 × 10^6 to 2.0 × 10^8 CFU/ml (strain U1106024) and from 2.5 × 10^6 to 2.7 × 10^8 CFU/ml (strain ATCC 25922) after 24 h. In contrast, in test catheters with ascorbic acid and nitrite, both strains tested were effectively killed. The NO donor (DETA NONOate, (Z)-1-[(N-(2-aminoethyl)-N-(2-ammonioethylamino)diazene-1-im-1,2-diolate]) also showed antibacterial activity in the same model, thereby supporting a central role of NO in achieving the observed effects. Future clinical trials will reveal whether this novel approach for the intravesical delivery of an antibacterial gas could be used to prevent catheter-associated infections.

Urinary tract infection (UTI) is the most common hospital-acquired infection, and the use of indwelling catheters is considered a major risk factor (12). In medical intensive care units in the United States, 95% of UTIs are catheter-associated UTIs (CAUTIs) (16). CAUTIs are also a major source of resistant nosocomial pathogens (10). With a Foley catheter, bacteriuria develops with a daily incidence of about 5 to 10%. Several attempts have been made to minimize catheter-associated infections, including the introduction of entirely closed drainage systems and prophylactic treatment with systemic antibiotics (12, 18). Another approach is to soak the catheter in an antibiotic compound or to cover the surface with a coating that prevents bacterial adhesion and the production of biofilms. Despite these efforts, CAUTIs are still very common, and the costs for these potentially serious complications are substantial (12). We have been studying a novel approach to prevent the growth of bacteria in urine (4, 5). When inorganic nitrite (NO_2^-) is acidified, a variety of reactive nitrogen species (RNSs) are generated, including nitric oxide (NO), a gas with antimicrobial properties (19). In the presence of the reducing agent ascorbic acid, NO generation from nitrite is greatly enhanced (19). Urinary pathogens such as Escherichia coli and Pseudomonas aeruginosa are killed if they are exposed to the combination of mildly acidified urine (pH 5 to 5.5) and nitrite, and the antibacterial effect is further enhanced by ascorbic acid (5). Because NO is a tiny uncharged gaseous compound, it diffuses readily over biological membranes. We therefore tested whether filling of the retention balloon of a urinary catheter with nitrite and ascorbic acid solution would generate measurable NO levels outside the membrane and whether this procedure would affect the growth of E. coli in a laboratory model of the urinary bladder.

MATERIALS AND METHODS

Bacterial cultures and media. The strains used in the study were an E. coli strain (strain U1106024) isolated from a patient with a urinary tract infection and a reference strain, E. coli ATCC 25922; both strains were obtained from the Department of Clinical Microbiology, Uppsala, Sweden. Urine was collected from four healthy male volunteers, pooled, and stored at −20°C until use. Before each experiment the bacteria were grown aerobically in Mueller-Hinton broth for 6 h at 37°C, resulting in 2 × 10^8 to 5 × 10^8 CFU/ml. This culture was added to 25 ml of urine to a final density of 10^8 CFU/ml.

The in vitro model of a catheterized urinary bladder. The inoculated urine was placed in long-necked 50-ml flasks with a shape resembling the urinary bladder and the urethra. An all-silicone catheter (Argyle; Sherwood Medical, Tullamore, Ireland) was placed in the flask, and the retention balloon was filled with 10 ml of saline containing ascorbic acid (10 mM) and sodium nitrite (5 mM) (Fig. 1). The acidity of the solution was adjusted to pH 2.5 by using hydrochloric acid (3 M). Ascorbic acid and nitrite were prepared and mixed immediately before administration. Ascorbic acid solution alone (pH 2.5) was used in control catheters. After the retention balloons were filled, the catheter was gently pulled outward and fixed at the neck, whereby the flask opening was sealed off. Then, the flasks were turned upside down and incubated at 37°C for 24 h. In separate experiments we also tested shorter exposure times of 5, 30, 60, and 120 min.

To compare the effects of nitrite with those of another NO-releasing compound, we also studied the antibacterial effect of DETA NONOate, which is considered a rather pure NO donor. In these experiments, the sodium nitrite was replaced with DETA NONOate (0.05, 0.5, or 5 mM), but all other test parameters were identical. DETA NONOate was prepared immediately before the
start of the experiment. To exclude any antibacterial effect of the parent compound (a polyamine), the same experiment was also performed with a DETA NONOate solution that had been prepared and stirred for 72 h in open air to release all NO (complete NO release was confirmed by chemiluminescence, which showed that the NO signal at 24 h was <0.1% of the initial peak level).

**Determination of bactericidal activity.** After incubation of the urine in the bladder model at 37°C, the urine was serially diluted with phosphate-buffered saline (pH 7.3) and the mixture was transferred to agar plates. The agar plates were further incubated for 24 h, and then a viable count was performed (counting of the CFU/ml). The effects were considered bactericidal when at least 99.9% (>3 log CFU/ml) of the original inoculum was killed. The effects were considered bacteriostatic when the bacterial counts (CFU/ml) did not change from those in the primary inoculum. All experiments were performed in quadruplicate. The pH of the urine was 6.1 and did not change significantly during the course of the experiments.

A portion of the urine was centrifuged (3,000 × g for 15 min) and then frozen for later determination of nitrate and nitrite concentrations by a chemiluminescence method described earlier (9). In preliminary experiments we noted that the nitrite accumulated up to about 2 mM in the urine outside the balloon containing nitrite and ascorbic acid. To rule out any antibacterial effects of the nitrite ion itself, we therefore grew bacteria in urine with the addition of 2 mM sodium nitrite and ascorbic acid.

In separate experiments (n = 4) the retention balloons were refilled with fresh solutions of nitrite and ascorbic acid on day 2 and were then incubated for an additional 7 days. After this period bacterial survival was determined as described above.

**Nitric oxide formation.** The kinetics of NO release from the retention balloon were monitored in separate experiments (n = 4). The catheter was placed in the bladder model (50-ml flask), and the retention balloon was filled with 10 ml of saline containing ascorbic acid (10 mM) and sodium nitrite (5 mM) at pH 2.5. The flask was then closed, and synthetic NO-free air was flushed via an inlet at a rate of 4 liters/min. The NO concentration in the headspace was continuously measured from an outlet with a rapid-response chemiluminescence system (Aerocrine AB, Stockholm, Sweden) at room temperature (20°C). Ambient NO levels were below 5 ppb in all experiments.

The NO release kinetics from solutions of sodium nitrite or the pure NO donor DETA NONOate were compared in separate experiments. Solutions of freshly prepared sodium nitrite (final concentration, 0.5 mM) or DETA NONOate (0.5 mM) were injected into a purge vessel (50 ml) placed on a magnetic stirrer. The purge vessel had been filled with 10 ml ascorbic acid solution (10 mM; pH 2.5) before the injection of nitrite or DETA NONOate. Air was flushed through the system via the inlet at a rate of 5 liters/min, and the NO concentration was measured (by chemiluminescence) and recorded continuously via a side arm on the outlet side.

**RESULTS**

**Effects on bacterial growth and survival.** In experiments with control catheters, the *E. coli* ATCC 25922 counts had increased from 2.5 × 10⁶ to 2.7 × 10⁸ CFU/ml (Fig. 2, top) and the *E. coli* (strain U1106024) counts had increased from 9.0 × 10⁵ to 2.0 × 10⁶ CFU/ml after 24 h (Fig. 2, bottom). In contrast, when test catheters with ascorbic acid and nitrite (5 mM) were used, both strains tested were effectively killed (Fig. 2). When the bacteria were exposed to nitrite for shorter periods of time (5 to 120 min), bacterial growth was completely prevented during the exposure period, but no killing occurred (Fig. 3). A 24-h exposure to DETA NONOate was completely bacteriostatic at 5 mM, while 0.5 and 0.05 mM had no effect on bacterial growth (data not shown).

In the separate experiments, when the retention balloons were refilled with fresh solutions of nitrite and ascorbic acid on day 2 and then incubated for an additional 7 days, no bacteria could be recovered when samples were cultured on the recovery plates (data not shown).

**Nitric oxide formation.** The first experiments were done to find out if the NO that formed inside the balloon could diffuse through the silicone membrane to the outside. NO was detected in the headspace gas outside the nitrite- and ascorbic
acid-containing retention balloons. The NO concentration initially peaked at ~10 ppm and then gradually decreased (Fig. 4). If the balloon was filled with nitrite alone (pH 7) or ascorbic acid alone (pH 2.5), no NO signal was noted (NO concentration, <0.001 ppm).

In additional experiments the amounts of NO released from equimolar (0.5 mM) amounts of nitrite or DETA NONOate were compared. This was done directly in solutions without the balloons. The amount of NO released from nitrite solutions initially peaked at 5 ppm and then declined rapidly, with a half-life of 3 min. The levels of NO released from DETA NONOate peaked at 1.5 ppm, with a half-life of about 30 min. The total amounts (areas under the curve) of NO released from nitrite and DETA NONOate (0.5 mM) were almost identical (data not shown).

When the balloons with acidified nitrite (5 mM) or DETA NONOate (5 mM) were placed in urine, nitrite accumulated in the urine at levels of 1.65 and 1.41 mM, respectively. In further experiments, the bacteria were grown in urine to which 2 mM sodium nitrite was added, and no inhibition of growth was noted (data not shown).

**DISCUSSION**

We show here that the urinary pathogen *E. coli* is effectively killed by a diffusible antibacterial compound generated from the combination of nitrite and vitamin C. A tiny uncharged gas such as NO can easily diffuse through the silicone membrane of the retention balloon, as shown here. Thus, in this setting the retention balloon was used as the source of an antibiotic agent. This approach is somewhat similar to that used in a study by Stickler and colleagues (17), in which the retention balloon was filled with the antisepsic agent triclosan, which dissolved in the silicone membrane, thereby inhibiting bacterial (*Proteus mirabilis*) adherence and encrustation. It is likely that the combination of the different RNSs produced from acidified nitrite accounts for the antibacterial effects observed in the present study. The exact mechanism whereby NO and related compounds inhibit bacteria is still not settled, but multiple cellular targets, including DNA, surface proteins, and key enzymes in the respiratory chain, are most certainly involved (6, 19). At least three facts favor the view that NO is involved at some stage in the process. First, only a small uncharged molecule such as NO is expected to pass the silicone membrane freely, whereas charged nitrogen oxides, e.g., NO$^+$ or NO$_2^-$, or free hydrogen ions will not. Indeed, we did detect NO outside of the membrane (Fig. 3). The fact that the pH outside the membrane did not change illustrates that protons traverse the silicone membrane poorly. Second, ascorbic acid strongly favors the formation of NO from acidified nitrite at the expense of most other RNSs (19). Third, the NO donor DETA NONOate also had antibacterial effects, and this NO donor is believed to release pure NO with minimal formation of other reactive nitrogen intermediates (7). It is also possible that the reactive nitrogen intermediates that pass the membrane can react with constituents of urine to yield other yet uncharacterized compounds with antibacterial activity. In addition, once NO has passed into urine, it could form other RNSs with antibacterial activity, for example, N$_2$O$_3$ or N$_2$O$_4$. In the model used here, large amounts of nitrite accumulated in the urine as a consequence of NO oxidation. This nitrite is an unlikely candidate, however, because it completely lacked antibacterial effects when it was placed directly in the urine, as shown here and earlier (5).

Bacteria have developed mechanisms to defend themselves against NO and other RNSs generated by phagocytic cells (8). Flavohemoglobins, an ancient family of proteins found in many bacteria, including *E. coli* (14, 15), are an example of such a defensive pathway. These proteins effectively bind NO, thereby protecting the bacteria from nitrosative stress. Alternatively, under low oxygen tensions a bacterial enzyme cytochrome c nitrite reductase (NrfA) may further reduce NO to other less toxic nitrogen metabolites (13). However, in this setting such defensive pathways were obviously insufficient, since the bacteria did not survive the exposure to RNSs generated from acidified nitrite. The reason for this is not entirely clear, but it could be related to the rapid kinetics of NO release (Fig. 4). Thus, with such large acute exposure to RNSs, there is probably no time for upregulation of the defensive pathway de-
scribed above. On the other hand, when the bacteria were exposed to acidified nitrite for shorter periods of time (5 to 120 min), the effect was not bactericidal; so clearly, the antibacterial effects are greater with longer exposure times, even though the amount of NO released from the active compounds had decreased markedly. This could support the notion mentioned above that other more long-lived antibacterial compounds are formed in the urine from the reactive nitrogen intermediates generated in the retention balloon.

The slower NO release kinetics of DETA NONOate compared to that of nitrite could explain why this compound had a bacteriostatic effect while nitrite was bactericidal. Indeed, the total amounts of NO released from these compounds were similar, as shown by the nitrite accumulation in urine and the NO release data.

In the present study we examined two different strains of E. coli, the most common cause of UTIs. Although they were not studied here, it is likely that other urinary tract pathogens are sensitive to this procedure. Thus, it is known that the RNSs generated from acidified nitrite have effects against several of the most common urinary pathogens, including E. coli, P. aeruginosa, and Staphylococcus saprophyticus, as well as other pathogenic bacteria (5, 8).

Toxicity is an important issue if this concept should be considered for clinical use. The concentrations of ascorbic acid (10 mM) used here were in the same range as those found in urine after the intake of 1 to 2 g of this vitamin (3), and a similar concentration of nitrite (3 mM) is allowed when this anion is used as a food additive, for example, in cured meat. Moreover, the stomach mucosa is continuously exposed to considerable amounts of endogenous nitrogen oxides from the natural acidification of nitrite in saliva (19). In fact, salivary nitrite levels can reach 1 to 2 mM after the ingestion of nitrate-rich food (2). Thus, these antibacterial compounds are expected to have rather low levels of acute toxicity to host cells. Whether a prolonged exposure of the bladder to these RNSs could be harmful, for example, by promoting the formation of potentially carcinogenic nitrosamines (1), must indeed be studied further.

We have done a first “proof-of-concept” study here to find out if this novel way of administering an antimicrobial drug to the urinary bladder can work. Naturally, further studies are needed before this method can be considered for clinical use. Important issues include toxicity studies, testing of the durability of the effects and exact dosing, and evaluation of the risk of bacterial resistance. Nevertheless, some features of this concept are potentially very attractive. The method allows repeated administration of active drug at chosen doses and intervals without disturbing the integrity of the closed drainage system. Considering the potent bactericidal effects, it is not unlikely that once-daily administration of new drug is sufficient to prevent the establishment of new bacteria, but again, this must be tested. Another attractive feature of this method is that the antibacterial mechanism mimics that already used by immunocompetent host cells. Indeed, nitric oxide and other RNSs are produced endogenously, for example, by activated white blood cells; and they play a central role in primary host defense through their antimicrobial actions (6, 8, 11).

The unspecific multitarget antibacterial actions of NO and related RNSs are a feature that likely reduces the risk for bacterial resistance. Indeed, NO has been a successful part of the endogenous host defense for millions of years (6, 19). An additional advantage with the concept described here when it is compared to most traditional systemic antibiotics is that there is likely no ecological effect on the intestinal microflora.

In conclusion, we describe here a novel concept which allows the intravesical delivery of antibacterial gases into the urinary bladder via the catheter retention balloon. Future clinical trials will elucidate whether this approach could have a place in clinical practice for the prevention of catheter-associated urinary tract infections.

REFERENCES