

# The Antimicrobial Effect of Nitric Oxide on the Bacteria That Cause Nosocomial Pneumonia in Mechanically Ventilated Patients in the Intensive Care Unit

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**BACKGROUND:** Nosocomial pneumonia is the second most frequent nosocomial infection and the leading cause of death from hospital-acquired infection. Endogenously produced nitric oxide is an important component of the body's natural defense mechanism. Recent studies have demonstrated that exogenous gaseous nitric oxide (gNO) is bactericidal and that inhaled gNO is beneficial to bacterial clearance. **OBJECTIVE:** Determine the antimicrobial effect of exogenous gNO in vitro against organisms from culture collections and pathogens derived from tracheal aspirates of mechanically ventilated patients with pneumonia in an intensive care unit. **METHODS:** Using bacterial isolates in pure culture, a 0.5 McFarland standard ( $10^8$  colony-forming-units [cfu] per mL) was prepared and further diluted to 1:1,000 with saline, to  $10^5$  cfu/mL. For each isolate tested, 3 mL was pipetted into each well of a 6-well plate, and placed in a specially designed incubator with compartments for both a treatment arm and a control arm. Both chambers received a continuous flow of heated, humidified gas. The treatment chamber had 200 ppm of gNO in the gas flow, which is higher than the clinically accepted concentration for gNO. Samples were drawn off at time intervals, plated onto Columbia agar base with 5% sheep blood, and placed in a traditional incubator at 35°C for a minimum of 24 h. All tests were performed in duplicate. The colony-forming units were visually counted to determine percentage kill. **RESULTS:** There was total kill (100% of all colony-forming units) of each bacterial strain subjected to the test conditions at between 2 and 6 h of exposure to 200 ppm gNO. **CONCLUSION:** gNO is bactericidal against various strains of bacteria suspended in saline, including both Gram-positive and Gram-negative organisms, and those that commonly cause nosocomial pneumonia in mechanically ventilated patients. Future work should focus on developing strategies that maximize the antimicrobial effect while minimizing the effect of these same interventions on host cells. *Key words: nitric oxide, bacteria, pneumonia, nosocomial, antimicrobial, bactericidal.* [Respir Care 2005;50(11):1451–1456. © 2005 Daedalus Enterprises]

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## Introduction

Nosocomial pneumonia is the leading hospital-acquired infection in the intensive care unit.<sup>1,2</sup> It is considered to be the main cause of infection-related death and is associated with significantly higher hospitalization costs.<sup>3-5</sup> Current management of nosocomial pneumonia usually includes obtaining a respiratory sputum sample for diagnostic workup, followed by initial empirical symptomatic treatment. *Staphylococcus aureus* is the most prevalent pathogen recovered from the airways of patients in the first 4 days of mechanical ventilation, and *Pseudomonas aeruginosa* is the most prevalent pathogen recovered after 4 days of mechanical ventilation.<sup>4,6,7</sup> These 2 organisms and other causative bacteria may be of antibiotic-resistant strains that can further complicate the treatment of nosocomial pneumonia.<sup>8</sup> At a time of increased antibiotic resistance in bacteria associated with nosocomial pneumonia, there is a need for new antimicrobial strategies that are microorganism-nonspecific.

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Nitric oxide (NO) plays a critical role in various bodily functions, including the vasodilation of smooth muscle, neurotransmission, regulation of wound healing, and non-specific immune responses to infection.<sup>9</sup> Currently, exogenous gaseous NO (gNO) is used clinically as an inhaled selective pulmonary vasodilator to treat persistent pulmonary hypertension of the newborn and to improve oxygenation due to ventilation-perfusion mismatching in acute respiratory distress syndrome.<sup>10</sup> We use the term gNO to maintain a clear distinction between exogenous gaseous NO delivered as a treatment and NO produced by the body in its various forms and distinct from NO-donor compounds. The potential uses for gNO may not be limited to only inhalation, and therefore the widely-used term "inhaled nitric oxide" (INO) might be confused with topical applications.

Experimental evidence is accumulating that NO, in various forms, has antimicrobial activity against a growing list of organisms. In vitro studies have shown that oxides of nitrogen inhibit the growth of or kill a number of fungi, parasites, helminths, protozoa, yeasts, mycobacteria, and bacteria.<sup>9,11-14</sup> NO may even play a role in killing tumor cells and in halting viral replication.<sup>15</sup> However, there is little consistency in NO delivery methodology, and reported results for any one species can range from minimal susceptibility to total bacterial toxicity.

More than 50 years ago, oxides of nitrogen were shown to prevent spoilage of meats.<sup>16</sup> The bacteriostatic effect was thought to be due to NO being released from sodium nitrite in an acidic environment.<sup>17</sup> There is strong evidence

throughout the literature that NO has a static effect on bacterial growth, and recent in vitro studies utilizing NO-donor compounds demonstrated that NO may even be bactericidal.<sup>9,11-15</sup> Hoehn et al<sup>18</sup> specifically used gNO in the lower range of clinically acceptable concentration to study gNO's effects on bacterial growth in vitro, with marginal success. In animal models of pneumonia, other investigators demonstrated that bacterial loads were decreased by inhaled gNO at 40 ppm and 10 ppm.<sup>19,20</sup> To our knowledge, there are no reports showing complete bactericidal effect on clinical strains of bacteria using gNO.

The purpose of this study was to investigate whether 200 ppm of gNO would kill various clinical strains of bacteria associated with ventilator-associated pneumonia, in an in vitro environment, and, if so, the time required for the gNO to be effective. The activity of gNO on other selected strains, including *S. aureus*, methicillin-resistant *S. aureus*, *Escherichia coli*, and Group B *Streptococcus* from culture collections, was also assessed.

## Methods

To validate the in vitro incubator/exposure chamber and sampling methods of the study, we performed an initial pilot trial using American Type Culture Collection (ATCC) strains of *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923). The methods and results of that validation were previously reported.<sup>21</sup> Clinical isolates were obtained from endotracheal aspirates, as described below.

The study was performed at Vancouver General Hospital, Vancouver, British Columbia, Canada, after institutional review board approval. Patients in a 24-bed multidisciplinary intensive care unit (ICU) were identified by the attending physician as having acquired nosocomial pneumonia, by radiographic evidence or by clinical presentation (ie, increasing oxygen requirement, elevated body temperature, increased heart rate, and/or decreased body temperature) and sputum culture. Informed consent was obtained from the patient or the patient's surrogate, and a respiratory sputum sample was collected via endotracheal-tube aspiration during routine endotracheal-tube suctioning. The sample was labeled and sent to the hospital microbiology laboratory, where it was cultured and potential pathogens were identified as per standard microbiology procedures, by a senior technologist. Patients were excluded who were under 18 years of age, pregnant, and/or did not consent to participate.

Strains of potential pathogens isolated from fresh overnight cultures were used to make a 0.5 McFarland standard with 10<sup>8</sup> colony forming units (cfu) per mL, which was further diluted to 1:1,000 with sterile saline, to 10<sup>5</sup> cfu/mL and a volume of 20 mL. Viable plate counts were performed to document the exact inoculum. Three-mL aliquots were pipetted into each well of a 6-well cell-culture

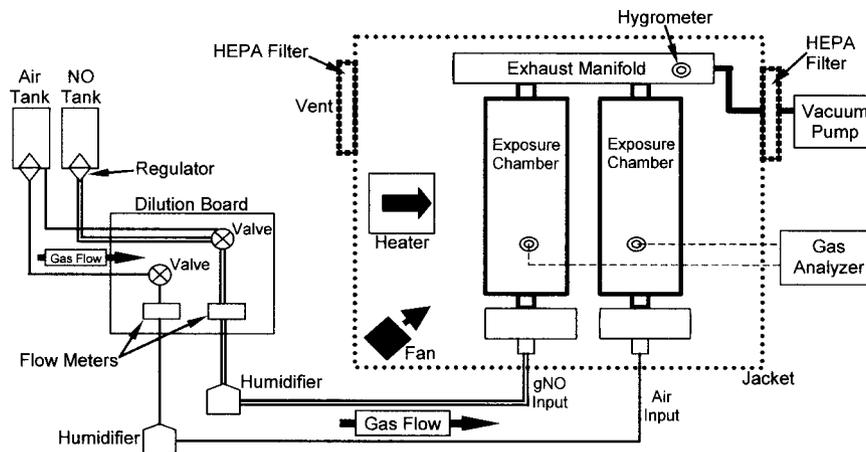


Fig. 1. Schematic of the test setup. NO = nitric oxide. gNO = gaseous nitric oxide. HEPA = high-efficiency particulate arrestor.

flat-bottom plate with lid (3516, Corning, Corning, New York). The culture plates were prepared and, with the lids on, transferred into either the control or the treatment exposure chamber.

All gases were supplied from high-pressure cylinders. These included 800-ppm medical-grade NO (ViaNOx-H, SensorMedics Corporation, Yorba Linda, California), medical air, oxygen, and carbon dioxide (Praxair, Mississauga, Ontario, Canada). Using an appropriate Compressed Gas Association approved gas regulator, the gases were delivered to the incubator/exposure system at a constant pressure (50 psig). Gases were then mixed together at pre-calculated concentrations in a dilution manifold (Fig. 1) that included digital mass flow meters (TSI Inc, Shoreview, Minnesota). The gas manifold allowed for up to 5 different gas mixtures and various flow rates.

Two identical channels in the manifold provided for 2 different sources of gas mixtures for simultaneously exposing samples in the control chamber and the test chamber. The gases mixed in the manifold were delivered through 2 separate pieces of 22-mm inner-diameter corrugated respiratory tubing, to 2 humidifiers (MR850, Fisher & Paykel Healthcare, Auckland, New Zealand) set for >60% relative humidity, using sterile water (Baxter Corporation, Clin-tec Nutrition Division, Deerfield, Illinois) at 40.0°C. Both the heated-and-humidified gas mixtures (treatment and control mixture independently) were delivered to each exposure chamber at a constant flow rate of 2.0 L/min during all experiments. The oxygen concentration was maintained at  $20 \pm 1\%$  in both exposure chambers. The gas mixtures were analyzed with a gas analyzer (AeroNOx, Pulmonox Medical, Tofield, Alberta, Canada).

Each exposure chamber could contain 3 culture plates. A low-pressure vacuum pump was placed at the exhaust port to create negative pressure throughout the system, and to prevent possible back-flow and ensure 1-way flow through the system. The exhaust gas mixture was filtered

through a double-layered high-efficiency-particulate-arrestor (HEPA) filter, to prevent contamination of exhaust air. The exhaust gas mixtures, composed mainly of NO, NO<sub>2</sub>, O<sub>2</sub>, and CO<sub>2</sub>, were safely vented to a class I biosafety cabinet through a vinyl duct.

When the temperature and gNO concentration (set ppm) were in steady-state conditions, the control plates were placed in the control chamber and the treatment plates were placed in the treatment exposure chamber. The gNO concentration for the experimental arm was 200 ppm. To measure the time-based effects of gNO, each plate was sampled at 1, 2, 3, 4, 5, 6, 12, and 24 h. Samples were taken in 3 volumes (0.1 mL, 0.01 mL, and 0.001 mL) and were plated onto blood/agar plates (Columbia agar base with 5% sheep blood, PML Microbiologicals, Willsonville, Oregon). The blood/agar plates were incubated in a conventional incubator at 35°C for a minimum of 24 h, after which the plates were removed and the colony-forming units were manually counted and reported independently by a senior technologist blinded to the experimental conditions.

Organisms isolated from the ICU patients were *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter aerogenes*, *Stenotrophomonas maltophilia*, and *Acinetobacter baumannii*. Additionally, *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), methicillin-resistant *S. aureus*, *S. aureus*, *E. coli*, and Group B streptococci source colonies were tested from laboratory culture collections.

### Statistics

The results were analyzed using the unpaired Student's *t* test for comparison between any 2 groups, and by non-parametric equivalents of analysis of variance for multiple comparisons. Differences were considered statistically significant when  $p < 0.05$ . Unless otherwise indicated, results are presented as mean  $\pm$  SD for at least 3 indepen-

dent experiments. A commercially available statistics/graphics package was used to analyze and graph the data (Prism, version 3.0, GraphPad Software, San Diego, California).

## Results

As previously reported, in the pilot trial we exposed the ATCC strains of *S. aureus* and *P. aeruginosa* to gNO at 200 ppm, and there were no viable *P. aeruginosa* colony-forming units at 2 h and no viable *S. aureus* colony-forming units at 4 h.<sup>21</sup>

Ten ventilated patients were recruited from the ICU of Vancouver Hospital between July 2003 and January 2004. The average age of the study participants was  $47.9 \pm 18.9$  years. There were 8 male and 2 female patients. Six patients had trauma as their primary admitting diagnosis, and 4 were admitted to the ICU after surgical procedures. Radiographic changes were observed as new or worsening infiltrates in each of the study subjects: 7 had infiltrates in the left lower lobe, 1 had infiltrates in the right lower lobe, and 2 had infiltrates in both lower lobes.

All samples collected from patients were purulent, and polymorphs were demonstrated on Gram-stain (1+ to 4+). The results of tracheal aspirates from 3 patients were reported as normal flora, and one showed no growth of organisms. Seven clinical isolates were recovered from the cultures of the remaining 6 patients, including *K. pneumoniae* (2 patients), *S. marcescens* (2 patients), *E. aerogenes*, *S. maltophilia*, and *A. baumannii*. The culture from one of these patients grew both *K. pneumoniae* and *S. marcescens*. The average exposure time to when there were no viable *S. aureus* (ATCC 25923), *P. aeruginosa* (ATCC 27853), fresh clinical strains of *S. marcescens*, *K. pneumoniae*, *S. maltophilia*, *E. aerogenes*, *A. baumannii*, or organisms from culture collections (including methicillin-resistant *S. aureus*, *S. aureus*, *E. coli*, and Group B streptococci) was following  $4.8 \pm 1.3$  h of exposure to gNO at  $200 \pm 5$  ppm. During the same time period, samples from the control arm maintained colony density at  $110 \pm 30\%$  cfu/mL, compared to the original inoculum (Fig. 2). The reduction in cfu/mL for each organism exposed to gNO was greater than  $10^4$  cfu/mL.

## Discussion

The results of this study demonstrate that gNO at 200 ppm is completely bactericidal ( $> 3$ -log reduction in bacterial cfu/mL) to strains of bacteria that commonly cause nosocomial pneumonia in mechanically ventilated patients in the ICU setting, and the bactericide was accomplished with a short exposure time: less than 5 h.

During the past 2 decades there have been tens of thousands of research papers on NO, but very few of these

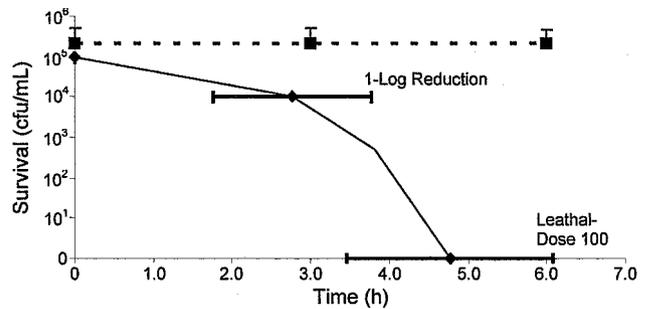


Fig. 2. Composite survival curve of the average of all microorganisms exposed to either room air (dotted line) or nitric oxide (solid line). The data points and error bars indicate the average time points to 1-log reduction in colony-forming units and the time to total kill (lethal-dose 100). cfu = colony-forming unit.

examined the potential use of gNO as a bacteriostatic or bactericidal agent. De Groote and Fang<sup>11</sup> summarized the antimicrobial activity of NO against parasites, fungi, viruses, and bacteria. Early studies used NO-donors, which needed to be metabolized to produce NO to target bacteria, and attempted to deliver NO in ranges that may have approached the concentration naturally produced by macrophages. Tamir et al<sup>22</sup> used this approach and delivered NO through a semi-permeable membrane and found activity against *Salmonella typhimurium*. O'Reilly et al found that human macrophages produce NO and use it to kill *K. pneumoniae*.<sup>23</sup>

Two prior in vitro models showed promise of gNO as a bactericide. Hoehn et al<sup>18</sup> plated bacteria on tryptic soy/agar plates and exposed them to 40, 80, and 120 ppm for 24 h, with mixed results. An effect was noted on *S. epidermidis* and Group B streptococci, but not on *S. aureus*, *E. coli*, or *P. aeruginosa*. Long et al<sup>24</sup> found a positive effect on *Mycobacterium tuberculosis* on agar plates exposed to 90 ppm gNO. Webert et al<sup>19</sup> examined the effect of gNO at 40 ppm on pulmonary bacterial load, leukocyte infiltration, and NO-synthase activity in a rat model of *P. aeruginosa* pneumonia. That study found that gNO had antimicrobial activity against *P. aeruginosa*, both in vitro and in vivo, and was also associated with reduced pulmonary leukocyte infiltration. Jean et al<sup>20</sup> also examined a rat model of *P. aeruginosa* pneumonia, but with an exposure of 10 ppm gNO for 24 h, and examined neutrophil function in in vivo and ex vivo experiments. They reported that gNO had a beneficial effect on bacterial clearance and that neutrophil function was not inhibited. Recent preliminary work by Ghaffari et al<sup>21</sup> and Miller et al<sup>25</sup> showed that high concentrations of gNO may not have a deleterious effect on host cells and tissues and may reduce bacterial load in nonhealing wounds.

For our experiment we chose to suspend the organisms in 0.9% saline and expose the suspension to 200 ppm gNO. We chose saline rather than nutrient broth media,

because saline maintains the bacteria in a stasis in which they neither multiply nor die. Recent evidence also suggests that substances found in bacterial laboratory support media may bind NO, and that interference may have masked the true effects of NO in previous studies.<sup>26</sup> Evidence of this static situation was the relatively stable cfu/mL in the control chamber, which at the end of the experiment was  $5.1 \log \pm 0.3 \log$  of the starting inoculum; when the bacteria were removed from the saline and transferred to a nutrient-rich media, they were viable, as evidenced by continued growth.

The previously cited studies used concentrations ranging from 10 ppm to 120 ppm gNO. We chose 200 ppm gNO based on previous dose-ranging studies (data not shown), which indicated that a dose  $>160$  ppm was highly effective as a bacteriostatic agent. At 5 h of continuous exposure to 200 ppm gNO, this represented an uptake of NO into the saline solution of  $192 \pm 56.6 \mu\text{M}$ . The 200 ppm dose used in this study is a supraphysiologic level and is above the currently accepted treatment range for humans. For the purpose of this study, this concentration was used to clearly evaluate if gNO is effective against bacteria associated with nosocomial pneumonia.

Differential analysis of the mechanism of kill to explain the results of these experiments required that we develop alternative hypotheses besides a direct effect of gNO. Two alternative hypotheses that could explain the results were (1) effects of NO<sub>2</sub> and (2) changes in pH. When gNO reacts with oxygen, a toxic by-product, NO<sub>2</sub>, is produced. In this study, which used low flow through the chambers, the NO<sub>2</sub> by-product measured in the gNO exposure chamber ranged up to 18 ppm. That level of NO<sub>2</sub> is well beyond the clinically acceptable level of 5 ppm. It is possible to reduce the NO<sub>2</sub> level by either scavenging or increasing the flow rate through the test apparatus. Several standard scavenging methods were tried but they also reduced NO concentrations, which led to an increase in NO consumption, which was the same result, as did increasing the flow rate. To test whether the NO<sub>2</sub> was the cause of the cytotoxic effect, the same protocol was used for *S. aureus* and *P. aeruginosa* using 20 ppm of NO<sub>2</sub> in the exposure chamber instead of gNO. No difference in survival between the microorganisms in the control chamber and those in the NO<sub>2</sub> exposure chamber was found. These results rejected the hypothesis that it was the NO<sub>2</sub> concentrations that were responsible for the cytotoxic effects.

To test the second alternative mechanism that could explain the results, a time study of pH versus bacterial survival was performed. It was observed that during a prolonged exposure to gNO, the pH of the saline did not fall substantially into the acidic range ( $\text{pH } 7.13 \pm 0.01$ ) before 5 h, but pH decreased to  $6.43 \pm 0.01$  following 10 h of exposure. In addition, the results demonstrated that saline exposed to 200 ppm gNO for up to 48 h did not

undergo a substantial change in pH (unpaired Student's *t* test,  $p > 0.05$ ), compared to saline exposed to medical air alone (control). Time measurements of the pH (ABL 500 blood gas analyzer, Radiometer Instruments, Copenhagen, Denmark) in the saline of microorganisms exposed to gNO were compared with their survival data. It was noted that the death of the bacteria occurred several hours prior to substantial changes in pH, which rejects the hypothesis that acidification contributed to the bactericidal effect of gNO observed.

Rejecting those 2 hypotheses points toward a direct effect of gNO being bactericidal. As seen in Figure 2, there is a prolonged time to reach a 1-log reduction in colony-forming units, followed by a sharp fall to no viable colonies (lethal dose for 100% of the colony-forming units). It is theorized that the bacteria have a defense mechanism to protect themselves but that that mechanism can be overwhelmed, leading to the demise of the bacteria. Further research is being done to identify the defense pathway(s) and bactericidal effect of NO.

We acknowledge that these preliminary data raise some interesting possibilities but may have some clinical limitations. A concentration of 200 ppm NO continuously for inhalation is much higher than the current clinically accepted dose. That high concentration increases the potential of deleterious effects from methemoglobin formation, which can arise from using gNO continuously at that dose. The rate of formation of methemoglobin in dogs exposed to 160 ppm is 2.07% per hour.<sup>27</sup> Young et al reported similar results in humans, and the half-life of methemoglobin in humans is approximately 1 h.<sup>28</sup> This would suggest that if 160 ppm were continuously inhaled for 4 h to help reduce the bacterial burden in nosocomial pneumonia, the methemoglobin level would reach 8%, and it would take approximately 5 h to reach baseline. We might further speculate that 30 min of continuous exposure to 160 ppm gNO would only increase methemoglobin to 1% and it would take only 3–4 h to reduce the methemoglobin to zero. This intermittent-dosing strategy could be repeated every 4 h, toward a reduction in the bacterial burden. However, additional *in vitro* studies are required to evaluate the antimicrobial effectiveness of an intermittent-dosing strategy. Once an effective strategy is identified, then additional studies would be to evaluate the effect of gNO on host cell lines.

This study evaluated only a limited number of bacterial strains, but they were chosen as representative strains of organisms actually associated with nosocomial pneumonia in mechanically ventilated patients. Further studies will be needed on additional bacteria strains to see if these conclusions can be more broadly applied. Further research also needs to be conducted on bacterial cells growing in a biofilm phenotype and not only planktonic cells growing in liquid media.<sup>29</sup> In addition to gNO's antimicrobial ef-

fects against bacteria associated with nosocomial pneumonia, future potential uses for gNO might include treatment of other pulmonary infections such as tuberculosis and respiratory-tract infections in cystic fibrosis, should the etiologic microorganisms be found susceptible to gNO.

### Conclusions

Continuous in vitro exposure of microorganisms to 200 ppm gNO was cytotoxic, within 5 h, to all the bacteria that cause nosocomial pneumonia in our ICU. Though the continuous use of 200 ppm gNO is above the clinically accepted level, this study was the first step in evaluating the potential of gNO as an antimicrobial treatment. Further research is required to evaluate strategies that would enable the safe use of gNO at levels required to effectively reduce the bacterial load and ultimately treat pulmonary infections.

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