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Gaseous nitric oxide bactericidal activity retained during intermittent high-dose short duration exposure

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ABSTRACT

Previously, we have shown that gaseous Nitric oxide (gNO) has great potential as an effective topical anti-infective agent for non-healing wounds due to its non-specific antimicrobial properties. These same antimicrobial attributes may be useful for pulmonary infections. However, gNO would have limited usefulness as an inhaled antimicrobial agent as continuous exposure to the concentration required for a bactericidal effect (160–200 ppm) leads to methemoglobinemia. To overcome this problem, we investigated whether a thirty minute exposure of 160 ppm every four hours would retain the same antimicrobial effect as continuous delivery.

In vitro, exposure of clinical multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains isolated from the lungs of nosocomial pneumonia patients and a lethal antibiotic-resistant strain of *Pseudomonas aeruginosa*, isolated from a deceased cystic fibrosis patient resulted in over a 5 $\log_{10}$ reduction in bacterial load after multiple thirty minute treatments (4 cycles) every four hours to 160 ppm gNO. The intermittent regimen required 320 (SD = 0) ppm h for 100% lethality whereas the continuous exposure required 800 (SD = 160) ppm h. We have also shown that selection for a gNO resistant phenotype did not lead to decrease sensitivity to gNO therapy ($p > 0.05$). In addition, no host cellular toxicity was observed in human THP-1 monocytes and macrophages following intermittent delivery of a high concentration of gNO, and the proliferation and migration of pulmonary epithelial cells was not adversely affected by the administration of intermittent high-dose gNO. These results justify further studies that should focus on whether intermittent delivery of 160 ppm of gNO every four hours can technically be administered while keeping inhaled NO_2 levels less than 2 ppm and methemoglobin saturation less than 2.5 percent.

Nitric oxide (NO) plays a key role in the non-specific innate immune system [1,2]. Based on murine research, an antimicrobial response to bacteria, viruses, and their byproducts is initiated within macrophages and neutrophils that involve IFN-gamma activation of NOS2, producing inducible nitric oxide synthase (iNOS). iNOS, a NADPH dependent enzyme, catalyzes oxidation of the terminal guanidine nitrogen atoms of L-arginine to produce citrulline and NO, a purported inorganic microbicidal molecule [3]. When NO production is blocked in infected and iNOS knock-out animal models with tuberculosis, pneumonia, or malaria, the disease is exacerbated [4–11]. *In vitro* studies utilizing a variety of NO donors suggest that NO possesses antimicrobial activity against wide variety of phyla including bacteria, viruses, helminthes, parasites and it may even be tumoricidal [9,12–14]. We have previously reported that continuous *in vitro* exposure to exogenous gaseous NO (gNO) is efficacious as a non-specific antimicrobial agent against a broad range of microorganisms including gram positive, gram negative and multi-drug resistant strains of bacteria, yeast, and mycobacteria [15]. These studies demonstrated that the minimum inhibitory concentration (MIC) is 160 parts per million (ppm) during five hours of continuous exposure. We have shown *in vivo* that this same gNO dose reduced bacterial load and concurrent inflammatory response [16] and that multiple eight hour daily exposures to 160–200 ppm gNO was neither toxic nor mutagenic to human cell lines used in modeling dermal wounds [17]. Those

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observations resulted in the successful use of gNO to treat a critically colonized, non-healing, lower leg ulcer in a human subject [18].

We hypothesized that gNO may also be effective as an inhaled antimicrobial treatment against pulmonary pathogens. Prior to testing this hypothesis in an animal model, a number of key issues need to be resolved. For instance, inhaled administration of continuous gNO at 160 ppm for 5 h would unequivocally lead to methemoglobinemia and unacceptable hypoxemia. To circumvent this problem, we have explored an inhaled gNO delivery regimen using a high-dose of short duration (160 ppm for 30 min) with sufficient time in between treatments (3.5 h) to avoid significant methemoglobinemia and reduce the potential of host cell toxicity. The purpose of this *in vitro* study was to verify that this dosing regimen would retain the desired antimicrobial effect as seen in continuous gNO delivery. This study also provides additional data regarding host cell tolerance to high-dose gNO therapy. Further, we desired to establish whether this intermittent regimen would have a propensity for selection of bacterial phenotypes less sensitive to NO over time.

Methods and materials

In vitro nitric oxide exposure device

The design and validity of the continuous horizontal-flow gNO delivery device used in this study has been described in detail elsewhere [19]. In brief, the device consisted of two cylindrical Plexiglas® exposure chambers with separate gas entry ports and a common exit port. These chambers were surrounded by an airtight Plexiglas® jacket to create a thermally isolated environment. This jacket enclosed an electrical heater unit controlled by an internal thermostat (Invensys Appliances Control, Carol Stream, Illinois, USA), that provided stable temperatures inside the chamber. Independent lines from each of the two exposure chambers provided samples of the gas mixtures to a $NO/NO_2/O_2$ electrochemical analyzer (AeroNOx, Pulmonox Medical Inc., Tofield, AB, Canada) to detect the exact composition of the gases in the mixture. Gases were supplied from pressurized cylinders at a constant pressure of 50 pounds per square inch. These included 800 parts per million (ppm) medical-grade NO diluted in N_2 (ViaNOx-H, Viasys Healthcare), medical air, O_2 and CO_2 (Praxair, Mississauga, ON, Canada). These gases were then mixed together at pre-determined concentrations using a dilution manifold and a digital mass flow meter (TSI Inc., Shoreview, MN, USA). The gas mixture was delivered to the exposure chamber at a rate of 2.0 L/min at 90% relative humidity through two independent humidifiers (MR850, Fisher & Paykel Healthcare, CA, USA).

In vitro nitric oxide delivery regimen

Bacterial cultures were obtained from American Type Culture Collection (ATCC) and from a cystic fibrosis clinic (British Columbia Children's Hospital). The bacteria used for this study were a clinical multi-drug resistant *Staphylococcus aureus* and *Escherichia coli* strains isolated from the lungs of nosocomial pneumonia patients and a lethal antibiotic-resistant strain of *Pseudomonas aeruginosa*, isolated from a deceased cystic fibrosis patient.

The organisms were grown according to the standard operating procedures of the certified main hospital laboratory. From these cultures, a 0.5 McFarland standard with 10^8 colony forming units per milliliter (cfu/mL) was prepared and further diluted 1:1000 with sterile saline to 10^5 cfu/mL in a volume of 20 mL. The concentration of 10^5 cfu/mL was chosen since it is an accepted threshold for determining wound infection [20]. We chose to suspend the bacteria in 0.9% saline rather than nutrient broth media, because saline maintains the bacteria in more representative *in vivo* static and more resistant state in which they neither multiply nor die. Further, we standardized to observations from similar *in vitro* NO studies which have shown that substances found in bacterial laboratory support media bind NO, and that this interference may have masked the true effects of NO in previous studies [21]. Aliquots were then pipetted, in triplicate, into six-well, flat bottom, cell culture dishes with lids (Corning 3516, Corning, NY).

When the temperature and gNO concentration were in steady state, the diluted cultures were incubated in the control and treatment arms of the chamber. To simulate a minimal and safe gNO pulmonary exposure with an acceptable bacteriocidal effect, an intermittent exposure protocol was designed. Bacterial suspensions in the treatment arm of the chamber were exposed to 160 ppm gNO for either 30 min followed by exposure to the diluent medical air for 3.5 h or continuous gNO exposure in a separate study. This gNO exposure cycle was repeated for a total of six complete cycles up to a 24-h period. Bacterial colonies in the control arm were exposed to continuous medical air only. To measure the effect of gNO on bacterial viability, cultures were sampled at 0, 4, 6, 8, 12, 16 and if needed 24 h, spread on Columbia agar containing 5% sheep's blood (PML Microbiologicals, Willsonville, OR), and incubated at 35 °C for 24 h. A technologist blinded to the exposure performed the colony counts.

The total ppm-hours for the intermittent regimen and continuous exposure regimens were calculated as follows:

$$\begin{aligned}\text{Total ppm-hours} = {} & [\text{gNO ppm concentration} \\ & \times \text{Duration of exposure}] \\ & \times [\text{number of exposures}]\end{aligned}$$

Thus, the intermittent regimen for six, thirty minute exposure cycles would be calculated as follows:

$$\begin{aligned}\text{Total ppm-hours} &= [160\text{ppm} \times 0.5\text{hours}][6\text{treatment cycles}] \\ &= 480\text{ppm-hours}\end{aligned}$$

Nitric oxide sensitivity assay

To determine if bacteria could develop resistance to gNO, *S. aureus* (ATCC 25923) was grown in Nutrient Broth at 37 °C with shaking over night then diluted with fresh media to an optical density at 600 nm of 0.5. The resulting bacterial suspension was equilibrated for 10 min at 37 °C with shaking, then diluted 1:1000 in normal saline, and equilibrated again. The bacterial suspension was incubated for 1 h in the presence of 160 ppm gNO or medical air. One hour exposure was the time required to achieve a 50% reduction in bacterial count as determined in previous experiments. Bacterial survival and calculation of colony forming units per milliliter (cfu/mL) were determined by sampling 0.001, 0.01, and 0.1 μl of the exposed suspensions and plating them onto Sheep's Blood Agar, incubating at 37 °C for 24 h and then counting the colonies. This experiment was repeated seven more times, each time preparing the inoculum with bacteria surviving the previous gNO treatment. After the eighth exposure cycle, in addition to the 1-h incubation in the presence or absence of gNO, the *S. aureus* suspension was also incubated for 2, 3, and 4 h in the presence and absence of gNO before determining viable counts. This was done to see if the resulting phenotype was as sensitive to gNO as its parent strain.

Lung epithelial cell culture

A549 cells (human lung epithelial carcinoma) were obtained from the ATCC. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum

(FBS), 100 units/ml penicillin, 100 units/ml streptomycin sulfate, 2 mM L-glutamine, 1% essential amino acids, and grown in a humidified 37 °C, 5% CO_2 incubator.

Growth and analysis of THP-1 cell cytotoxicity

The monocytic cell line THP-1 (ATCC 202, Rockville, MA) was cultured in RPMI 1640 (Hyclone, Logan, UT) supplemented with 5% fetal calf serum (FCS), (Hyclone, Logan, UT), and 2 mM L-glutamine (StemCell Technologies, Vancouver, BC). Cells that were three passages or younger were used for all experiments. The cells were seeded in 96-well microtiter dishes at a density of 3×10^5 per well, and incubated at 37 °C, 5% CO_2 for 24 h, either continuously in the presence of 200 ppm gNO or intermittently in the presence of 160 ppm gNO. The negative control consisted of THP-1 cells incubated in the presence of medical air. As a positive control for cytotoxicity, THP-1 cells were treated for 6 h with 5% H_2O_2, and stained with PI, confirming the correlation between PI staining and THP-1 cell death during this assay. Cytotoxicity analysis was performed by flow cytometry as described earlier [22].

Lung epithelial cell proliferation assay

Colorimetric methyl thiazolyl tetrazolium (MTT) assay was used to evaluate the cytotoxic effects of gNO on lung epithelial cell proliferation [23,24]. A549 cells were seeded at a density of 5×10^4 per well in a 12-well plate (Costar). Cells were treated with 160 ppm gNO for 30 min followed by 3.5 h of medical air. This intermittent treatment was repeated for up to 72 h. The control group was continuously exposed to medical air for 72 h. Following each exposure period the MTT assay was carried out to determine the number of viable cells. MTT was dissolved in phosphate-buffered saline (PBS) at 2 mg/ml, and 200 μl was added to each well. After 5 h incubation at 37 °C, MTT end products were solubilized by replacement of the culture medium with dimethyl sulfoxide (DMSO) and the optical density at 540 nm was recorded. The MTT analysis is dependent on the reduction of the tetrazolium salt, MTT, by the mitochondrial dehydrogenase of viable cells to form a blue formazan product.

Lung epithelial cell migration assay

To analyze the effect of 160 ppm gNO on cell migration and attachment, A549 cells were seeded at 1×10^6 cells per 60 mm culture dish and incubated at 37 °C in DMEM containing 10% FBS to form a confluent monolayer. Monolayers were scratched with a plastic pipette tip to mimic a wound with a uniform width. Suspended cells were washed away and the media was replaced by DMEM containing 2% FBS. These cultures were then exposed continuously to 160 ppm gNO or medical air for up to 72 h. Serial light micrographs of the wound areas were taken daily for the next 3 days. Individual frames were stored, calibrated, and measured using an image analyzer (Quartz PCI, Pleasanton, CA, USA) for the distance migrated across the wound. Migration distance was expressed as a percentage of the wound width on day 0.

Clonogenic cell survival assay

Exponentially growing A549 cells were seeded in 60 mm culture dishes at a density of 200–600 cells per dish. The dishes were incubated overnight at 37 °C to allow the cells to attach. The cultures were then subjected to different doses of γ-radiation (^{60}Co gammacell; Atomic Energy of Canada Limited, Mississauga, ON, Canada), 160 ppm of gNO, continuously, for 24 h at 37 °C, or air alone as a control. Following each treatment, the culture medium was replaced with fresh DMEM supplemented with 2% FBS, and incubated at 37 °C for 10–14 days. Cell colonies were fixed in 70% ethanol, stained with 10% methylene blue, and counted. The surviving colonies were plotted as a function of treatment received.

Statistical analysis

The results were analyzed using the unpaired Student's *t*-test for comparison between any two groups. Group means were statistically tested by least squares means (two-tailed *t*-test). Data analysis and graphical presentation were done using a commercial statistics package (Graphpad-Prism V 3.0, GraphPad Software Inc., USA). Unless otherwise specified, $p < 0.05$ indicated statistical significance. Results were reported as the means ± standard error of the mean from at least three independent measurements.

Results

Antimicrobial effect of intermittent gNO in vitro

Previous studies demonstrated that continuous delivery of 160 ppm gNO resulted in a 100% lethal dose (LD_{100}) representing a 5 $\log_{10}$ reduction in colony forming units per milliliter (cfu/mL) at 5 (SD = 1) h. On the other hand, the proposed intermittent delivery regimen in this study showed that the average LD_{100} was 16 (SD = 0.0) h for a 5 $\log_{10}$ reduction in cfu/mL. The resulting survival curves for each organism comparing the continuous versus the proposed intermittent regimen are shown in Fig. 1. This intermittent delivery strategy resulted in a 100% bactericidal effect (5-log reduction in concentration) against *S. aureus, P. aeruginosa*, and *E. coli*. Bacteria that are associated with human pulmonary pathogenesis. These experiments demonstrated that four cycles of 160 ppm gNO for 30 min followed by air exposure for 3.5 h resulted in complete cell death for the bacteria tested.

Nitrogen dioxide (NO_2) levels were measured during the studies and were analyzed at a concentration of 15–18 ppm at flows of 2 L/min.

Bacterial selection and sensitivity to exogenous gNO

Because of bacterial propensity for developing phenotypic resistance to antimicrobial drugs, an attempt to enrich for organisms that might be less sensitive NO was performed. This was accomplished by repeatedly subculturing *S. aureus* in the presence of gNO for a sub-lethal exposure time of one hour. *Staphylococcus aureus* surviving serial gNO exposures were isolated after a series of incubations in the presence of 160 ppm gNO. Survivors of the 8th round of gNO exposure were further incubated in the presence of gNO to determine if they were more resistant to gNO cytotoxicity compared to *S. aureus* treated with eight rounds of exposure to medical air. The results in Fig. 2 demonstrate that the organisms surviving eight previous rounds of gNO exposure did not differ significantly from the control organisms in the rate of killing by gNO ($p > 0.05$). This suggests that selection for a less sensitive gNO phenotype did not lead to decrease NO sensitivity.

Proliferation, migration and mutagenesis of pulmonary cells in the presence of exogenous gNO

The effect of gNO on pulmonary epithelial cell proliferation was measured by MTT assay. Proliferation data were expressed as OD_{540} readings representing formazan production in metabolically active and viable cells (Fig. 3). Cytotoxic and cytostatic effects were not observed in epithelial cells exposed intermittently to 160 ppm gNO. In fact, cells demonstrated a slightly higher growth rate in the presence of gNO after 72 h of exposure. Interestingly, this dose of gNO exhibited significant antimicrobial properties in previously

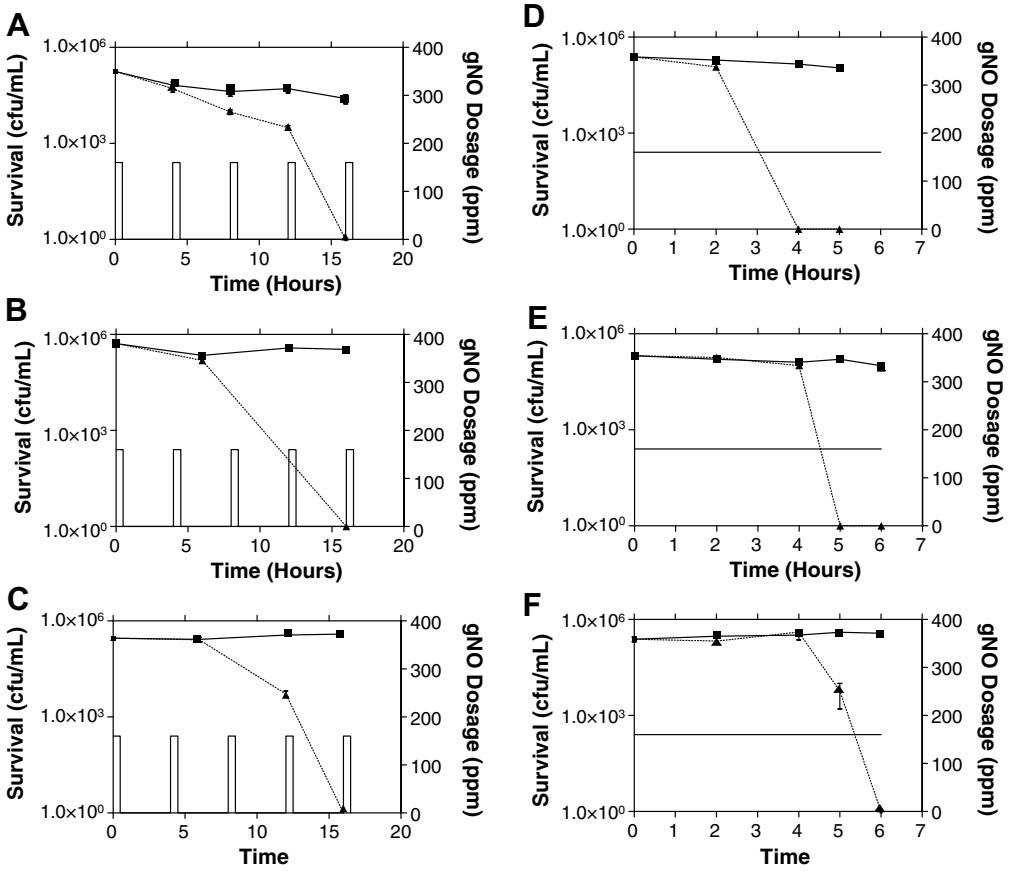

Fig. 1. Bacterial survival comparison curves comparing intermittent high-dose and short duration gNO (20 h scale) to continuous high-dose gNO (7 h scale). Bacteria were exposed to repeated cycles of 160 ppm gNO for 30 min followed by medical air for 3.5 h (A–C triangles) or continuous 160 ppm gNO (D–F triangles). Treatment regimens were compared to medical air continuously as a control (squares), for up to 24 h. The gNO dose regimen is also shown (bars). (A and D) *S. aureus*; (B and E) *P. aeruginosa* (cystic fibrosis isolate); (C and F) *E. coli*.

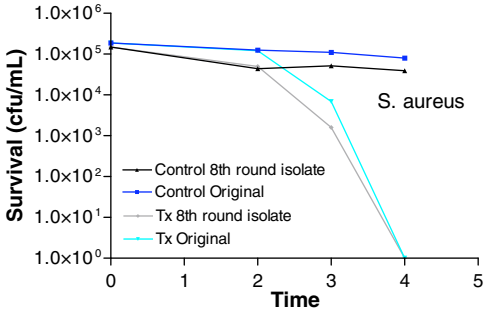

Fig. 2. Enriched *S. aureus* survival curves after continuous exposure to a high-dose of gNO. Bacteria were exposed to 160 ppm gNO or medical air for 1 h, subcultured, and exposed to gNO or medical air again, for a total of eight rounds of exposure. These gNO-enriched and air control cultures were exposed to 160 ppm gNO or medical air for up to 4 h to determine bacterial survival. Symbols: gNO-enriched *S. aureus* exposed to medical air, triangles; gNO-enriched *S. aureus* exposed to 160 ppm gNO, diamonds; air-enriched *S. aureus* exposed to medical air, squares; air-enriched *S. aureus* exposed to 160 ppm gNO, inverted triangles.

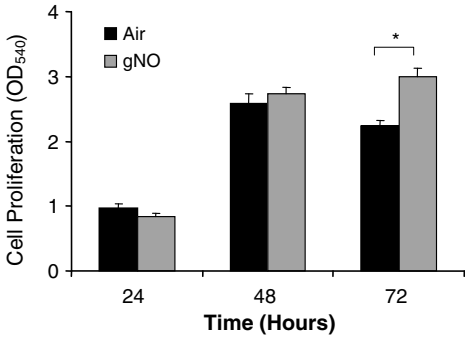

Fig. 3. Effect of gNO on proliferation of epithelial cells. Following intermittent exposure to 160 ppm gNO for up to 72 h, an MTT proliferation assay was carried out to determine the number of viable cells in culture. Data represents the amount of formazan production by metabolically active cells at OD_{540}. The asterisk ($^{*}p < 0.01$) denotes a significant difference in proliferation between gNO and control group at 72 h.

reported *in vitro* studies [15,16]. The result of a migration assay revealed that epithelial cells did not lose their ability to migrate in presence of gNO (Fig. 4A). Cell migration was monitored and photographed for 3 days post exposure. The distance migrated by epithelial cells was measured and expressed as a relative percentage of the original injury size. Cells in both treated and control groups were able to repopulate 70% of a 300 μm gap by day 3 (Fig. 4B). The sensitivity of A549 lung epithelial cells to 160 ppm gNO was determined using the clonogenic survival assay. Various doses of gamma (γ) radiation, a genotoxic agent, were used as positive controls. As shown in Fig. 5, epithelial cells were not sensitive to 160 ppm intermittent gNO exposure compared to gamma radiation. In fact, a significant difference noted between the control and the gNO treated cells. Of interest, it is purported that NO orchestrates wound and influences cell behavior [25]. It is possible that NO may act as an effector molecule in epithelial cell proliferation.

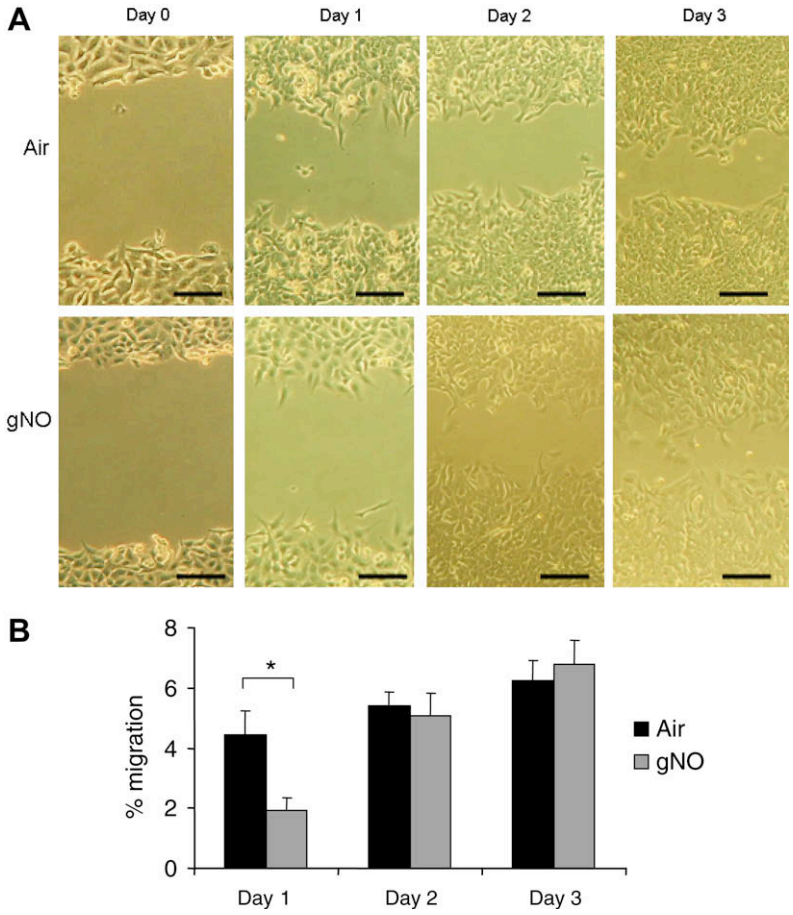

Fig. 4. Effect of 160 ppm gNO on A549 cells migration. (A) Digital photographs illustrating epithelial cell migration behavior under exposure of gNO or medical air. (B) Quantitative comparison of percent migration relative to baseline (day 0) in both groups. The asterisk ($*p < 0.01$) denotes significant difference between migration rate of gNO and control group in day 1. Scale bar represents 100 μm.

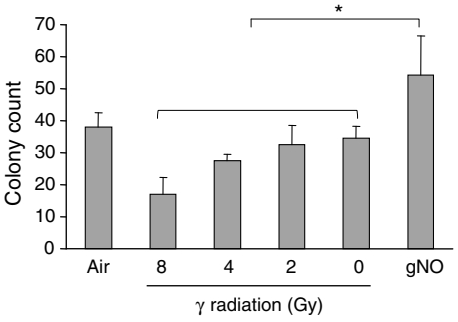

Fig. 5. Lung epithelial cell clonogenic survival assay. A549 cells were exposed to air or to 160 ppm gNO for 24 h. Cells were exposed to 0, 2, 4, or 8 gray of gamma radiation, a genotoxic agent. Cell colonies were counted on day 10 post-treatment. Each bar represents the mean values from a minimum of three independent determinations ± standard deviation. The asterisk denotes a significant difference ($p < 0.01$) between gNO and gamma radiated group colony counts.

To determine if gNO exposure was harmful to human immune cells important in lung defense, survival of THP-1 monocytes and macrophages was measured after continuous exposure to 200 ppm gNO or medical air as a negative control. The dose of 200 ppm was used to test for a 25% higher dose and continuous duration of exposure to simulate long term multiple exposures of up to 10 time greater duration threshold exposure to gNO than during the proposed exposure regimen. After 24 h of exposure, almost 75% of undifferentiated THP-1 monocytes survived, whether they were treated with gNO or air (Fig. 6A). In addition, there was no significant difference between survival in the presence of gNO or air only. When THP-1 cells were first differentiated into macrophages before exposure to gNO or air, they had a slightly lower survival rate compared to the monocytic cells, at approximately 55% for treatment with air and 60% for treatment with gNO (Fig. 6B). In this case, survival of gNO-treated macrophages was increased compared to the macrophages exposed to air only.

Since the undifferentiated THP-1 monocytes were not sensitive to treatment with the continuous, long term, high end doses of gNO, these same cell lines were further tested for differences in survival when treated with an intermittent regimen of 160 for 30 min and 3.5 h of 20 ppm gNO (repeated for 5 cycles) or 20 ppm continuously for 24 h. A control of 20 ppm gNO was used as this is the indicated dosage for continuous delivery of gNO to treat full term infants with persistent pulmonary hypertension of the newborn [26]. The histograms of the two PI-stained populations are shown in Fig. 6C. Except for a small population of PI-stained cells in the 160/20 ppm gNO treatment group, no significant population shifts were observed between the cells exposed to 160/20 ppm and the cells exposed to 20 ppm gNO. These observations were consistent with the previous results, indicating that high-dose gNO delivered in an intermittent regimen for a prolonged period of time to representative pulmonary host cells does not induce the same cytotoxicity seen for bacteria at similar doses and durations.

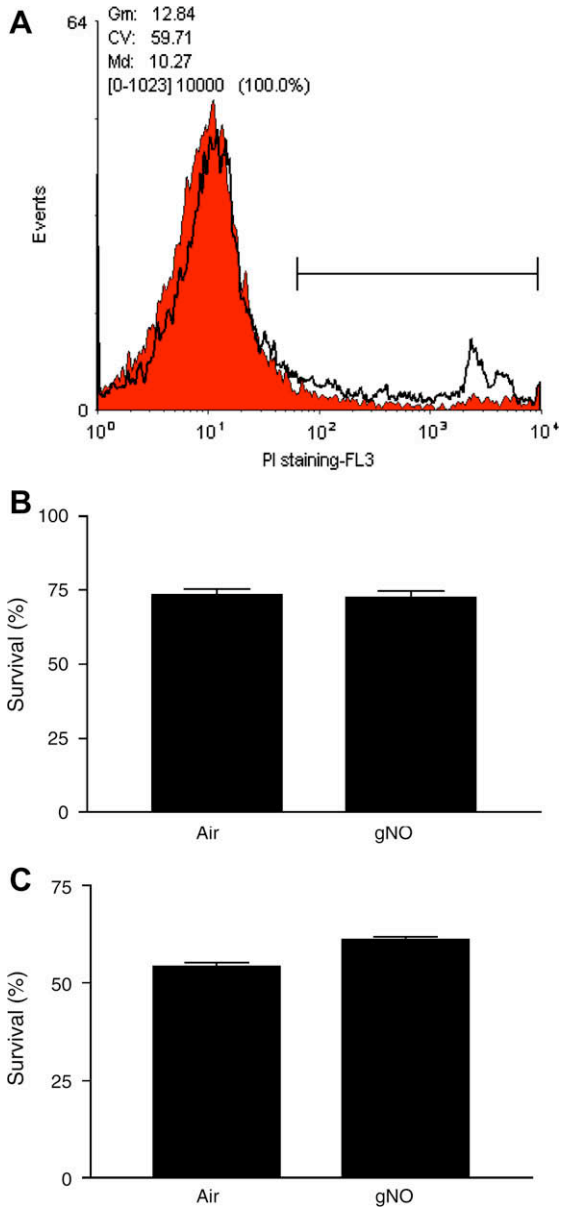

Fig. 6. Cytotoxic effects of nitric oxide *in vitro*. (A) FACS histogram of the monocytes survival study. (B) Graphical representation of gNO effect at 200 ppm (24 h) on human monocytes survival. (C) Graphical representation of gNO effect at 200 ppm (24 h) on human macrophage survival. Toxicity was measured as death rate by flow cytometry with PI (propidium iodide). Black outline represents THP-1 monocytes exposed to intermittent 160/20 ppm nitric oxide. Solid red represents THP-1 monocytes exposed to continuous (24 h) 20 ppm nitric oxide. One representative of three is shown. The black line represents area of dead cells. (For interpretation of the references to colours in this figure legand the readers referred to the web version of this paper).

Discussion

Results from this study suggest that the bactericidal effect of 160 ppm gNO was preserved during intermittent delivery as compared to continuous delivery of the same concentration. This is a highly relevant finding if gNO is to be considered for inhalational use as an antimicrobial agent to treat pulmonary infections.

A different approach from topical administration is required for inhaled delivery of 160 ppm gNO. NO has a high affinity for metal ions and reacts readily with hemoglobin to create methemoglobin. At high levels, methemoglobin can interfere with oxygen transport. Due to the larger gas-blood surface area in the lungs, rapid conversion of hemoglobin to methemoglobin by continuous exposure to gNO will cause hypoxemia. This may be especially pertinent as the target populations for gNO antimicrobial therapy are likely already to have pulmonary complications. Instead of using continuous delivery of gNO, with its higher potential for inducing methemoglobinemia, an alternative high-dose (160 ppm), short duration (30 min) strategy was hypothesized for a pulmonary application model of gNO.

This study was designed to evaluate whether an intermittent high-dose gNO delivery regimen that would preserve the antimicrobial effect of continuous gNO delivery yet, theoretically, avoid elevated methemoglobin levels. When gNO was administered to three bacterial strains associated with pulmonary disease at 160 ppm for 30 min, repeated every 4 h, the same potent antimicrobial activity was observed as when 160 ppm gNO was delivered continuously. Despite taking about 10 h longer to achieve the same antimicrobial effect as with continuous delivery of gNO, this intermittent regimen might allow for a safer approach in order to reduce potential side effects. However, this gNO delivery regimen may not be as effective in an *in vivo* setting where bacteria might replicate to unacceptable levels between gNO exposures. We speculate that the continuous exposure to gNO is not needed to kill bacteria and an intermittent exposure is effective because of the affinity of NO to bind target cells. NO is a gas that passes unhindered through cell membranes targeting a wide range of macromolecules. As previously reported, after thiol detoxification sites are overwhelmed, the free NO in the cytosol binds with iron complexes such as aconitase interrupting cellular respiration. NO can further react with reactive oxygen species resulting in production of peroxynitrite that is known to destroy DNA [27]. It appears that this regimen is of a significant burden to bacteria, not allowing the bacteria time to sufficiently replenish or up-regulate thiol production. However, a dynamic *in vivo* biological model is required to verify this.

The rationale for the 30 min of 160 gNO therapy was based on published methemoglobin kinetic studies [28–30]. The predicted half-life of methemoglobin in humans is approximately 1 h. The anticipated rise in methemoglobin during the 30 min, 160 ppm gNO treatment is calculated to be approximately 1%. The 3.5 h interim period would allow the methemoglobin concentration to return to baseline, at which time another 30 min gNO treatment of 160 ppm could be given. The total 24 h NO metabolic burden for this six cycle regimen is calculated as 480 ppm h. The reality is, that in more than 10 years of successfully treating term infants with inhaled NO, methemoglobinemia levels have not been shown to be a reported risk factor, even in the presence of rare cases where methemoglobin reductase is impaired. Of interest, 80 ppm for 24 continuous hours as used in early neonatal and adult clinical trials results in 1920 ppm h [26]. However, in clinical practice, the usual accepted dose is 20 ppm and the exposure to the lung in 24 h is 480 ppm h. Thus, the metabolic burden for the resulting effective antimicrobial intermittent regimen of six cycles of 160 ppm gNO for 30 min is the same (480 ppm h) as that currently being used in neonatal practice. We hypothesized that this treatment combination could be a safe therapeutic regimen from the standpoint of methemoglobinemia and NO metabolic burden but this needs to be confirmed in an animal model.

An ever-increasing clinical problem and of significant concern is the ability of disease causing organisms metabolically and genetically adapt to the drugs used to treat them. Nowhere is this more evident than in the development of antimicrobial resistance by bacteria. Bacteria that are not intrinsically resistant to an antimicrobial drug may develop resistance through *de novo* mutation or through the acquisition of resistance genes from another organism by horizontal transfer [31]. Specifically, it has been suggested that bacteria such as *S. aureus*, *P. auruginosa*, and *E. coli* have the ability to metabolically adapt to nitrosative stress and to decrease their

sensitivity to nitric oxide antimicrobial actions [32]. Although it appears from previous studies that high-dose gNO is effective as an antimicrobial agent, the proposed intermittent delivery of gNO could potentially increase the probability of developing a phenotype resistant to gNO or inducing metabolic adaptation to nitrosative stress. The study attempted to enrich for organisms adaptive response to the toxic effects of gNO. The data showed that the organisms surviving multiple gNO exposures did not have a decrease in sensitivity to the cytotoxic effects of cyclic high-dose gNO exposure. As an additional test (results not shown), these strains were successfully evaluated to confirm a continued antibiotic (10 μg gentimycin disk) susceptibility as compared to the original strain before enrichment. However, this *in vitro* model is limited compared to a dynamic biological system. We speculate that the mechanism of antimicrobial action of high-dose gNO is so basic to the biochemistry of the bacteria that it is unlikely that mutations result in viable offspring. These results seem to substantiate this.

A series of *in vitro* mechanistic studies has previously demonstrated the effectiveness of gNO as a bacteriocide. This antimicrobial activity was dependent on 160 ppm gNO overwhelming bacterial thiol-based detoxification mechanisms and maintaining pressure on the thiol pathways so that bacteria could not replenish this defense reservoir [33,34]. Eukaryotic cells (human) have much higher thiol levels and can cope with high levels of NO better than prokaryotes/microbes [35]. Thus, host cells should tolerate NO stress more effectively than bacteria. The data reported herein provides evidence for this in several ways: the proliferation of A549 cells which are sensitive pulmonary epithelial carcinoma cell lines in the presence of 160 ppm gNO was equivalent or better than in the presence of medical air for up to 72 h. A549 cells exposed to gNO also exhibited normal function and survival as demonstrated in the healing process. In this study, A549 cells were used as a surrogate for primary (normal) lung epithelial cells. Previous studies have also used these cell lines to predict the behavior of primary cells, since cell lines are already established by others and much easier to grow in culture [24]. A549 cells along with gNO-treated THP-1 monocytes (undifferentiated) and macrophages, were shown to survive as well as, or better than control cells exposed to medical air. Together these data support our hypothesis that the host will tolerate a high-dose, short duration gNO treatment strategy.

In earlier work exploring the mechanisms of the antimicrobial action of NO, it was found that 40 and 80 ppm gNO was not an effective dose for killing bacteria. Other *in vitro* and *in vivo* work reported in the literature has concluded that doses below 90 ppm would not be clinically efficacious [36,37]. This has also been confirmed clinically by Long et al., who showed that inhaled 80 ppm gNO was ineffective at reducing the bacterial load in patients with TB [38]. We speculate that had they used a higher dose their results may have shown a significant reduction in bacterial load.

Administration of NO donors, such as polyethyleneimine cellulose NONOate polymer, *S*-nitroso-*N*-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), and molsidomine (*N*-ethoxycarbomyl-3-morpholinyl-sidnonimine), to deliver NO may be effective as an antimicrobial agent [9,12–14]. Acidified nitrite derivatives have been shown to kill mucoid *P. aeruginosa* and decrease bacterial load in cystic fibrosis conditions [39]. The challenge with donors and nitrite derivatives is whether or not a consistent and effective antimicrobial delivery dose could be achieved. For instance, nitrites require a specific pH level in order to release NO. Donors require various conditions to release NO and this is further exacerbated by the potential toxicity of these NO carrier compounds [40]. gNO is not compounded like other drugs and is carried within inert nitrogen as a 0.0160% active ingredient. As such there are no toxicity issues other than directly as a molecule. Aerosolization of any compound is problematic with regard to particle size and deposition to all target sites within the lung. Because gNO is a gas, diffusion and dispersion of a uniform concentration are more predictable within the known lung model.

NO_2 formation during gNO therapy is a concern and is exacerbated at high levels as suggested by these results. Occupational safety and health standards limit the NO_2 exposure of workers to 5 ppm [41]. Adverse effects of high NO_2 have been reported throughout the literature [42]. Nitric oxide is an approved drug for term infants and is inhaled continuously and may be inhaled for weeks [26]. The acceptable NO_2 levels for inhaled NO delivery have been established at 2 ppm [43]. Previous *in vitro* work has shown that high levels of NO_2 are not bactericidal and further concomitant high levels of 18 ppm NO_2 did not have deleterious toxic effects on a wide range of host cells [16,17]. Nevertheless, 18 ppm NO_2 is unacceptable from a clinical perspective and the attitude adopted should be to keep the inhaled NO_2 level as low as possible during gNO therapy. NO is a free radical and formation of NO_2 is concentration and time dependent and is further accelerated as the fractional inspired oxygen concentration increases such as in the case of the compromised pulmonary patient. This is a significant challenge should gNO therapy be considered for further study as an inhaled antimicrobial agent. To date, there are no approved delivery devices capable of delivering 160 ppm gNO let alone having the ability to keep the inhaled NO_2 below 2 ppm. We hypothesize that increasing the total flow of gas from the 2 L/min used in the exposure chamber to that required for ventilation will reduce the dwell time and result in a significant reduction to 2–3 ppm NO_2 formation. Prior to this regimen being extrapolated to human testing it would be prudent to ensure that inhaled NO_2 levels are kept below 2 ppm.

These results give promise to the use of high-dose, intermittent gNO as an inhaled antimicrobial agent. We have calculated that the resulting metabolic burden would be within normal safety levels for methemoglobin. Further, we have shown this treatment regimen to be non-toxic to representative cells residing in the host airway. These results suggest that gNO should be explored for use against pathogens associated with pulmonary infections. However, it still remains to be seen whether or not devices can be engineered to deliver high-dose gNO to animal models while keeping the concomitant NO_2 levels below 2 ppm. If this challenge can be overcome, animal models could be examined to evaluate, prove out these findings and further examine histopathologic and toxicological sequelae. Should these studies prove to be effective and safe, we speculate that high-dose (160 ppm), intermittent (every four hours; Q4h) gNO administration may be useful as a first line inhaled antimicrobial treatment to augment the innate defense system or possibly as an adjuvant to antibiotics.

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