Slow Release of Nitric Oxide from Charged Catheters and Its Effect on Biofilm Formation by *Escherichia coli*[▽]

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Catheter-associated urinary tract infection is the most prevalent cause of nosocomial infections. Bacteria associated with biofilm formation play a key role in the morbidity and pathogenesis of these infections. Nitric oxide (NO) is a naturally produced free radical with proven bactericidal effect. In this study, Foley urinary catheters were impregnated with gaseous NO. The catheters demonstrated slow release of nitric oxide over a 14-day period. The charged catheters were rendered antiseptic, and as such, were able to prevent bacterial colonization and biofilm formation on their luminal and exterior surfaces. In addition, we observed that NO-impregnated catheters were able to inhibit the growth of *Escherichia coli* within the surrounding media, demonstrating the ability to eradicate a bacterial concentration of up to 10⁴ CFU/ml.

Urinary tract infection (UTI) is the most prevalent cause of nosocomial infections, 80% of which involve catheter-associated urinary tract infection (CAUTI). The risk of acquiring CAUTI depends on the method and duration of catheterization, the quality of catheter care, and host susceptibility (27, 32). Free-floating (planktonic) bacteria can adhere to surfaces on catheters and colonize, creating a tenacious milieu called a biofilm (6). These sessile microcolonies consist of bacteria that are highly differentiated and extremely resilient against standard antibiotics (35). Several innovative approaches have focused on inhibition of biofilm formation in order to prevent CAUTI. These include antiseptic lubricating gels applied at the catheter insertion point, the use of a taped seal applied to the catheter drainage tubing junction, and utilizing an antireflux valve (14, 34). A new approach has recently been emerging whereby catheters are coated with various antiseptic materials (14, 17, 28). For example, catheters coated with silver or silver-containing compounds show clinical promise, but clinical effectiveness varies and appears to be dependent on the silver matrix used. (34). Hydrogel- or silver-hydrogel-coated catheters have been suggested to provide an antiseptic benefit by creating a physical barrier to bacterial infection, thereby preventing adhesion of the bacteria to the catheter (14, 26). The synergistic combination of compounds such as chlorhexidine and protamine sulfate has also been evaluated with some success (8). Antibiotic-coated catheters containing ciprofloxacin, gentamicin, norfloxacin, and nitrofurazone have also been designed (14, 17, 26). However, the risk of developing an antibiotic-resistant strain of bacteria is high (26, 34).

Nitric oxide (NO) is a small, naturally produced, hydrophobic, free-radical gas that has a major role in innate immunity.

NO exhibits broad reactivity and rapid diffusive properties through biological liquids and lipid membranes, with a short half-life in a physiological milieu (33). Overproduction of NO induced by the enzymatic activity of inducible nitric oxide synthase (iNOS) in various cell types has been shown to play a vital role in several inflammatory and immunoregulatory processes (1). Most notably, NO has been shown to play important roles in vasodilatation, neurotransmission, angiogenesis, modulation of wound healing, and nonspecific responses to infection (19, 29).

The antimicrobial activity of NO was demonstrated more than 50 years ago (31), with later in vitro studies showing inhibition of a wide variety of Gram-negative and Gram-positive bacterial species (16). NO was shown to be bacteriostatic (3, 9, 10, 23), with recent in vitro evidence demonstrating bactericidal effects (20). We have shown previously that multiple 30-min treatments of 160 ppm nitric oxide results in over a 5 log₁₀ CFU/ml reduction in the bacterial load of *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (21).

A variety of methodologies have been attempted to deliver and study NO therapeutically. They include the use of polymers containing a diazeniumdiolate NO donor (16, 24), exposure chambers for direct topical application of NO (11, 20), and filling a urinary catheter retention balloon with nitrite and ascorbic acid to release NO (4).

In this paper, we present a novel approach that creates an antiseptic barrier on urinary catheters by impregnating the catheters with gaseous NO (gNO) using a proprietary technology (2). We show that NO-impregnated Foley urinary catheters slowly release NO into urine over 14 days and are stable under various clinical models and storage. We also provide data showing that these NO-impregnated catheters are rendered antiseptic, prevent bacterial colonization on their exterior and luminal surfaces, and are able to eradicate up to 10⁴ CFU/ml of *E. coli* in the surrounding media under both stagnant and dynamic conditions.

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MATERIALS AND METHODS

Technique for impregnating catheters with gaseous nitric oxide. A 6-mm-diameter Folsyl silicon Foley catheter (catalog no. AA6118; Coloplast Corp. Minneapolis, MN) was taken as a whole or aseptically cut into 3-cm sections and impregnated with NO (Airgas Specialty Gases, Chicago, IL) in a previously described exposure chamber under proprietary conditions and using a proprietary technique (2, 12). Unexposed catheter sections, used for controls, were kept in sterile sealed vials. Following impregnation of the catheters, four 1-cm lengths of the NO-treated and untreated pieces were put in separate vials with 5 ml of sterile water. One-hundred- μ l aliquots were taken at time intervals for NO analysis. To ascertain accumulated NO release over a 48-h period, the nitrite and nitrate contents for each time point were measured using Griess reagent (13).

We used the following formula to convert reported nitrite production into parts per million of NO: $\text{NO}_{\text{ppm}} = (46 \times [\text{NO}_2^-]) \times 0.65 \times 10^{-3}$.

Each μM ($\mu\text{mol/liter}$) was multiplied by the nitrite's molecular mass (46 g/mol). This value was converted to ppm of NO, taking into account the difference in molecular weight (MW) between nitrites and NO (0.65) and the multiplication factor between grams and milligrams (10^3). All calculations were done per 1 cm of catheter.

To follow the release of NO per day from impregnated catheters, four sections were processed following 24 h of NO impregnation, under the following conditions: (i) immersion in 5 ml of sterile water, (ii) storage for 1 week in air and then placement in 5 ml of sterile water, and (iii) placement in 5 ml of sterile urine. The sections were transferred to new vials with fresh sterile water/urine every 24 h. Each day, 100- μ l aliquots were taken for measurement of nitrite and nitrate contents using Griess reagent. Urine was collected every day from a male volunteer. The urine was filtered (with a sterile syringe filter [0.2 μm]), and 50 μg per ml ampicillin was added (as a final concentration).

Bacterial preparation. The *E. coli* bacterial culture was obtained from the American Type Culture Collection (ATCC 25922). Bacteria were grown to 0.5 McFarland standard, and 1-ml aliquots of these preparations containing approximately 2.5×10^8 CFU/ml were stored at -70°C . On the day of the experiments, the fresh stock was removed from the freezer and thawed, and 2 ml of Luria Broth (LB) was added. The cultures were further diluted with LB to 10^4 CFU/ml in volumes (inocula) specific to the experimental conditions. The concentration of 10^4 CFU/ml was chosen because it is 1 $\log_{10}$ CFU/ml below an accepted threshold for determining bacterial colony infection that is below the accepted threshold for determining significant bacteriuria (18).

Planktonic antimicrobial activity of NO-impregnated catheters. Three different concentrations (10^2 CFU/ml, 10^3 CFU/ml, and 10^4 CFU/ml) of *E. coli* inocula were incubated with 2 ml inoculum and 1 cm of catheter section at 37°C for 24 h. At time points of 0 and 24 h, the tubes were vortexed and aliquots were plated on LB agar and then incubated at 37°C for 24 h. The CFU were counted, and the final bacterial load was calculated as CFU per ml.

Presence of *E. coli* colonization on the surfaces of NO-impregnated catheters. Catheter sections were immersed for 24 h in 10^2 CFU/ml, 10^3 CFU/ml, or 10^4 CFU/ml of *E. coli*. The catheter sections were washed twice with sterile double-distilled water (ddH_2O) and then aseptically transferred to LB agar plates. Each section was rolled once on the plate and then incubated at 37°C overnight.

Dynamic urine flow model system. Urine (2 liters) was collected from male volunteers and placed in two sterile plastic containers (1 liter each). Two ml of 10^2 bacteria was inoculated into each container. Impregnated and control catheters were placed separately into each container. Urine from the containers was recirculated in a closed system, using a flow rate of 1.5 ml/min (Rabbit-Plus peristaltic pump; Rainin) through the catheters, for 24 h at 37°C . The catheters were aseptically removed, washed with sterile water, cut into 3-cm pieces, and processed as follows. (i) A biofilm formation assay was performed as previously described (25). Briefly, each catheter piece was cut lengthwise and placed with 4 ml of 1% (wt/vol) crystal violet for 15 min. The vials were washed, and the solution was replaced with 4 ml of 95% ethanol. The extracted color was measured at 595 nm. (ii) In order to measure biofilm-embedded bacteria, each piece (cut lengthwise) was washed and put into 4 ml sterile water. Following sonication for 30 s, samples were plated on LB plates and incubated for 24 h at 37°C .

RESULTS

Release of NO from NO-cured urinary catheters. NO has a short half-life in vivo of a few seconds. Therefore, the levels of more stable NO metabolites, nitrites and nitrates, were used for indirect measurement of NO in biological fluids (13). We impregnated Foley catheters as described in detail

FIG. 1. Accumulation of NO production from impregnated catheters. Shown is the total accumulation of nitrites and nitrates, in water, produced from catheters impregnated with NO. Nitrites and nitrates were measured using Griess reagent. The nitrite concentration was calculated per 1 cm of catheter. The error bars indicate standard deviations.

elsewhere (2). During the 48 h following NO impregnation, NO was slowly released from the catheter section (Fig. 1), with the largest amounts released during the first hour and decreasing slowly for 48 h. After 24 h, approximately 46 μM of nitrites was accumulated for every 1 cm of catheter. This equals 2.2 ppm nitrites, the equivalent of 1.4 ppm NO being released. Figure 2A shows that NO release decreased with time, declining from 60 μM at day 1 to about 2 μM per day after 14 days. Two μM is equivalent to 0.06 ppm for a 1-cm section. Storage of the impregnated catheter in air for 1 week (Fig. 2B), caused some loss of NO, but still, as from day 3, the amount of NO released was about the same as that from the nonstored sections.

To simulate a static clinically relevant milieu, we immersed the catheters in urine. Fresh urine was used every 24 h to control for nitrites. Urine nitrites remained constant and served as the baseline. Figure 2C shows that the initial amount of nitrites detected at day 1 was four times higher in urine than in water. Nevertheless, from day 3 onward, the same amount of nitrites was detected in urine and water.

NO-impregnated catheters prevent biofilm formation. As illustrated in Fig. 3, although the impregnated catheter section was immersed in bacterial culture for 24 h, no bacteria were attached to it. Even at a bacterial concentration of 10^4 CFU/ml, there was still an apparent reduction of bacteria attached to the NO-treated section versus the nontreated section. Thus, biofilm formation was prevented with up to 10^3 CFU/ml bacteria and minimized with 10^4 CFU/ml.

Even though NO does not travel far in solution, it showed antimicrobial activity when immersed in an *E. coli*-containing solution. An antimicrobial effect was shown in all three concentrations tested, with a stronger effect at 10^2 and 10^3 bacteria (Fig. 4). After 24 h, all three control concentrations tested were at about 10^8 CFU/ml bacteria, while levels were about 50 , 10^2 , and 10^5 CFU/ml of bacteria for the treated section when their starting concentrations were 10^2 , 10^3 , and 10^4 CFU/ml, respectively. The NO effect was observed for up to 12 days of immersion in a liquid. The slow release of NO (Fig. 2A) and its antimicrobial effect were clearly demonstrated (Fig. 5) to show a 90% reduction in planktonic bacterial growth even after 12 days of continuous release of NO immersed in liquid.

FIG. 2. NO production per day from impregnated catheters. Shown is production of nitrites and nitrates per 24 h, in direct correlation with NO production, during a 14-day period. (A) Production after storage of the catheter in sterile water. (B) Production after storage of the catheter in air for 7 days after impregnation and then immersion in sterile water. (C) Production after storage of the catheter in sterile urine. All storage media were changed each day. The nitrite and nitrate contents were measured using Griess reagent. The nitrite concentrations were calculated per 1 cm of catheter. The error bars indicate standard deviations.

In a dynamic situation where urine was flowing for 24 h, bacterial levels in the control and treated containers reached 10^8 and 10^7 CFU/ml after 24 h, respectively. However, using crystal violet, we showed that more than twice the amount of biofilm matrix was formed on the luminal surface of the control catheter as on the impregnated catheter (Fig. 6A). After the removal of matrix-bound bacteria, using sonication, there were no bacteria on the impregnated

FIG. 3. Presence of *E. coli* on the surfaces of impregnated catheters. Comparison of *E. coli* colonization on NO-impregnated catheters and on control catheters after immersion of the catheters for 24 h in suspensions comprising 10^2 CFU/ml (I), 10^3 CFU/ml (II), or 10^4 CFU/ml (III) *E. coli*. Each of the images shows a three-compartment petri plate in which a selected immersed catheter was rolled over the surface of each compartment and then incubated at 37°C overnight.

catheter surface compared to an average of 1.2×10^4 CFU/ml on the control piece (Fig. 6B).

The effect of storage conditions on NO catheters and their antimicrobial activity in suspensions of *E. coli*. After a week of storage in either water or air, bacterial colonization on the surfaces of the catheter sections was still prevented. After catheter sections were immersed for 60 s in a suspension of 10^3 CFU/ml bacteria and then incubated for 24 h in phosphate-buffered saline (PBS), no bacteria were found to attach to the impregnated sections, and thus, no bacteria were transferred to the PBS (Fig. 7).

To assess the effect of catheter storage on antimicrobial activity after impregnation, we tested the effect of 1 week of storage in water or air. Figure 8 shows that after a week of storage, whether in air or in water, NO-cured catheters still showed significant antimicrobial activity. These stored catheter sections were immersed in suspensions of 10^3 CFU/ml bacteria for 1 min. After being transferred to PBS and incubated for 3 or 24 h, aliquots did not grow any bacteria, whereas the controls grew as many as 10^8 CFU/ml bacteria. This indicated that the gNO-cured catheters, after being stored for 1 week in

FIG. 4. Antimicrobial activity of impregnated catheters. Shown is a comparison of the growth of *E. coli* in media from catheters impregnated with NO versus media from control catheters after immersion of the catheters for 24 h in suspensions comprising 10² CFU/ml (I), 10³ CFU/ml (II), or 10⁴ CFU/ml (III) *E. coli*. Each of the images shows a three-compartment petri plate in which each compartment was surface plated with an aliquot of suspension from a selected catheter previously immersed in a selected *E. coli* suspension and then incubated at 37°C overnight. (A) Representative plates of samples. (B) Viable counts of the triplicate CFU. The hatched bars are data from the control, while the checkered bars are data from the impregnated catheters. The error bars indicate standard deviations.

either air or water, still released sufficient quantities of NO into the surrounding solution to act as a planktonic antimicrobial agent.

DISCUSSION

The results of this study demonstrated that we were able to impregnate urinary catheters with NO, which prevented biofilm formation on their surfaces and might even have formed a zone of bacterial inhibition around the catheter. The amount of NO that was released was approximately 5 to 150 ppm per catheter, starting from 150 ppm (5 mM) per 24 h on the first day after impregnation and decreasing to 5 ppm (166 μM) after the catheters were immersed in solution for 14 days. This is consistent with the effective antimicrobial dose of NO re-

FIG. 5. Persistent antimicrobial effects of NO-impregnated catheters immersed in water. The data describe CFU formation with treated and untreated catheter sections that were previously immersed in water (changed each day) over 2 weeks. The catheter sections were immersed in 10³ CFU/ml of *E. coli* for 1 min and transferred to PBS for 24 h, and then samples were plated. The hatched bars are data from the control, while the checkered bars are data from the impregnated catheters. The error bars indicate standard deviations.

ported elsewhere in the literature (20, 21). Specific to a clinical application, these results are in the range of those shown by Carlsson et al., who found 10 ppm NO (produced by the reaction between sodium nitrite and ascorbic acid) to be an efficient antimicrobial concentration in urinary bladder infection (4). Storing the impregnated catheter in air for a week resulted in 50% reduction of NO released on the first day compared to the nonstored catheter. During the 14 days measured, a lower release rate was observed in the stored catheters. However, the same amount of NO was released after 14 days. These results suggest that impregnated catheters are stable and can be packed and stored before use. Immersion of the impregnated catheter in urine for 14 days resulted in four times the amount of NO released in the first 24 h after impregnation. NO was released more rapidly in urine, probably because of the highly acidic environment, which facilitates NO dispersion from the catheter surface. Nevertheless, after day 3, the amount of NO released in urine was similar to that released in water.

We further showed that, even after being stored in water or air for a week, the amount of NO released from the catheter was sufficient to have an effect on the surrounding planktonic bacteria (Fig. 8B). This strengthens our previous findings implying that the effect of NO released from a catheter is not immediate. Nitric oxide is dose dependent and needs time to “start working” (21). Biofilms are communities of sessile bacteria covered in an extracellular matrix of secreted proteins and carbohydrates. Biofilms assume phenotypes distinct from those of planktonic cells, demonstrating increased intrinsic resistance to antimicrobial drugs compared to planktonic bacteria (5, 15, 26). Based on our findings, impregnating the catheter with NO prevented the adhesion of bacteria to the catheter in a concentration of 10⁴ CFU/ml of bacteria and significantly eradicated the surrounding planktonic *E. coli* bacteria. Critical colonization is defined clinically as a bacterial concentration of less than 5 log₁₀ CFU/ml (4). Without associated clinical symptoms (e.g., inflammation, pain, edema, and

FIG. 6. Biofilm formation and biofilm-embedded bacteria on the inner lumen of the catheter after 24 h of urine flow. Shown is a comparison of colonized biofilm formation. (A) Biofilm formation showing the absorbance at 595 nm of the crystal violet that was attached to the catheter pieces after extraction with ethanol. The hatched bars are data from the control, while the checkered bars are data from the impregnated catheters. The error bars indicate standard deviations. (B) Biofilm-embedded bacteria. The image compares bacteria within the biofilms on control and impregnated catheters. Following 30 s of sonication, 1 μ l (bottom) and 10 μ l (top) of water surrounding a selected catheter piece were plated (in triplicate) and incubated for 24 h.

suppuration), this concentration of bacteria, by itself, is not considered an infection requiring antibiotic therapy (30). Our data suggest that impregnated NO catheters are useful in preventing bacterial levels associated with biofilm formation, but these findings may not apply to an already clinically infected catheter scenario. The mechanism by which NO prevents bacterial adhesion is still unknown. One hypothesis is that NO, through reactive intermediate species such as N_2O_3 and N_2O_4 ,

destroys the function of bacterial adhesion proteins that mediate surface adhesion (16). Another hypothesis is that NO creates a physical barrier on the surface of the catheter. Darling and Evans have suggested that NO produces a chemical modification of surface thiols or metal centers involved in critical enzymatic or regulatory function. Inactivation of cysteine proteases was suggested as a general mechanism of NO-related antimicrobial activity (7). It has also been suggested that gelatinase, controlled by the gene *gelE*, plays a key role in the formation of a biofilm (5). We hypothesize that suppression of *gelE* expression may be involved in the NO^- inhibition mechanism. However, this requires further research.

This study suggests that NO associated with the surface of a standard urinary catheter can act as an antimicrobial agent in an environment of up to 10^4 CFU/ml of *E. coli* for up to 24 h. Moreover, even after 12 days of immersion in water, the NO-impregnated catheter was still effective, with only 3% of bacteria present compared to the control. A variety of hypotheses that attempt to explain this in vitro effect are found in the literature. Recent work by our laboratory has identified and demonstrated the mechanism of thiol-dependent depletion associated with the antibacterial action of NO (22). Bacterial cells have evolved detoxification mechanisms against NO cytotoxicity in which NO reacts with the low-molecular-weight thiols. Once the protected thiol is depleted, the cells become highly vulnerable to NO, which reacts with a large number of cellular and biochemical targets.

We acknowledge that the limitation of an in vitro model of this type is that it does not immediately translate to the clinical environment. For instance, NO-charged catheters are exposed to large volumes of urine flow in patients, up to 2 to 3 ml/min. This constant flow of urine may wash NO away or reduce the amount of NO associated with the catheter lumen and thus ameliorate the purported zone of bacterial inhibition observed in this study. As we measure NO into 5-ml aliquots of fluid, it would be beneficial if we could determine experimentally the NO concentrations released when urine is flowing through catheters at normal flow rates. On the other hand, in a clinical setting, high urinary flow may be synergistic to the NO effects observed here by preventing bacteria from adhering easily to the catheter. In this study, we addressed this limitation in two

FIG. 7. Presence of *E. coli* associated with the surfaces of impregnated catheters after 1 week of storage. Shown is a comparison of colonization of *E. coli* on control catheters and on catheters impregnated with NO after both sets had been stored for 7 days in 5 ml sterile air (I) or 5 ml sterile water (II). After storage, the catheter sections were immersed in 10^3 /ml *E. coli* for 1 min, followed by 24 h of incubation in PBS, after which a selected section was rolled on the surface of a compartment in a selected petri plate and incubated at 37°C overnight.

FIG. 8. Effect of catheter storage conditions on the antimicrobial activity of planktonic *E. coli* in surrounding catheter fluids. Comparison of colonization of *E. coli* on control catheters and on catheters impregnated with NO after both sets were stored for 7 days in 50 ml sterile air (I) or 5 ml sterile water (II). After storage, the catheter sections were immersed in 10^3 CFU/ml *E. coli* for 1 min, followed by 24 h of incubation in PBS. Each of the images shows a three-compartment petri plate in which each compartment was surface plated with an aliquot of suspension from a selected catheter previously immersed in a selected *E. coli* suspension and then incubated at 37°C overnight. (A) Representative plates of samples. (B) Viable counts of the triplicate CFU. The hatched bars are data from the control, while the checkered bars are data from the impregnated catheters. The error bars indicate standard deviations.

ways. In the stagnant experiment, we replaced the fluid surrounding the catheter sections every 24 h and showed antimicrobial activity of NO-impregnated catheters. Furthermore, in the dynamic experiment, we were able to show inhibition of biofilm formation on those catheters when urine was flowing for 24 h.

To summarize, this work showed that NO can be impregnated into urinary catheters, released slowly over 2 weeks, and have the ability to prevent the formation of biofilm on the surface of the catheter and to create a zone of bacterial inhibition in solution. Moreover, it appears that these catheters may be stored without losing these properties.

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