

# Discovery and Development of Gaseous Nitric Oxide Under Increased Atmospheric Pressure as an Antimicrobial

## In Vitro and In Vivo Testing of Nitric Oxide Against Multidrug-Resistant Organisms

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### KEYWORDS

- Nitric oxide • Pressure • Antimicrobial • Infection • Wounds
- Antibiotic-resistant bacteria • Multidrug-resistant bacteria

### KEY POINTS

- Gaseous nitric oxide under increased atmospheric pressure has shown the ability to kill multidrug-resistant bacteria in an in vitro model.
- Increasing the concentration of the gaseous nitric oxide reduced the testing time needed to kill multidrug-resistant bacteria in the in vivo model.
- Using successful in vitro parameters, gaseous nitric oxide under increased atmospheric pressure showed multilog reduction of bacteria in a live mammalian (pig) model.
- Delivering gaseous nitric oxide while increasing the pressure at the wound site, and maintaining an appropriate flow of NO gas, shows promising antimicrobial capabilities that should be studied further.

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## INTRODUCTION

### *Wound Healing and Infection*

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It has been well established that in acute and chronic wounds microbial contamination and infection play significant roles in delaying or preventing wound healing. In fact, it is infection that is the leading cause of wound deterioration, leading to hospital stays, surgical treatment, and in many cases, lower-limb amputation.<sup>1</sup> Diabetic foot ulcerations (DFUs) and subsequent infection are a significant source of morbidity with the potential of limb loss and mortality. The lifetime risk of foot ulceration in patients with diabetes is 15% to 20%.<sup>2</sup> The significant problem in chronic wounds, specifically DFUs, is the lack of consistent healing. Less than 25% of DFUs heal within 12 weeks.<sup>3</sup> If these wounds remain open for extended periods of time, they are more susceptible to infection. Hence, more than half of all DFUs become infected, and 20% of those infected DFUs end in amputation.<sup>4</sup> Sadly, after years of decline, the rate of amputations increased by 50% between 2009 and 2015 to 4.6 for every 1000 adults.<sup>5</sup>

To put this in perspective, the United States sees more than 80,000 lower-extremity amputations to Medicare beneficiaries annually.<sup>6</sup> That number does not take into account the many such procedures that occur on the patient population under 65+ years of age in the United States. The total number is well over 100,000 amputations each year.<sup>5</sup> This problem is a worldwide problem as well, with the World Health Organization acknowledging the diabetic foot complications issue stating, “One lower limb is lost to diabetes every 30 seconds [worldwide].”<sup>7</sup> This situation continues to worsen in large part because specific diseases, such as diabetes, show a dramatic increase in prevalence with projections increasing.<sup>8,9</sup> Currently, diabetes affects more than 30 million individuals in the United States and more than 425 million people worldwide with those numbers projected to increase to more than 40 million and more than 629 million, respectively, by 2045.<sup>8</sup>

### *Economic Cost*

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In 2018, *Value in Health* published an article examining the economic impact of chronic nonhealing wounds. The investigators discovered that nearly 15% of US Medicare beneficiaries were diagnosed with a wound or wound infection based on 2014 data. Approximately \$98 billion was spent on wound care across the board. In terms of infection-specific spending, this totaled ~\$29 billion. Diabetic foot infection-related spending was nearly half of the total infection spending at ~\$14 billion.<sup>10</sup> Once again, it should be emphasized that these numbers do not account for the non-Medicare population, so the real scope of this epidemic is even greater.

In a surprising statistic, costs of diabetic foot care and its related limb complications are higher than even the most costly form of cancer in the United States, breast cancer.<sup>11</sup> Of all the insurance costs related to DFUs, nearly two-thirds are inpatient care related.<sup>12</sup> In the cases of patients undergoing amputation, the 2-year costs associated with initial hospitalization, rehospitalizations, postacute care, and prosthesis-related costs were more than \$90,000. These patients with amputations additionally face lifetime health care costs projected at more than \$500,000.<sup>13</sup> Importantly, this is just the United States alone. Costs related to DFUs are similar throughout the world and are expensive regardless of the health care system.<sup>14</sup> It is important to note that infection precedes amputation in at least 75% of the cases.<sup>4,15,16</sup>

## **Resistant Infection**

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Many of the bacteria species that infect wounds have developed resistance to antimicrobial agents. In recent years, bacterial resistance to systemic antibiotics has increased despite the formation of new and improved drugs to help reduce infection.<sup>2,9</sup> Bacterial infections showing antibiotic resistance more than doubled from 2002 to 2014 from 5.2% to 11.0% and result in cost of infection treatment increasing by 165% when facing antibiotic resistance.<sup>17</sup> More than 2 million people suffer antibiotic-resistant infections annually.<sup>18</sup>

The antibiotic-resistant bacteria tested with gaseous nitric oxide under increased atmospheric pressure (gNOp) were the following: *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and methicillin-resistant *S aureus* (MRSA). As of 2019, the Centers for Disease Control and Prevention (CDC) have classified *A baumannii* as threat level: urgent; *P aeruginosa* and MRSA classified as threat level: serious; and *S aureus* classified as threat level: concerning. It should be noted that *Staphylococcus* bacteria are a common cause of health care-associated infections. There are more than 400,000 MRSA infections per year leading to more than 10,000 deaths and costing an estimated \$1.7 billion annually. *P aeruginosa* is identified in more than 32,000 infections per year. The CDC states that “some types of multidrug-resistant *P. Aeruginosa* are resistant to nearly all antibiotics, including carbapenems.” *A baumannii* infections are fewer in number annually than the previously discussed bacteria; however, *A. baumannii* is “resistant to nearly all antibiotics and few new drugs are in development.”<sup>19</sup> The US Department of Defense identified these 4 bacteria as the pathogens to study to evaluate the antimicrobial potential of gaseous nitric oxide (NO) under pressure. Each of these bacteria can be found in diabetic foot ulcers.<sup>20</sup> Therefore, eradicating these pathogens that contribute to the epidemic of chronic, infected wounds is integral to achieving successful outcomes in wound healing in addition to developing new ways of attacking multidrug-resistant organisms. This set of studies evaluates NO as a potential solution to resistant infection in wounds. NO has antimicrobial properties along with numerous wound-healing properties, such as enhancing blood supply, increasing fibroblastic activity, and serving as a potent vasodilator.<sup>21–28</sup>

## **Nitric Oxide as an Antimicrobial**

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Gaseous NO is a universal antimicrobial.<sup>21–28</sup> The bactericidal properties of gaseous NO are even more exciting when coupled with avoiding resistance issues. It is unlikely that the infecting organisms develop bacterial resistance against exogenous NO owing to “the multiple mechanisms by which NO presents toxicity toward microbes.” Privett and colleagues<sup>23</sup> identify NO’s small molecule size and hydrophobicity as key to moving through “bacterial lipid membranes where a number of nitrosative and oxidative reactions may occur,” killing the pathogen. Dr Chris Miller and his team have studied the antimicrobial properties of NO since 2004 and have demonstrated that NO, delivered topically, is an effective nonspecific antimicrobial agent against a broad range of microorganisms, gram-positive, gram-negative, and multidrug-resistant strains of bacteria, yeast, viruses, and mycobacteria with no evidence of resistance development.<sup>21,22,28,29</sup> Gaseous NO is thus an exciting potential topical antimicrobial.

One main concern regarding topical antimicrobials is their ability to penetrate tissues to resolve infection within granulation tissue and subcutaneously. The authors’ hypothesis for the initial study of gNOp, conducted through a Defense Advanced

Research Projects Agency (DARPA) grant proposal, was that additional atmospheric pressure is needed to allow the gaseous NO to penetrate tissue.

The development of gNOP is reviewed from beginning in vitro testing, through optimization of therapeutic parameters, to an initial in vivo mammalian (porcine) wound testing model. In the initial study, an in vitro testing system was developed using the EpiDerm-FT full-thickness skin model (EFT400), a stem cell grown skin created by MatTek Corporation (Ashland, MA, USA). This tissue was used to develop an infected wound model for the 4 bacteria strains: *A baumannii*, *P aeruginosa*, *S aureus*, and MRSA. A custom-built testing system was developed to control pressure and gas flow inside of a modified Franz cell apparatus. This system was used for all in vitro testing whereby the therapeutic parameters of NO concentration, pressure, gas flow, and time were evaluated and optimized. An in vivo mammalian (porcine) wound model was then developed by Bridge PTS, Inc (San Antonio, TX, USA) to test gNOP against gram-positive and gram-negative bacteria, *S aureus* and *Pseudomonas*, respectively. A partial-thickness wound model, through the dermis but not through the facial layer, was used to mimic diabetic foot wounds. Results were evaluated using colony forming units (CFUs) and respective log-reduction comparing control samples to tested samples.

## MATERIALS AND METHODS

### *In Vitro Testing*

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#### *Tissue culturing*

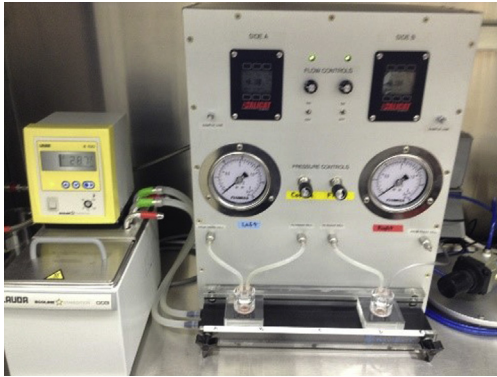
MatTek EFT-400 skin cultures were obtained from MatTek Corporation. Upon receipt, the tissues were immediately taken out of the growth agar and placed in new sterile 6-well plates. About 2 mL of fresh culture media in liquid form was placed below the tissue inserts, and the tissue was left to stabilize for 24 hours in an incubator set to 37°C and 5% CO<sub>2</sub>. Every 24 hours, culture media were replaced to ensure growth of the MatTek EFT400 tissues. The tissue was ready for experiments after the initial 24-hour stabilization period.

#### *Infection assay and growth curves*

The in vitro studies used 4 bacteria common with infections and amputations: *A baumannii* (ATCC #BAA-747), *P aeruginosa* (ATCC #BAA-47), *S aureus* (ATCC #12600), and MRSA (ATCC #33591). All work was performed on MatTek epidermal full-thickness skin tissues (EFT-400). Growth curves were established for all bacterial strains in order to develop parameters for the tissue infection model. The final parameters for infection were 15 µL of bacterial suspension grown to optical density (OD) of 1 (10<sup>8</sup> CFU). A single colony of bacteria was isolated from a nutrient agar plate and placed into 4 mL autoclaved nutrient broth. The bacteria-laden broth was then placed on a Thermo Scientific MaxQ 4450 incubated shaker for 18 hours at 37°C and 200 rpm and allowed to grow. After the initial 4 hours on the shaker, 200 µL of this growth was then transferred to 20 mL of fresh nutrient broth and allowed to continue growth for 4 hours at 37°C and 200 rpm. After this latest growth phase, the bacteria were collected and diluted, and the OD was read with a SpectraMax 384 PLUS to obtain an OD<sub>600</sub> of 1.00. A 3-mm punch biopsy on the MatTek EFT-400 was made, and 15 µL of the bacteria suspension was used to inoculate the tissue wound and allowed to grow for 24 hours.

#### *Exposure setups with nitric oxide*

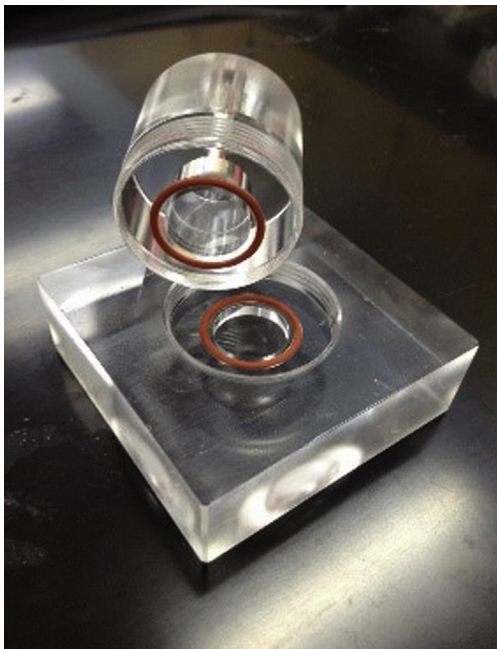
Infected tissues were set up in modified Franz cell exposure chambers with a custom-built manifold, as seen in [Fig. 1](#), capable of adjusting pressure, flow,



**Fig. 1.** Manifold for NO delivery, flow adjusted to 100 cc/min.

and length of exposure with gaseous NO. Exposure chambers custom made for the tissue inserts, as seen in [Fig. 2](#), were fabricated and used to seal the tissue with the gas. Gaseous NO was then delivered to the exposure chamber from the gas canister, through the manifold. As flow is maintained and pressure is held stable, gas exits the chamber and returns through the manifold to an exhaust system.

In the initial DARPA study, it was determined that a flow rate of 100 cc/min (0.1 L/min) was required for effective use of gaseous NO *in vitro*. Higher flow rates did not change results; however, lower flow rates did not achieve bacterial kill. Flow is important to maintain a constant new amount of NO gas so that there is



**Fig. 2.** Modified Franz cell exposure chamber for NO delivery.

no conversion of NO to NO<sub>2</sub>, which is both toxic and ineffective as an antimicrobial. Hence, all testing moving forward was completed with a 100 cc/min (0.1 L/min) flow rate.

In the DARPA testing, 1% (10,000 ppm) gaseous NO was floated over the tissue for 90 minutes of exposure against *S aureus*. Pressure was added and set to 14.7 psi (1 atm) for this duration. These parameters were compared with air at no pressure, air under 14.7 psi (1 atm), and 1% gaseous NO at no pressure. Then, 1% gaseous NO under pressure at 14.7 psi (atm) was tested against the remaining bacteria wound models. Each experiment was run 3 times, with a total of 3 infected tissues each for a total of 9 treated infected tissue models.

The next set of in vitro testing was conducted to evaluate whether lower pressures could achieve a similar result. *S aureus* had previously proved the most difficult pathogen to kill, so it was selected for evaluation at 4.4 psi (0.3 atm) and 3.7 psi (0.25 atm). Concentration, flow, and time were all held constant at 1% gaseous NO, 100 cc/min, and 90 minutes, respectively.

Once understood that lower pressures could maintain an antimicrobial effect with gaseous NO, a new goal of lowering procedure time was adapted. In order to achieve a lower time, it was hypothesized that an increase in concentration of the gaseous NO might achieve that intended result. This testing primarily evaluated the following parameters: 2% (20,000 ppm) gaseous NO, a flow rate of 100 cc/min (0.1 L/min), pressure of 3.7 psi (0.25 atm), and an exposure time of 40 minutes. This testing evaluated these parameters against all 4 bacteria strains. After these studies, NO above atmospheric pressure was then referred to as gNOP.

### ***In Vivo Testing: Mammalian (Porcine) Model***

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#### ***Infection assay***

Infections were completed at Bridge PTS using their approved wound infection protocols for porcine experimental subjects. This infection included creating a partial-thickness wound, through the dermis but not through the fascial layer, to mimic typical diabetic foot wounds, infecting the wounds with *S aureus* and *P aeruginosa* (Gram positive and Gram negative, respectively) for subsequent exposure to gNOP.

#### ***Exposure setup with nitric oxide***

Fabricated exposure devices made of material nonreactive to NO were developed to hold this additional pressure at the wound site for the required time, while the 2% gaseous NO was delivered to the wound interface via a custom-designed manifold. Exposure parameters were set up on this manifold capable of adjusting pressure, flow, and length of exposure with 2% gaseous NO. During the exposure of gNOP, flow rates were set to 100 cc/min (0.1 L/min) for all the experiments. To test the effectiveness of the multiple parameters, testing included varying the pressure and the time of exposure. This study primarily focused on 3.7psi (0.25 atm) and 4.4 psi (0.3 atm) for timed intervals of 40 and 50 minutes.

#### ***Thiazolyl Blue Tetrazolium Bromide Tissue Viability***

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During the in vivo experiments, thiazolyl blue tetrazolium bromide, or methylthiazolyl-diphenyl-tetrazolium bromide (MTT), viability assays were conducted to assess the effect of pressure, concentration of gaseous NO, and time on viability of the MatTek tissue and the healthy porcine tissue.

## Histologic Samples

Histologic samples were taken during the in vivo mammalian wound model to assess the effect of gNOp on the wounded and healthy porcine tissue.

## RESULTS AND DISCUSSION

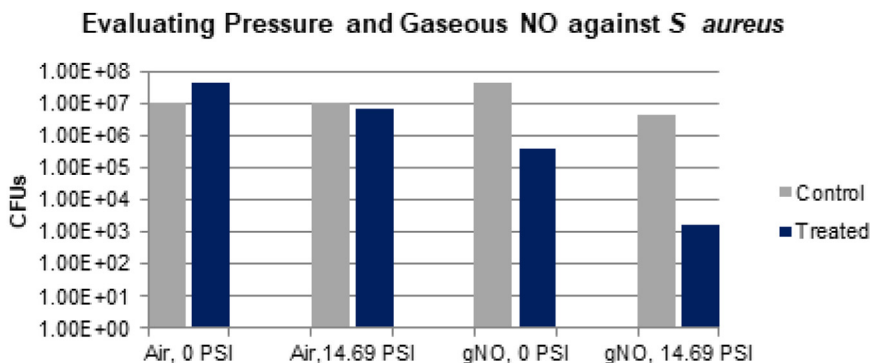
### In Vitro Testing

#### Initial Defense Advanced Research Projects Agency proof of concept study

The results of the DARPA study experiments showed a clear indication that gNOp has a powerful time, pressure, and concentration antimicrobial effect. **Fig. 3** demonstrates that although the gaseous NO itself has an antimicrobial effect, the addition of pressure enhances this property drastically. **Fig. 3** also shows that pressure alone with the presence of air has no desirable effect on the bacteria load: the gaseous NO is a necessity.

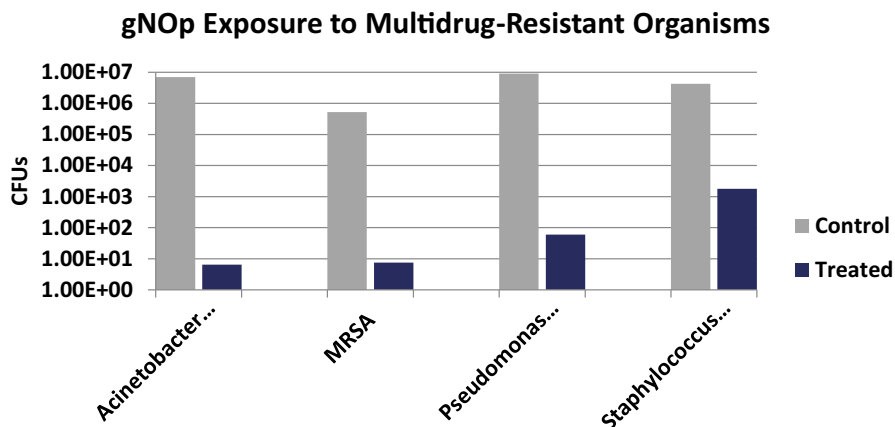
The results also show a reduction in bacteria after gNOp treatment of the different species. **Fig. 4** indicates the bacteria reduction versus a nontreated control (without pressure or gaseous NO) after 90 minutes of exposure with gNOp at 14.7 psi (1 atm) of pressure. The effectiveness of the gNOp treatment between both gram-positive and gram-negative bacteria was similar in this study. The *S aureus* (ATCC #12600) is a biofilm-forming strain that proved the most difficult to eradicate with gNOp exposure. Thus, *S aureus* was selected as the infection control model to determine the specific ranges for the experimental design. These results demonstrate NO's ability to universally eradicate bacteria, whether Gram positive or Gram negative. Based on these in vitro studies, gNOp has significant potential in wound care applications given most infections are not single species.

Next, regarding the MTT assays, the results in **Fig. 5** demonstrate that tissue viability is not affected by the infection itself (columns 3–6) when compared with wounded only and noninfected tissues (columns 1 and 2). Pressure itself also does not meaningfully affect viability (columns 7 and 8). The combination of pressure and NO concentration however is significant. There is a large decrease in viability as NO concentration increases, first without pressure (columns 11, 13), and even more greater with added pressure (column 12 1% NO, +1 atm pressure). In addition, gaseous NO concentration levels are also significant, and the higher the concentration (10,000–20,000 parts per million), the greater the decrease in viability. As



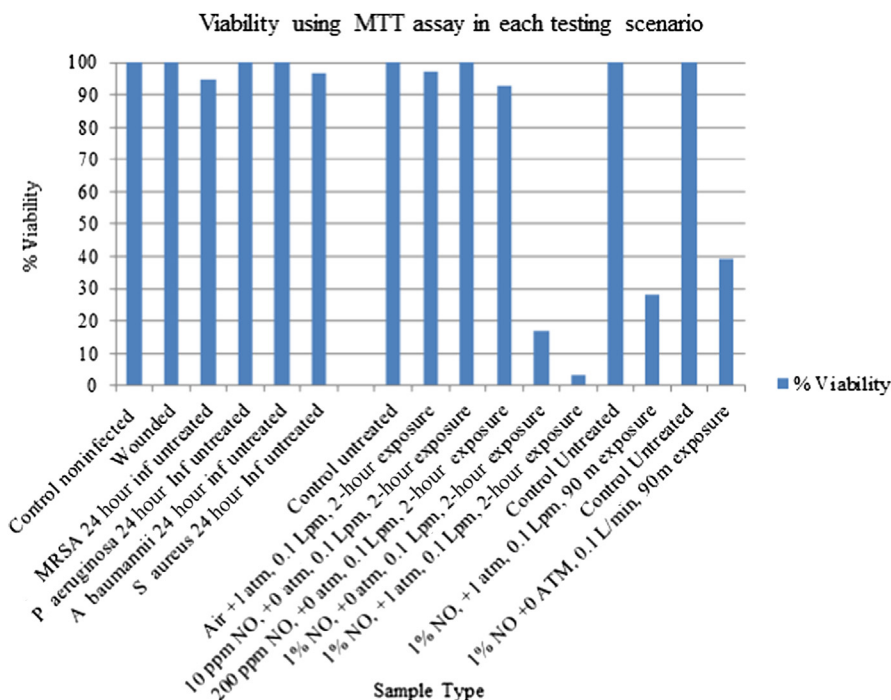
**Fig. 3.** Determine significance of pressure with and without gaseous NO on reduction of bioburden. 24-hour infection, 90 minutes; 0 or +14.69 psi, flow 0.1 L/min, pathogen *S aureus*. Control = infected, untreated tissue, 3 sets of triplicates per experiment.





**Fig. 4.** Exposure of gNOp (10,000 ppm) for 90 minutes with 14.69 psi to all 4 organisms ( $\log_{10}$  CFU/g).

exposure time is decreased, viability increases in the presence of 1% NO with or without pressure (columns 13–16). An important factor to consider in relating the in vitro model to an in vivo model is the fact that the tissue has no way of breaking down the gaseous NO by-products (nitrites and nitrates) that cells in a living system



**Fig. 5.** Viability using MTT assay study evaluating effect of infection, pressure, and NO on cell viability. Inf (Infection); Lpm (L/min).



are able to carry out without difficulties nor can they regulate their pH within a normal level. Compared with bacteria, host cells have a highly evolved nitrosative thiol detoxification pathway, which may be hampered in a closed in vitro experimental system.

With this set of information and positive data regarding the antimicrobial effect of gNOp, understanding requirements for potential human application was the next step. The feasibility of holding 14.7 psi (1 atm) of pressure onto the dermal surface around a wound seemed unlikely at best. Thus, the next set of testing would need to evaluate the antimicrobial effect of gNOp at lower pressures.

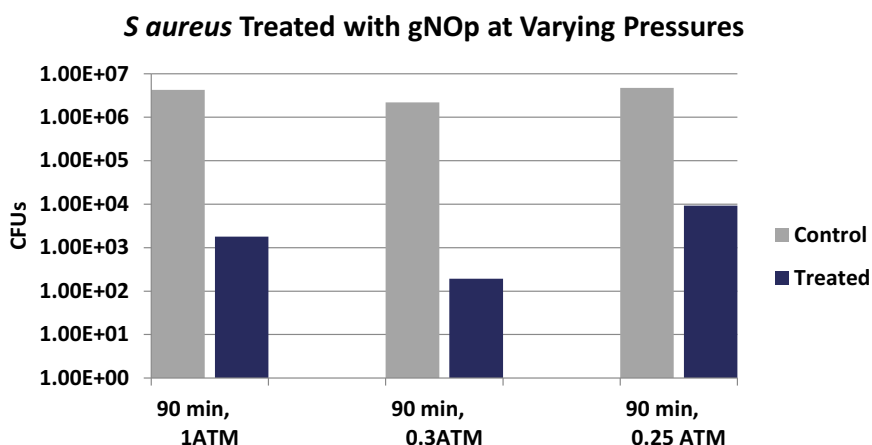
#### ***Defense advanced research projects agency study follow-up: reduced pressures***

The next set of tests indicated that lower pressures could achieve the desired antimicrobial effect. Fig. 6 shows the bacterial log-reduction of *S aureus* against gNOp at lower pressures. In the previous testing, *S aureus* proved the most difficult of the pathogens to kill (see Fig. 4). Given this previous result, it was used as the benchmark for evaluating whether gNOp at a lower pressure could retain its antimicrobial effect.

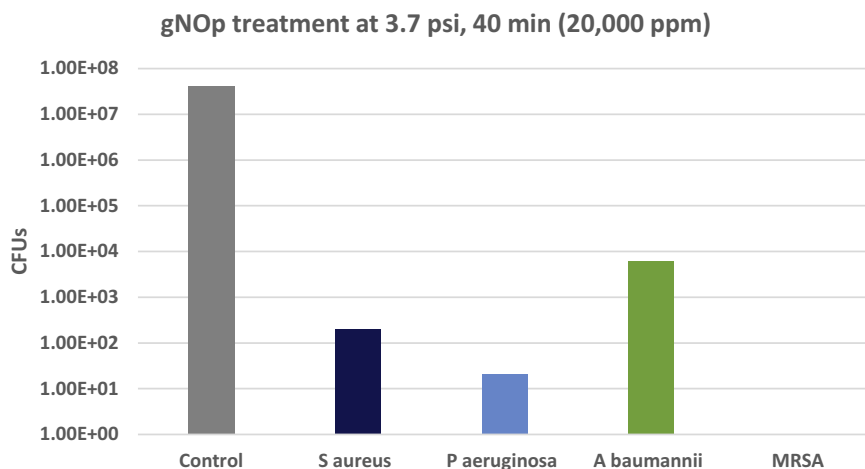
With these promising results of lowering the effective pressure, attention was shifted to the time of the treatment. Although 90 minutes is similar to other wound-healing treatments, such as hyperbaric oxygen therapy, quicker treatment time, if achievable, would be more feasible for patient and medical providers. Consequently, the next phase of testing was to look into an experimental design in which the shortest length of time, with the least amount of pressure added, could achieve the same results for bacteria kill.

#### ***Increased nitric oxide concentration testing***

In this third set of in vitro testing, the concentration of gaseous NO was increased to 2% (20,000 ppm). With the same testing approach, gNOp with the higher NO concentration showed a powerful bactericidal effect. Fig. 7 shows the bacterial log-reduction of the 4 multidrug-resistant bacteria tested. An important additional note is that there was complete eradication of bacteria in at least 1 sample of each pathogen. The minimum log-reduction across all bacteria strains was 3-log reduction, whereas most samples showed complete eradication of bacteria. Results specifically indicated a



**Fig. 6.** Reduction of pressure and effects on bioburden. 24-hour infection, 90 minutes, 0.1 L/min flow. Control = untreated tissues, 3 sets of triplicates per experiment. Pathogen: *S aureus*.



**Fig. 7.** Increased concentration of gaseous NO and effects on bioburden. 24-hour infection, 3.7 psi, 40 minutes, 0.1 L/min flow. Control = untreated tissues, 3 sets of triplicates per experiment. Pathogens: *S aureus*, *P aeruginosa*, *A baumannii*, MRSA.

$10^5$  to  $10^7$  log<sub>10</sub> CFU/g reduction for strains of *S aureus*, *P aeruginosa*, with MRSA completely eradicated, whereas *A baumannii* achieved a  $10^4$  CFU/g reduction.

The results indicate gNOp, at the higher concentration, was successful in eradicating multidrug-resistant organisms. **Fig. 7** shows the effectiveness of the gNOp treatment greatly decreases bacteria load in as little as 40 minutes. **Fig. 7** also shows that even in certain bacterial strains, a 40-minute treatment is still capable of a minimum of a 3-log reduction in bioburden or greater. Both gram-negative and gram-positive bacteria appear to be susceptible to eradication using gNOp with the increase in gas concentration. One limitation is that these studies did not test anaerobes, but others have reported that gaseous NO has a similar antimicrobial effect on anaerobic bacteria.<sup>30</sup> These tests suggest that gNOp could be an innovative antimicrobial. The next step was to evaluate gNOp in an in vivo mammalian model to mimic typical wounds.

### ***In Vivo Testing***

#### ***Mammalian (porcine) model***

The in vivo mammalian (porcine) partial-thickness wound model, as seen in **Fig. 8**, was used to mimic typical diabetic foot ulcers. The results in **Figs. 9** and **10** show a greater than 2-log antimicrobial effect against both gram-negative and gram-positive bacteria strains using gNOp in this testing model.<sup>a</sup> This log-reduction of bacterial load is consistent with that of powerful systemic antibiotics, such as vancomycin.<sup>31</sup> In addition, the results in **Figs. 9** and **10** indicate that both pressure and time are important for bacterial kill. There appears to be an added antimicrobial effect with greater pressure in the *Pseudomonas* testing, and the best results against *S aureus* are with the higher pressures. In addition, the 50-minute tests showed greater kill in both pathogens. Furthermore, the histology images taken after exposure, **Figs. 11** and **12**, show that most of the cells and the surrounding tissue appear to be unaffected by the exposure to gNOp in this model. Overall, the in vivo results seem consistent with the in vitro data.

<sup>a</sup> During the testing, the test animal expired. Standard postmortem laboratory work was conducted, including measurements for methemoglobin and known toxicology for exposure to NO. All laboratory data were within normal limits, and necropsy failed to identify the cause of death.



Fig. 8. Full gNOp setup in porcine mammalian partial-thickness wound model.

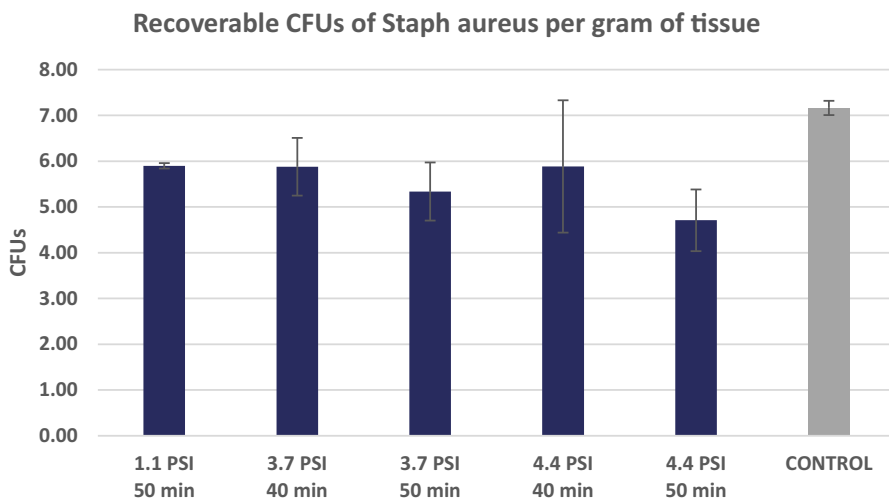
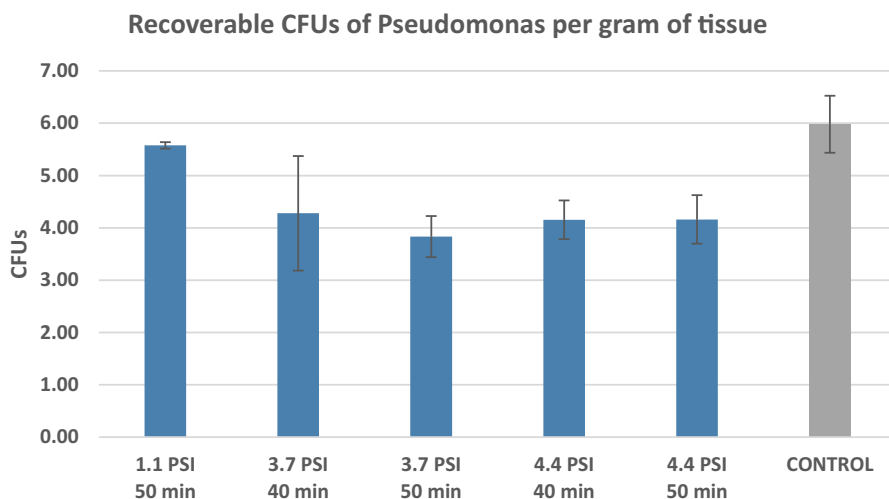


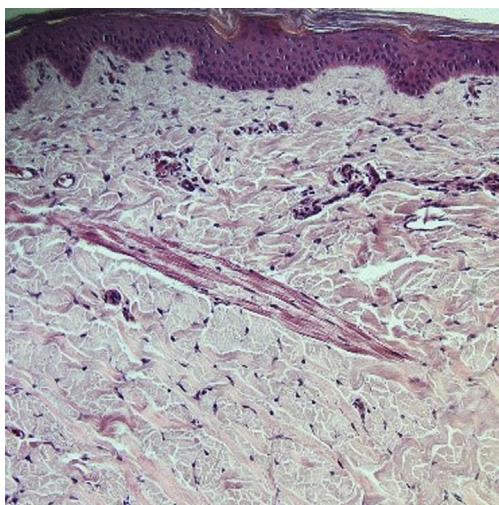
Fig. 9. In vivo mammalian model (porcine); gNOp treatment (20,000 ppm), varying pressures and times, 0.1 L/min flow; Control = untreated wounds; Pathogen: *S aureus*.



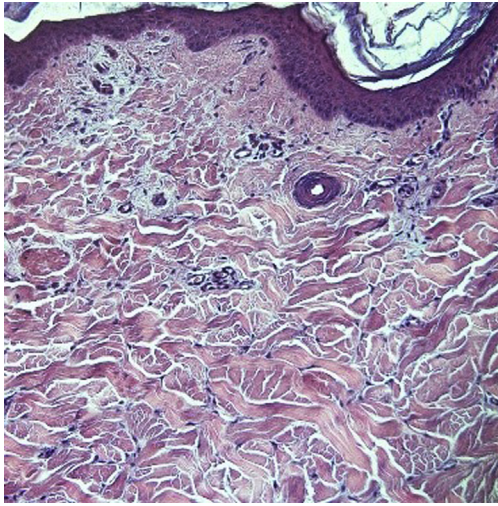
**Fig. 10.** In vivo mammalian model (porcine); gNOP treatment (20,000 ppm), varying pressures and times, 0.1 L/min flow; Control = untreated wounds; Pathogen: *P aeruginosa*.

### Results summary

The initial DARPA proof-of-concept in vitro testing showed that gaseous NO (10,000 ppm) with added pressure (14.7 psi; 1.0 atm) was more effective killing bacteria than gaseous NO delivered without added pressure or air with pressure over a 90-minute treatment. Constant flow of gaseous NO was also essential for the bactericidal effect (0.1 L/min). With these parameters, the follow-up in vitro testing looked to achieve the same results at lower pressures. The bactericidal effect was seen at 3.7 psi (0.25 atm) and 4.4 psi (0.3 atm) over the 90-minute treatment time with the same flow and gaseous NO concentration as the initial DARPA testing. In the



**Fig. 11.** Stain of pig skin that has not been exposed to gNOP (hematoxylin-eosin, original magnification  $\times 200$ ).



**Fig. 12.** Stain of pig skin that has been exposed to gNOp (hematoxylin-eosin, original magnification  $\times 200$ ).

increased concentration in vitro testing, gaseous NO at 20,000 ppm was administered with the goal of reducing the treatment time. Bactericidal effects were seen in the testing across pathogens at 3.7 psi (.25 atm) and a 40-minute treatment period. Finally, the parameters used in the increased concentration in vitro testing were applied in the in vivo mammalian (porcine) model. The bactericidal effect ( $\sim 2$ -log bacterial reduction) was seen in both the gram-positive and the gram-negative pathogens.

## SUMMARY

The in vitro and in vivo results show the development of gaseous NO under pressure from initial idea conception through an in vivo mammalian model. The data suggest gNOp has a powerful antimicrobial effect with potential for application in chronic and acute infections, specifically diabetic foot ulcers, or other mild to moderate skin and skin structure infections. Given the concern over antibiotic resistance, gNOp may provide an alternative solution for skin and skin structure infections over the use of standard systemic antibiotics. Traditional use of systemic antibiotics that are not organism or site specific can cause deleterious effects, propagate resistance, and require extended periods of treatment. NO presents a unique combination of properties that enable an antimicrobial effect, while reducing the likelihood of resistance issues, in a topical, localized treatment. With added pressure, gNOp enhances the bactericidal effect, especially subcutaneously where many topical treatments fail to penetrate tissues.

The histologic results in the in vitro models showed a significant viability issue with the addition of gaseous NO and the added pressure. In the in vitro model, the tissue had no way of breaking down the gaseous NO by-products (nitrites and nitrates) that cells in a living system are able to carry out without difficulties nor can they regulate their pH within a normal level. The histology images taken after exposure in the in vivo mammalian model show that most of the cells and the surrounding tissue appear to be unaffected by the exposure to gNOp. Thus, these

data suggest that tissue viability in potential human models is of less concern now, after the in vivo testing, than post the in vitro testing where viability seemed to be an issue.

Looking to the future development of gNOp, additional testing will be done to optimize parameters for the best therapeutic effect and to evaluate the safety of gNOp in order to progress into potential human trials. It may also be beneficial to evaluate gNOp effect on anaerobic bacterial species. An innovative topical approach to chronic and acute wounds, like DFUs, or other skin and skin structure infections would add value to the array of existing treatment options. Avoiding the prescription of systemic antibiotics that may be of little value to certain patients, such as those with severe vascular issues, is important. A topical solution that could be used in substitution of antibiotics for these types of wounds or infections would give the medical provider an additional tool in their infection treatment tool kit. Gaseous NO under pressure has the potential to be a novel topical antimicrobial treatment for infections and may even have the ability to prevent critical colonization of bacteria before the development of an infection for any patient presenting with a chronic wound.

### ACKNOWLEDGMENTS

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### DISCLOSURE

The authors have a significant interest in Hansen Pharmaceutical, LLC, of which J. Jensen, J. Hanft, and S. Jensen are material owners. Hansen Pharmaceutical, LLC has paid C. Miller for consulting work. The development of gaseous NO under pressure was initially funded by a DARPA grant at Barry University, where J. Jensen, C. Miller, D. Packert, and G. Packert began work to determine the antimicrobial effects of NO. Hansen Pharmaceutical, LLC bought the intellectual property from Barry University and has continued the development of gaseous NO under pressure, which includes a significant portion of the data and information in this article. Hansen is funded through private means.

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