Nitric oxide nanoparticles
Pre-clinical utility as a therapeutic for intramuscular abscesses

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Nitric oxide (NO) is a critical component of host defense against invading pathogens; however, its therapeutic utility is limited due to a lack of practical delivery systems. Recently, a NO-releasing nanoparticulate platform (NO-np) was shown to have in vitro broad-spectrum antimicrobial activity and in vivo pre-clinical efficacy in a dermal abscess model. To extend these findings, both topical (TP) and intralesional (IL) NO-np administration was evaluated in a MRSA intramuscular murine abscess model and compared with vancomycin. All treatment arms accelerated abscess clearance clinically, histologically, and by microbiological assays on both days 4 and 7 following infection. However, abscesses treated with NO-np via either route demonstrated a more substantial, statistically significant decrease in bacterial survival based on colony forming unit assays and histologically revealed less inflammatory cell infiltration and preserved muscular architecture. These data suggest that the NO-np may be an effective addition to our armament for deep soft tissue infections.

Introduction

Pyomyositis is a suppurative infection of the large skeletal muscles without an apparent origin from contiguous structures. The most commonly involved muscles are those in the thigh and gluteal region, though infection of multiple other muscles have been reported.1 In recent years, the incidence of muscle abscesses has increased.2 Staphylococcus aureus is responsible for greater than 70% of these infections.2 Cases of methicillin-resistant S. aureus (MRSA) pyomyositis have been increasingly reported to date, and inappropriate use of broad-spectrum antibiotics has further spurred the emergence of many antibiotic-resistant strains.3,4 Patients with MRSA intramuscular abscesses are often hospitalized for intravenous antibiotics and may require interventional radiological or surgical intervention to drain abscesses and fluid collections, adding to the overall cost of care and ultimately associated suffering with this condition. With both the rising incidence of resistant isolates and cost of care, new therapies are needed in order to unburden the strain and difficulty of preventing the progression of and treating these deep tissue infections.

Nanoparticles can be used to transport biologically active agents that in most clinical settings are quite difficult or not feasible to deliver, such as nitric oxide (NO). NO is known to possess impressively broad antimicrobial activity due to both its inherent ability to inhibit growth and kill pathogens as well as its function as a potent immunostimulatory signaling molecule.5,6 Our data and work by other investigators show that NO is a potentially effective therapeutic for severe skin and soft-tissue infections.7-13 However, as a highly reactive gas, NO has proven difficult to deliver in a convenient format and this has largely precluded its use, even in hospital settings.14 We have recently described a new nanoplatform comprised of nitric oxide releasing nanoparticles (NO-np).15 NO is generated within the particles through the thermally induced reduction of nitrite to NO. The unique makeup of the nanoparticles allows for long range electron and proton transfer, facilitating the redox chemistry. This technology is a novel NO generator without many of the shortcomings of organic nitrates such as nitroglycerin, the most commonly used NO-donor in clinical practice. The main limitation of organic nitrates is decreased efficacy with prolonged continuous use; a so-called “nitrate tolerance,” resulting from depletion of tissue thiols, an
acquired desensitization of soluble guanylyl cyclase to NO, or increase in breakdown of cyclic guanosine monophosphate (GMP) by phosphodiesterases. 14 As NO-np do not require an external reducing agent, it bypasses many of these limitations while retaining and perhaps improving efficacy.

The antimicrobial activity of the NO-np has been previously demonstrated in vivo, 15-17 and has also been evaluated in several infection pre-clinical models including MRSA 13 and A. baumannii 19 infection pre-clinical models including MRSA 12 and standard np has been previously reported. 15,19 Briefly a hydrogel/glass murine MRSA intramuscular abscess model to the current antimicrobial impact, we investigated the comparative efficacy of and investigate the reach with which the NO-np can exert their antimicrobial impact, we investigated the comparative efficacy of both topically (TP) applied and intraleusal (IL) NO-np in a murine MRSA intramuscular abscess model to the current “gold standard” for invasive MRSA disease, systemic vancomycin.

Methods and Materials

Ethics statement. All animal studies were conducted according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at the Albert Einstein College of Medicine.

NO nanoparticle (NO-np) synthesis. The generation of NO-np has been previously reported. 15,19 Briefly a hydrogel/glass composite was synthesized using a mixture of tetrarmethylthiosilicate (TMOS), polyethylene glycol (PEG), glucose, diastase, and sodium nitrite in a 0.5 M sodium phosphate buffer (pH 7). The nitrite was reduced to NO within the matrix because of the glass properties of the composite effecting redox reactions initiated with thermally generated electrons from glucose. Subsequently, the ingredients were combined and dited using a lyophilizer, resulting in a fine powder comprising nanoparticles containing NO. Once exposed to an aqueous environment, the hydrogel properties of the composite allow for an opening of the water channels inside the particles, facilitating the release of the trapped NO over extended time periods. Control nanoparticles (np) were generated in a similar manner without the inclusion of nitrite.

In vivo abscess model and NO-np treatment. To investigate the antimicrobial efficacy of NO-np for intramuscular abscesses formed by MRSA, female Balb/c mice (6 to 8 weeks old, National Cancer Institute, MD) were anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, the hair on their flanks removed, and the skin disinfected with iodine. Then, a suspension of 100 μL with 105 colony forming units (CFU) MRSA 6524 in PBS was inoculated intramuscularly in each flank of the animals (two abscesses per mouse). At times points 24, 48 and 72 h following infection, 5 mg np or NO-np were applied (TP) to the skin overlying the abscess or a suspension of 100 μL with 5 mg/mL of NO-np or np dissolved in PBS was injected into (IL) the abscesses. The topical application was aided by the use of a cone with a 5 mm opening, applied over the targeted tissue. The nanoparticles were applied into the distal cone followed by 50 μL PBS, which resulted in the formation of a gel that was absorbed within 30 min. For the vancomycin group, mice received 5 mg/kg vancomycin subcutaneously (mid back) at 2 h post-infection and then subsequently at 24, 48 and 72 h. Vancomycin dosing was based on previous studies utilizing animal models of soft tissue MRSA infections. 20,21 The np (without NO) were used as a control at the same TP and IL doses according to the NO-np schedule. Untreated, infected mice were used as an additional control. On days 4 and 7, mice were euthanized and the abscesses were excised, homogenized and cultured quantitatively by plating on tryptic soy agar. The experiment was performed twice, each experiment consisting of six animals per experimental arm. The percentage of CFU survival was determined by comparing the survival of the treatment arms relative to the survival of untreated bacteria.

Histological processing. At day 4 and 7 following MRSA intramuscular abscess formation after infection and treatment as above, abscess tissues from one hind limb were excised from euthanized mice, six per treatment group, fixed in 10% formalin for 24 h, processed, and embedded in paraffin. Four micron vertical sections were fixed to glass slides and subjected to H&E staining to observe the muscular morphology and pathology. Slides were examined by light microscopy with an Olympus AX70 microscope, and images were obtained (QImaging Retiga 1300 digital camera) with QCapture Suite V2.66 software (QImaging).

Statistical analysis. All data were subjected to statistical analysis using GraphPad Prism 5.0 (GraphPad Software). P values were calculated by analysis of variance and were adjusted by use of the Bonferroni correction. P values of 0.05 were considered significant.

Results

Intramuscular and topical administrations of NO-np decreased MRSA burden in intramuscular abscesses. The effect of NO-np on intramuscular MRSA abscesses in Balb/c mice was investigated. TP and direct intramuscular injections into abscesses of NO-np decreased the size and purulence of abscesses clinically on day 4 following initial infection. Animals treated with subcutaneous vancomycin also demonstrated some clinical improvement as compared with the control group (Fig. 1), whereas lesions in mice treated with either IL or TP np (without NO) were similar to controls (data not shown). Intramuscular abscesses on day 7 in all groups were not clinically or grossly visibly evident. Histological examination of excised tissue from the various groups of mice differed significantly. In the control animals, which included untreated mice and topically np or intraleusal np treated mice, lesions at day 4 were large and well circumscribed with intense, neutrophil rich inflammatory infiltrates and scattered macrophages resulting in central caseous necrosis destroying the normal architecture (Fig. 2A; np controls not shown). Sections from vancomycin treated animals revealed a similar pattern of inflammation and tissue destruction, though not as intense as the control groups. In contrast, abscesses from mice topical treated with NO-np demonstrated an interstitial inflammatory cell infiltrate in proximity to a poorly defined and comparatively smaller neutrophilic collection which did not
resemble the well circumscribed inflammation appreciated in the control groups as well as vancomycin treated groups. Even less inflammation was appreciated in the mice that received NO-np intralesionally in which neutrophils were splayed between muscle fiber bundles and there was no evidence of a defined abscess. Day 7 histologic examination demonstrated reduced inflammation in all groups, with near complete restoration of the tissue architecture at the sites of NO-np treated lesions (data not shown).

Tissue cultures from abscesses at both days 4 and 7 following infection demonstrated that vancomycin, TP NO-np and intramuscular NO-np significantly decreased bacterial burden as compared with controls (Fig. 2B). At days 4 and 7 following infection, control intramuscular abscesses contained 4.13 ± 0.53 log_{10} CFU/mg and 2.03 ± 0.43 log_{10} CFU/mg, respectively. There were no significant differences between untreated controls and the TP and IL np (without NO) groups (data not shown). On day 4 (Fig. 2Ba), the CFUs in the vancomycin treated group decreased to 1.36 ± 0.62 log_{10} CFU/mg and 0.80 ± 0.32 log_{10} CFU/mg at day 4 and 7, respectively (Fig. 2B, p < 0.001). The CFUs in the abscesses from mice treated topically with NO-np decreased to 0.34 ± 0.13 log_{10} CFU/mg and 0.23 ± 0.08 log_{10} CFU/mg at day 4 and 7, respectively. The topically delivered NO-np was significantly more effective at both intervals compared with vancomycin (p = 0.0001). The IL NO-np treatment was the most effective treatment approach against MRSA abscesses resulting in CFUs of 0.17 ± 0.03 log_{10} CFU/mg and 0.09 ± 0.02 at day 4 and 7, respectively. Compared with vancomycin and TP NO-np, IL NO-np administration was significantly more effective at both time points (day 4: p = 0.0001 and p = 0.0001, respectively; day 7: p = 0.001 and p = 0.045, respectively).

Discussion

The present study investigated the ability of NO-np to effectively clear intramuscular abscesses as compared with vancomycin. Based on previous findings from our in vivo studies, moistened 5 mg of NO-np was applied topically or 5 mg/ml of NO-np was delivered intramuscularly. The results demonstrate a significant decrease in CFU/mg of infected tissue in the NO-np treated animals as compared with control and vancomycin treated, which is consistent with past investigations using this NO-releasing technology. Similarly, the histology of involved muscle from the NO-np treated arms demonstrated limited inflammation and damage to muscle fibers.
Although a seemingly simple diatomic molecule, NO exhibits a wide array of functional activities. NO can interact directly with pathogenic microbes due to the ease with which it can transverse the lipid bilayer; reaching important metabolic enzymes and DNA to cripple essential biological processes. NO can be oxidized to reactive nitrogen species (RNS) such as peroxynitrite. RNS themselves exert antimicrobial effects via a variety of reactions, including the nitrosation of protein thiols and the nitrosylation of metal centers (Fe-S), ultimately modifying the functions of proteins that are essential to cellular processes. Peroxynitrite specifically can disrupt the microbial membrane through lipid peroxidation, accelerating degradation of cellular integrity. Given these antimicrobial properties, in concert with the ability of NO to accelerate wound healing, there has been tremendous interest in the development of NO donors and delivery systems. The use of nanoparticles as delivery vehicles for bactericidal agents represents a new paradigm in the design of both topical and systemic agents. Over the last few decades, the applications of nanotechnology in medicine have been extensively explored in many medical arenas. Nanotechnology concerns the understanding and control of matters in the 1–100 nm range, at which scale materials have unique physicochemical properties including ultra-small size, large surface to volume ratio, high reactivity and unique interactions with biological systems. By loading bioactive agents through physical encapsulation, adsorption, or chemical conjugation, the pharmacokinetics and therapeutic index of the drugs can be significantly improved in contrast to the free drug counterparts. Many advantages of nanoparticle-based drug delivery have been recognized, including releasing drugs at a sustained and controlled manner. The use of nano-vehicles also allows for the delivery of highly reactive and short-lived biomolecules such as nitric oxide.

In the present study, NO-np was found to be effective at accelerating the resolution of MRSA intramuscular abscesses when applied topically or intralesionally, and both approaches were more effective than systemic treatment with vancomycin. Both
the biofilm-like nature of and poor perfusion associated with bacterial abscesses often undermine the efficacy of conventional antibiotic therapy such as vancomycin. However, as a small, lipophilic gas, NO can transcend physical barriers such as biofilms, as well as even prevent their formation.10

Vancomycin is a branched tetacyclic glycosylated nonribosomal peptide that inhibits proper cell wall synthesis in Gram-positive bacteria such as S. aureus by two mechanisms—it prevents the synthesis of the lip polymers of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) that form the backbone strands of the bacterial cell wall, and it prevents the backbone polymers from doing so through cross-linking with each other.26 Unlike most antibiotics, NO exerts its antimicrobial effects through several mechanisms. By targeting protein thiol and metal centers, NO can block essential microbial physiologic processes including respiration and DNA replication. NO can also directly damage microbial DNA through the generation of peroxynitrite. Moreover, NO in an immunomodulatory signaling molecule, enhancing and accelerating the host’s own immune response by recruiting macrophages and T lymphocytes.14,27 Interestingly, the histopathology of tissue from the NO treated arms demonstrated decreased inflammatory infiltrate as compared with controls and vancomycin groups. The decrease in neutrophilic infiltrate is likely a result of the rapid clearance of bacterial burden from tissue due to the sustained release of NO. However, it is known that NO can limit neutrophil migration by downregulating the expression of ICAM-1 which is required for neutrophil diapedesis from the vasculature.28 Not only does NO aid in clearing invading pathogens, their robust generation and showing superiority to vancomycin. The data presented suggests that this technology could be used as an adjunctive therapy prior to or in addition to surgical drainage of bacterial abscesses, or in the current standard approach to treatment of an abscess. The antibacterial effects witnessed utilizing the limited dosing schedule in this study suggests that clinically relevant and realistic dosing would also yield a similar outcome. Both NO’s direct bactericidal and immunomodulatory properties provide several advantages over antibiotics that frequently have one mechanism of action, and ultimately limit the risk of resistance developing against this agent. It is further possible that IL NO-np could be effective in the setting of other deep abscesses, such as lesions in the lung or liver. Together, these data suggest that the NO-np platform has the potential to serve as a novel, easily administered class of topical or injectable antimicrobials for the treatment of deep tissue infections and abscesses.

Declaration of Potential Conflicts of Interest

A.J.F, J.M.F, and J.D.N. report serving on the scientific advisory board of Makefield Therapeutics, Inc.

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