High-dose Nitric Oxide as an Antibacterial Agent Against *Mycobacterium abscessus*

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BACKGROUND

Nontuberculous mycobacterium (NTM) species *Mycobacterium abscessus* is an increasingly more common pathogen in cystic fibrosis patients and associated with decreased lung function, worsened quality of life, and increased mortality [1]. Antibiotic therapy for *M. abscessus* is frequently ineffective at eradicating the mycobacteria and poorly tolerated[1].

Burst of nitric oxide (NO) production, a small lipophilic free radical, by alveolar macrophages plays a key role in host defense against airway pathogens including NTM [2]. NO displays broad-spectrum antibacterial activity in preclinical models, including NTM such as *M. smegmatis* [3,4].

Reduced airway NO levels has been associated with poor clinical outcome in cystic fibrosis patients. This has prompted investigation of inhaled NO therapy to supplement endogenous NO production in pulmonary diseases with chronic airway infection (**Fig. 1**).

In this study, we investigate anti-mycobacterial activity of high-dose NO *in vitro* against various drug-resistant clinical isolates of *M. abscessus*.

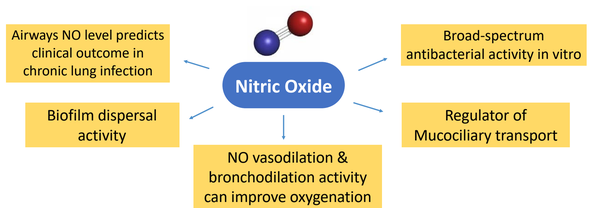

Fig. 1. Inhaled NO therapy potential benefit in lung disease.

METHODS

Bacterial Culture: *M. abscessus* strains were provided by the Microbiology Service, Department of Laboratory Medicine, National Institutes of Health. *M. abscessus* clinical strains B1 (smooth), B5 (smooth), B8 (rough) are multidrug-resistant serial isolates from sequential time points in a CF patient with worsening clinical status [5]. *M. abscessus* strain MRD (rough) is a multidrug-resistant isolate from a patient currently enrolled in a compassionate use trial at the National Institutes of Health.

NO Exposure: A custom designed continuous horizontal-flow NO Delivery System [6] was used to deliver NO at specific concentrations (**Fig. 2**). *M. abscessus* was then inoculated at 10^6 CFU/ml in 0.85% saline or artificial sputum [7] and treated with humidified medical air (control) or high-dose NO (>160ppm) for up to 10hr. Bacterial survival was assessed through quantitative Time-Kill assay by cultures on 7H11 agar and CFU analyses.

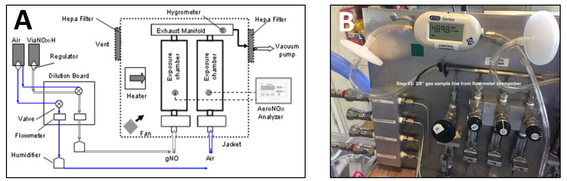

Fig. 2. Nitric Oxide Delivery System for Preclinical Studies. (A) Schematic design of NO chamber. (B) The NO, medical air, N_2, and CO_2 gas dilution manifold with regulators and digital flowmeters to allow accurate blending and delivery of up to 4 different gases. NO is blended from either a 5000ppm cylinder or AIT NO Generator (NOGen).

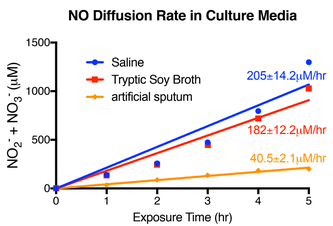

Fig. 3. NO diffusion rate in culture media. Various bacterial culture media were treated with 250ppm NO to assess diffusion rate in aqueous phase. Samples were taken every hour to measure measure NO_2/NO_3 (NO byproducts) by Griess reagent assay (Cayman Chemicals) and pH. Steady uptake of NO into the Artifical Sputum media was confirmed by linear and constant increase of NO_2/NO_3 levels.

RESULTS

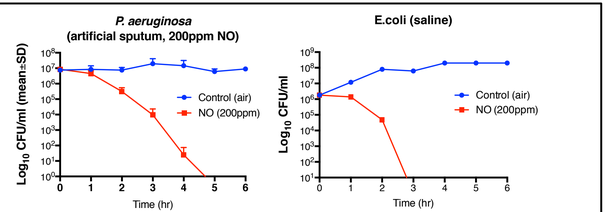

Fig. 4. Efficacy of NO *Delivery System* was confirmed against *Pseudomonas aeruginosa* and *E.coli*. Bacteria were cultured at 10^6 CFU/ml in artificial sputum (2ml, planktonic), and treated continuously with 200ppm NO for up to 10hr. Samples were plates on appropriate agar to obtain CFU count.

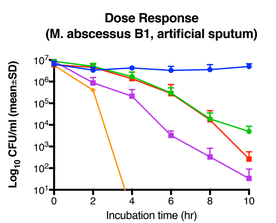

Fig. 5. Dose response anti-mycobacterial effect of high-dose NO against *M. abscessus*. Time-kill assays were performed in *M. abscessus B1* cultured in artificial sputum. Bacteria were treated with NO continuously up to 10hr and sampled every 2hr to obtain CFU counts. Controls were exposed to humidified medical air alone inside the NO chamber.

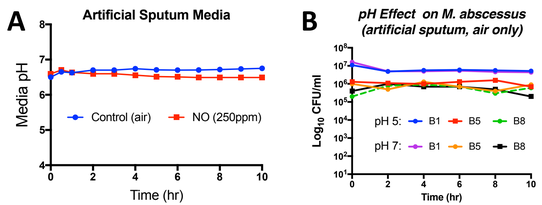

Fig. 6. Effect of culture media pH on *M. abscessus* viability. **A**) Exposure to exogenous NO has been shown to reduce the pH of culture media. Data showing effect of NO exposure on artificial sputum media pH levels in 10hr. **B**) Effect of reduced pH on *M. abscessus* viability was tested. Artificial sputum at pH 5.0 had minimal effect on viability and growth of *M. abscessus* B1, B5, and B8 clinical strains. This confirms the fact that anti-mycobacterial activity of NO is not caused by minute reductions in pH in artificial sputum media.

RESULTS

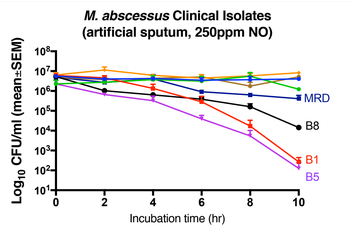

Fig. 7. Antibacterial activity of high-dose NO against multidrug-resistant *M. abscessus* clinical isolates. **A**) Time-kill curves show susceptibility of *M. abscessus* B1, B5 (smooth), B8 (rough), and MRD (rough) clinical isolates, cultured in artificial sputum, to continuous 250ppm NO treatment. All *M. abscessus* strains show susceptibility to NO treatment. Controls were exposed to humidified air alone inside the NO chamber. **B**) Non-linear regression fit of individual time-kill assays performed on B1, B5, and B8 strains in control (C) and 250ppm NO (NO) is plotted. Numbers in legend indicate biological replicates for each strain. Unpaired t-test of regression line slopes shows significant difference between control and NO-treated *M. abscessus* clinical isolates (p values shown in table).

CONCLUSION

- **Several multidrug-resistant clinical isolates of *M. abscessus* show significant susceptibility to 250ppm NO treatment (dose dependent).**
- ***M. abscessus* with rough morphology appear to be more resistant to NO treatment.**
- **In future, we will assess NO activity against intracellular *M. abscessus* (NTM-infected macrophages) and NO synergy in combination with anti-NTM drugs.**

1. Meng-Rui L, et al. Emerg Infect Dis (2015); **2**. Jamaati H, et al., Frontier for Microbiology (2017); **3**. Ghaffari A, et al. Nitric Oxide (2006); **4**. Miller CC, et al. Antimicrob Agents Chemother (2007); **5**. da Silva JL et al., Res Microbiol. (2018); **6**. Ghaffari A., et al. Nitric Oxide (2005); **7**. Sriramulu DD et al.. J. Med. Microbiol. (2005). **Acknowledgement**: We would like to thank AIT Therapeutics for providing the NO Exposure Chamber and expertise in conducting these studies.