Nitric oxide releasing nanoparticles are therapeutic for *Acinetobacter baumannii* wound infections

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*Acinetobacter baumannii (Ab)* is a frequent cause of hospital acquired pneumonia and recently has increased in incidence as the causative agent of severe disease in troops wounded in Afghanistan and Iraq. Ab clinical isolates are frequently extremely resistant to antimicrobials, significantly complicating our capacity to treat infections due to this pathogen. Hence, the development of innovative therapeutics targeting mechanisms to which the bacteria are unlikely to evolve resistance is urgently needed. We examined the capacity of a nitric oxide-releasing nanoparticle (NO-np) to treat wounds infected with Ab. We found that the NO-nps were therapeutic in an experimental *Ab* murine wound model. Treatment with NO-nps significantly accelerated healing of infected wounds. Histological study demonstrated that NO-np treatment reduced suppurative inflammation, decreased microbial burden and reduced the degradation of collagen. Furthermore, NO-np treatment alters the local cytokine milieu. In sum, we demonstrated that the NO-nps are an easily administered topical antimicrobial for the treatment of *Ab* wound infections, and our findings suggest that NO-nps may also be ideal for use in combat or disaster situations.

**Introduction**

The Gram negative coccobacillus *Acinetobacter baumannii (Ab)* has become an increasingly prevalent cause of hospital-acquired infections during the last 15 years. This pathogen is a frequent cause of pneumonia and recently has been identified as the etiologic agent of complicated infections, especially wound infections, in troops injured in Afghanistan and Iraq. Moreover, the majority of clinical *Ab* isolates display high-level resistance to antimicrobials, which severely compromises our capacity to care for patients with *Ab* disease.

The topical application of nitric oxide (NO) has the capacity to prevent and treat skin infections, including Methicillin-resistant *Staphylococcus aureus* (MRSA) infections. NO modulates immune responses and modifies wound healing. Our laboratories have formulated a silane hydrogel nanoparticle capable of sustained NO release. Further, chitosan is admixed in the nanoparticle, adding to the antimicrobial potency of the particle. Chitosan is a cationically charged polymer derived from the exoskeletons of crustaceans that is capable of disrupting cellular membranes and damaging cell walls.

We have applied the nitric oxide-releasing nanoparticles (NO-np) to study their efficacy in the setting of *Ab* skin infection. Amperometric studies have previously demonstrated that NO is immediately released upon the addition of NO-np to liquid. After an initial peak, the stable release of NO is rapidly achieved and ongoing release occurs for ~24 hours. Based on our previous work and the fact that NO has broad antimicrobial activity, we theorized that NO-np could be therapeutic in the setting of *Ab* disease. Hence, we examined the therapeutic potential of NO-np in mouse *Ab* wound infection model.

**Results**

**NO-np inhibits *Ab* growth.** *Ab* 0057 growth with and without exposure to NO-np was determined in real-time for 24 h (Fig. 1). NO-np significantly reduced bacterial growth after 12 h co-incubation when compared with *Ab* grown with 2.5 (NO released from nps corresponding to an initial peak of 37.5 nM and a steady state of 25 nM), 5 (initial peak of 75 nM and steady state of 50 nM) and 10 (initial peak of 150 nM and steady state of 100 nM) mg/mL of np or medium alone. Notably, 20 (initial peak of 300 nM and steady state of 200 nM) mg/mL of NO-np completely inhibited *Ab* growth. Interestingly, *Ab* grown with concentrations of 10 or 20 mg/mL of np had significant reductions in bacterial growth at 12 h, an effect that is likely due to the
chitosan in the control np.16 However, these concentrations of np did not inhibit Ab growth after this time.

The rate of wound-healing in mice increased with NO-np. We found that NO-np significantly impacted the rate of wound healing (Fig. 2). Mice receiving NO-np healed more rapidly (Fig. 2B). The average diameter of NO-np treated Ab wounds were ~5.33 mm after 2 days, but the control wounds were ~6.15 and ~6.3 mm in untreated or np-treated mice, respectively, (p < 0.05; compared with NO-np treatment). By day 3 (Fig. 2B), the diameter of the wounds of the NO-np group was ~4.7 mm compared to ~5.95 and ~6.15 mm in untreated or np-treated mice, respectively, (p < 0.001), respectively. Consistent with our prior work, NO-np-treated uninoculated wounds healed to a significantly greater extent (complete healing occurred by ~7–8 days) than control mice (~10–12 days) (data not shown).

Microbicidal activity of NO against Ab in wounds. The microbicidal activity of NO-np for Ab in the wounds was determined by measurements of tissue bacterial burden. The bacterial counts in Ab-infected wounds subject to NO-np treatment were significantly lower than in control animals (p < 0.01) (Fig. 3A). Pathological studies showed that the infected tissues from Ab-infected untreated or np-treated animals displayed dense, neutrophil-rich infiltrates, extensive bacterial penetration and cellular necrosis (Fig. 3B and C). Gram stains confirmed the presence of numerous Gram-negative cocccobacilli (Fig. 3C; insets). In contrast, NO-np treatment significantly reduced the inflammatory response and fewer bacteria were visualized (Fig. 3B and C; insets). Increased fibrin deposition was also present. Notably, in contrast to the untreated or np treated animals, tissue Gram stains revealed that bacteria were only seen at the wound margins.

Collagen degradation was impeded in the presence of NO-np. Bacterial infection of tissue frequently leads to collagen degradation. The amount of collagen in Ab wounds subjected to NO-np treatment was significantly greater than the collagen content in the lesions on control animals (Fig. 4A). Furthermore, the collagen in NO-np treated wounds was more mature, consistent with maintenance of tissue architecture. The quantitative analysis of collagen is provided in Figure 4B.

Figure 1. NO-np inhibits Ab growth in vitro. The effect of NO on Ab growth kinetics was determined using Bioscreen C analysis. Ab was grown in the absence (untreated) or presence of nanoparticles with NO (NO-np) or without NO (np). Each point represents the average of four measurements.

Figure 2. NO-np increased wound healing rate in mice. (A) Wounds of Balb/c mice untreated Ab-infected, np-treated Ab-infected, and Ab-infected NO-np-treated, 3 days post-infection. Scale bar: 5 mm. (B) Wound size analysis of Balb/c mice skin lesions. Time points are the averages of the results for five measurements, and error bars denote standard deviations. *p < 0.05; **p < 0.001 in comparing the NO-np-treated group with untreated and np-treated groups.

NO-np induces cytokine expression by the host. We measured the cytokine response in the wounds of mice intradermally infected with Ab. At day 3 post-infection, wound tissues from mice treated with NO-np displayed significantly greater concentrations of IL-12 and IFNγ than that of np or untreated mice (Table 1). Furthermore, wound tissue of infected mice treated with NO-np exhibited reduced levels of IL-4, TGFβ and IL-1β. Notably, np-treated mice produced significant quantities of TNFα, IL-4, TGFβ and IL-1β whereas untreated mice did not show evidence of a consistent pattern of cytokine expression.

Discussion

Ab is a nosocomial pathogen of increasing importance.17 Although pneumonia and sepsis have been well recognized sequella to Ab acquisition, skin and soft tissue infections have increasingly been recognized as a major complication of Ab in the settings of battlefield injuries, surgical wounds and ulcers.18 The organism possesses an impressive armamentarium of resistance mechanisms that can lead to resistance to all, or almost all, commercially available antibiotics.19 In fact, treatment of patients infected with multi-drug resistant Ab has become a formidable challenge worldwide. Since therapeutic options are limited for multi-drug resistant Ab diseases, the development or discovery of new therapies is imperative.

The use of nanoparticles as delivery vehicles for bactericidal agents represents a new paradigm in the design of antibacterial therapeutics. NO, a diatomic free radical that modifies the natural immune system response to microbial challenge, represents an alternative approach in the design of antibacterial nanoparticles.20-25 Moreover, we recently showed the applicability...
replication and differentiation correlates with collagen synthesis in healing tissues. We recently suggested that topically applied NO-np might also prevent collagen degradation by bacterial collagenases. For instance, Momboisse et al. purified a collagenase from Acinetobacter spp. that is highly activated during exponential growth at 37°C. Bacteria interfere with host healing responses by degrading tissues and impacting normal host cell processes by restricting resources, such as oxygen, iron and glucose.

The success of wound healing by NO-np stimulation likely depends on cytokines and growth factors involved in a complex integration of signals that coordinate cellular processes. For instance, the presence of high levels of pro-inflammatory IL-12 might be related to NO’s anti-angiogenic activity, resulting in the suppression of angiogenesis.

**Figure 3.** NO-np reduces bacterial burden in superficial skin lesions. (A) Wound bacterial burden (CFU; colony forming units) in NO-np-treated mice infected intradermally with 5 x 10^7 Ab is significantly lower than untreated or np-treated mice (n = 10 per group). Bars are the averages of the results, and error bars denote standard deviations. p value significance was calculated by analysis of variance and adjusted by use of the Bonferroni correction. *p < 0.001 in comparing the NO-np-treated group with untreated and np-treated groups. (B and C) Histological analysis of Balb/C mice untreated Ab-infected, np-treated Ab-infected, and Ab-infected NO-np-treated, day 3. Mice were infected with 5 x 10^7 Ab bacterial cells. (B) Low and (C) high magnification images representing H&E-stained sections of the skin lesions. Insets represent Gram staining specific for Ab cells (shown in red-pink). Pictures were taken using the stratum corneum of the skin as a reference. Scale bars: 20 μm.
this study because it has been sequenced and it is resistant to multiple antibiotics.38 The strain was collected from the bloodstream of a soldier in 2004 at Walter Reed Army Medical Center, Washington DC (Hujer KM, et al. 2006). The isolate was stored at -80°C in blood heart infusion broth with 40% glycerol. Frozen stocks were grown in Tryptic Soy broth (TSB) with rotary shaking at 150 rpm overnight at 37°C. Optical density (OD) measurements were taken at 600 nm (Bio-Tek, Winooski, VT) to monitor growth.

NO-np synthesis.

We recently described the methods for the synthesis of NO-np.10,13 Briefly, a composite hydrogel/glass particle was generated from tetramethylorthosilicate (TMOS), polyethylene glycol (PEG), chitosan, glucose, and sodium nitrite in 0.5 M sodium phosphate buffer (pH 7). NO was produced by reduction of the nitrite within the matrix. The nanoparticles were lyophilized for storage. The release of NO occurs upon exposure to an aqueous environment, which induces the opening of channels in the nanoparticles. Control nanoparticles (np) were identical to the NO-nps, except sodium nitrate was not added to the formulation. Transmission electron microscopy has demonstrated that the diameter of individual NO-nps is ~10 nm.10,13

Susceptibility of Acinetobacter baumannii (Ab) 0057 to NO-np.

To determine the impact of the NO-np on Ab, TSB was inoculated with a fresh colony grown on BHI plates and suspended in 1 mL of medium. A suspension of 100 μL of Ab was transferred to a 200-well plate with 100 μL of TSB per well containing NO-np or np (1.25, 2.5, 5, 10 or 20 mg/mL). Bacteria and nanoparticles were incubated for 24 h at 37°C. Controls included wells containing bacteria with TSB alone. Growth was assessed at OD 600 nm every 30 min using a microplate reader (Bioscreen C, Growth Curves USA, Piscataway, NJ). Moreover, high concentrations (5–20 mg/mL) of the inhibition of neovascularization and a reduction in bacterial dissemination. Furthermore, the NO-np increases production of IFNγ, which in turn inhibits endothelial cell motility and vascularization. In fact, the downregulation of TGFβ, which can antagonize IFNγ effects, suggests that the NO-np might modulate the impairment of Ab to disseminate within immune cells.34 Furthermore, downregulation of the pro-inflammatory cytokine, IL-1β by the NO-np are consistent with the fact that this cytokine can be expressed in late-stage of wound healing and the wounds in our experiments were excised in the early to middle stages of this coordinated process.35 Incidentally, high levels of IL-1β promote Ab replication within phagocytic cells.36 In addition, down-regulation of IL-4 suggests that the NO-np induces a protective Th1-biased immune response that facilitates the clearance of Ab.

In summary, the NO-nps have significant therapeutic properties in the setting of Ab wound infections. The NO-nps represent a significant advance for the treatment of this drug resistant pathogen. We did not detect any adverse tissue sequelae with the application of the NO-nps, which is consistent with prior findings that topically applied NO can produce local immune activity with nominal inflammation.37 Furthermore, the simplicity of the treatment and the stability of the product suggests that NO-nps can be applied to combat or disaster situations. The microbicidal activity of the NO-nps to resistant pathogens currently impacting the US and international troops in the Middle East increases their potential utility.

**Materials and Methods**

*Acinetobacter baumannii (Ab) 0057.* Ab 0057, a clinical isolate acquired from Mark D. Adams (Cleveland, OH), was chosen for this study because it has been sequenced and it is resistant to multiple antibiotics.38 The strain was collected from the bloodstream of a soldier in 2004 at Walter Reed Army Medical Center, Washington DC (Hujer KM, et al. 2006). The isolate was stored at -80°C in blood heart infusion broth with 40% glycerol. Frozen stocks were grown in Tryptic Soy broth (TSB) with rotary shaking at 150 rpm overnight at 37°C. Optical density (OD) measurements were taken at 600 nm (Bio-Tek, Winooski, VT) to monitor growth.

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of NO used in this study to evaluate bacterial susceptibility were not toxic to human fibroblasts or reconstituted human epithelial tissues (SkinEthic, Nice, France) (data not shown).

In vivo wound infection model and NO-np treatment. To investigate the antimicrobial efficacy of NO-np against Ab 0057 wound infections, female Balb/c mice (6–8 weeks; National Cancer Institute, Frederick, MD) were shaved along the back and iodine applied. Five-mm diameter full-thickness excision wounds were created using a punch biopsy (Tru-Punch, Sklar Instruments, West Chester, PA). Immediately after wounding, a 5 x 10^7 Ab 0057 in PBS was inoculated onto the wound. Twenty-four hours after infection, 5 mg of NO-np or np powder was applied. Applications were similarly performed at 48 h after wounding. Untreated, wounded mice were used as additional controls. To monitor healing, photographs were taken daily. Also, calipers were used to measure the wounds daily. Seventy-two hours after infection, mice were euthanized and biopsies were taken. Uninfected, wounded mice that were either untreated or treated with np or NO-np were used as additional controls.

Histological processing. Three days after infection, wound tissues were excised, fixed in 10% formalin and embedded in paraffin. Tissue sections were submitted to staining with either H&E, Gram or Gomori’s trichrome to assess morphology, bacteria, or collagen deposition, respectively. The slides were viewed under light microscopy.

CFU determinations. At day 3 after Ab infection, wound tissues were excised and homogenized in sterile PBS. Samples were plated on tryptic soy agar and bacterial colonies were determined. The results were normalized by tissue weights.

Cytokine determinations. Five mice per group were killed at day 3 of infection. Wound lesions were excised and homogenized in PBS with protease inhibitors (Complete Mini; Boehringer Ingelheim Pharmaceuticals Inc., Ridgefield, Connecticut, USA). Cell debris were removed from homogenates by centrifugation at 6,000 g for 10 minutes to remove cell debris. Samples were stored at -80°C until tested. Supernatants were tested for IFNγ, TNFα, IL-1β, TGFβ and IL-4 by ELISA (Becton Dickinson Biosciences Pharmingen, San Diego, CA; and eBiosciences, San Diego, CA). The limits of detection were 31.3 pg/mL for IFNγ, 15.6 pg/mL for TNFα and IL-1β, 62.5 pg/mL for IL-12p70r, 60 pg/mL for TGFβ, and 7.8 pg/mL for IL-4.

Light microscopy. An Olympus AX70 (Melville, NY) microscope was used to obtain histological analyses and images were digitally obtained (QImaging Retiga 1300 digital camera; Burnaby, BC, CA) and processed (QCapture Suite V2.46 software; QImaging).

Statistical analysis. GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) was used for statistical analyses. p values were determined by analysis of variance and adjusted by use of the Bonferroni correction. p values of <0.05 were considered significant.

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Table 1. Cytokine levels in wounds of mice

| Cytokine levels (pg/mL) (Average ± SD) | IL-12 | IFNγ | TNFα | IL-4 | TGFβ | IL-12p70r |
|--------------------------------------|-------|------|------|------|------|-----------|
| Untreated                            | 9.26 ± 1.32 | 640.73 ± 2.06 | 487.67 ± 1.9 | 167.15 ± 0.85 | 3067.67 ± 15.03 | 176.07 ± 0.45 |
| np                                   | 1366.3 ± 7.7 | 700.33 ± 3.46 | 616.38 ± 2.8 | 219.38 ± 1.01 | 5527.67 ± 16.81 | 219.46 ± 0.51 |
| NO-np                                | 1957.41 ± 12.7 | 723 ± 7.17 | 503.67 ± 2.42 | 86.01 ± 0.68 | 1543 ± 9.88 | 79.85 ± 0.33 |

n = 5 mice per group. *Value significantly greater than the value for control mice (p < 0.05). †Value significantly less than the value for control mice (p < 0.05).

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