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Potential application of gaseous nitric oxide as a topical antimicrobial agent

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Abstract

The presence of bacterial colonization in non-healing wounds and burn injuries interferes significantly with the normal process of healing. Recent evidence suggests that nitric oxide (NO) plays an important role in host defense against infection and regulates wound healing and angiogenesis. We investigated the potential application of a medical-grade gaseous form of NO (gNO) as a novel antibacterial agent in wound infection. Using a continuous horizontal-flow delivery system, the antibacterial activity of gNO was tested in vitro against a range of pathogens, including clinical isolates of *Staphylococcus aureus,* methicillin-resistant *S. aureus*, *Escherichia coli*, Group B *Streptococcus*, *Pseudomonas aeruginosa*, and *Candida albicans*. To probe the effect of topical application of gNO on the human skin, the proliferation and extracellular matrix gene expression of human dermal fibroblasts in culture were also analyzed by ^{3}H-thymidine incorporation assay and Northern blot techniques, respectively. Potent bacteriocidal activity was observed at 200 ppm gNO with an average of 4.1 ± 1.1 h to completely stop bacterial growth. Interestingly, this dose of gNO did not show any cytotoxic effect in human dermal fibroblasts in culture exposed for up to 48 h. Analysis of gene transcription in fibroblasts revealed a significant increase in MMP-1 mRNA expression as early as 2 h post-exposure to gNO. Although to a lesser degree, a significant reduction in type I procollagen was also observed in the same fibroblasts. The results of this study suggest that exogenous gaseous NO has potent significant antibacterial properties that can be beneficial in reducing bacterial burden in infected wound in burn injuries or non-healing ulcers.
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One of the major factors affecting non-healing wounds and burn injuries is the presence and persistence of bacterial burden, which can interfere with the normal process of healing [1]. Annually, over five million people in the United States alone suffer from chronic leg ulceration or burn injuries [2,3]. Conventional antibacterial treatments for wound infection are becoming less effective, partly due to the emergence of antibiotic-resistant strains. In addition, the relatively avascular nature of these wounds, as well as the presence of a complex network of bacterial biofilm, further impairs the effect of systemically administered antibiotics [4,5].

Recent evidence suggests that nitric oxide (NO) plays an important role in infection, vasodilation, angiogenesis, and modulation of wound healing [6–12]. NO is a short-lived free radical produced enzymatically by nitric oxide synthase (NOS) in the body. NOS converts the precursor amino acid L-arginine to NO and L-citrulline with the help of co-factors such as tetrahydrobiopterin, flavine mononucleotide (FMN), flavine adenine dinucleotide (FAD), nicotinamide–adenine–dinucleotide phosphate (NADPH), and an oxygen molecule [13,14]. NO is synthesized and released by various cells resident in the skin such as melanocytes, adipocytes, endothelial cells, macrophages, neutrophiles, fibroblasts, and keratinocytes [15–21]. NO, a hydrophobic molecule with a stoke radius of about 3–4 Å, can readily diffuse across most biological membranes [22].

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The possible role of NO as a non-specific defense mechanism against pathogen invasion was first raised in the early 1990s. Since then, it has become evident that this small molecule exerts its antimicrobial effects on a wide range of microorganisms such as bacteria, viruses, fungi, and yeast [8,10]. Infection in humans is often associated with a significant increase in NO production, which provided early evidence for the antimicrobial role of NO [23]. Production of NO has also been directly correlated with the host's ability to suppress microbial proliferation and contain the infection [24]. It was later demonstrated that mice lacking the inducible form of NOS were more susceptible to infection than were their wild-type counterparts [25]. Recently, through topical application of an acidified nitrite, Weller and colleagues [6] achieved some success in demonstrating the antimicrobial ability of NO against common cutaneous pathogens.

The indirect modulatory role of NO in wound healing was implicated well before its discovery as a signaling molecule in the body. The beneficial effect of arginine supplement, the only substrate in NOS enzymatic production of NO, in a rat incisional wound was studied over 25 years ago [26]. The ability of arginine to improve wound healing was later linked to NOS activity in an iNOS-deficient mice model, where the loss of NOS function abrogated the positive effect of arginine [27]. In a similar animal model, iNOS knockout mice showed significant delay in the closure of full thickness excisional wounds when compared with a control group of wild-type mice. More important, this delay was reversed by adenoviral-mediated expression of human iNOS cDNA at the wound site of the knockout mice [28].

The antibacterial, angiogenic, and regulatory role of NO in wound healing and infection has led investigators to study the potential of topical NO therapy in enhancing wound healing and reducing wound bacterial burden. A variety of NO donors, such as polyethyleneimine cellulose NONOate polymer, *S*-nitroso-*N*-acetylpenicillamine (SNAP), sodium nitroprusside (SNP), and molsidomine (*N*-ethoxycarbomyl-3-morpholinyl-sidnonimine), have been studied in vivo and as therapeutic agents for various clinical conditions. Topical application of NO donors has proven to be relatively successful in controlling infection and partially reversing impaired healing [29–32]. In aqueous media, the release of NO by donors is dependent on factors such as temperature, pH, and the nature of the carrier molecule. This variation can be somewhat problematic in the application of donors as a source of exogenous NO in wound healing [33]. It is also difficult to predict the cytotoxicity of the end products at the wound or infection site after the release of NO [34]. To overcome some of the difficulties in the prolonged topical application of NO donors, we investigated the potential application of air dilutions of medical-grade gaseous form of pure NO (800 ppm) in nitrogen as a source of exogenous therapeutic NO. Utilizing pure exogenous NO gas (gNO) can potentially overcome some of the limitations in a NO donor system, such as prolonged and consistent delivery of NO, the dose control, and

toxicity of the carrier molecules. The safety and efficacy of utilizing low doses of this gas were recently reiterated by the American Food and Drug Administration, which approved its use as an inhaled vasodilatory agent for treating pulmonary hypertension [35]. The goal of this study was therefore, to test the antimicrobial properties of gNO against a wide range of pathogens common in skin infection and to assess the effects of gNO on the proliferation of the human dermal fibroblasts and their capacity to express the key ECM such as collagenase (MMP-1) and collagen type I in an in vitro system.

Methods and materials

Nitric oxide exposure device

A continuous horizontal-flow delivery device was designed specifically for this study, to provide an ideal growth environment for both bacteria and human cell cultures while exposing them to various concentrations of nitric oxide gas. The application and efficacy of the in-house designed exposure chamber has been described in detail elsewhere [36]. 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 system enclosed an electrical heater unit controlled by an internal thermostat (Invensys Appliances Control, Carol Stream, IL), which provided stable temperatures inside the chamber. Independent lines from each of the two exposure chambers provided samples of the gas mixtures to a nitric oxide/nitrogen dioxide/oxygen electrochemical analyzer (AeroNOx, Pulmonox Medical, Tofield, AB, Canada) to detect the exact composition of the various gases in the mixture. With overall dimensions of $84 \times 66 \times 44$ cm, the apparatus was small enough to be kept on a countertop or inside a biological cabinet.

Gases were supplied from pressurized cylinders at a constant pressure of 50 psi. These included 800 ppm medical-grade NO, balance nitrogen (ViaNOx-H, Pulmonox Medical), medical air, oxygen and carbon dioxide (Praxair, Mississauga, ON, Canada). Gases were then mixed together at pre-calculated concentrations using a dilution manifold and a digital TSI mass flow meter (TSI, Shoreview, MN, USA). The gas mixture was delivered to the exposure chamber at 2.0 liters per minute (L/min) through two independent humidifiers (MR850, Fisher & Paykel Healthcare, CA, USA) set at 90% relative humidity (RH%). A vacuum pump at the exhaust port created negative pressure throughout the system to prevent possible back flow of gas and contaminants. Finally, the gas mixture was exhausted through a double-layered HEPA filter (Sentry Air Systems, Houston, USA) and vented to a Class I Biosafety Cabinet to avoid any possible contamination.

The NO level in the exposed media was confirmed by measuring its stable end products nitrite (NO_2^-) and nitrate

(NO_3^-) at various exposure times with the previously described Griess reaction and spectrophotometry (Helios Gamma & Delta, Unicam UV–Visible Spectroscopy, Cambridge, UK) [37].

Bacterial culture preparation

Culture of clinical isolates of *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli*, Group B *Streptococcus* (GBS), *Pseudomonas aeruginosa*, and *Candida albicans* were supplied by the Medical Microbiology Laboratory at Vancouver General Hospital and used in all studies. For clinical isolates, samples were obtained from the multidisciplinary intensive care unit (ICU) of the Vancouver General Hospital after institutional review board approval and informed consent from the patient.

On the day of the experiment, 3–5 isolated colonies from the freshly cultured tryptic soy agar (TSA) plates were selected and aseptically transferred to 5 ml of tryptic soy broth medium (TSB; Dalynn Biologicals, Calgary, AB, Canada). The bacterial suspension was adjusted to approximately 10^8 colony forming units per ml (cfu/ml) by visual comparison with the appropriate McFarland standard (# 0.5). Colony counts were performed as a control as per standard laboratory protocol. Inoculums were placed in a conventional incubator (UltraTech WJ301D, Baxter, USA) for 20 min to acclimatize. A number of preliminary serial dilution studies were performed to determine the optimal dilution to achieve approximately 30–200 colonies per agar plate. The inoculated TSA plates were then transferred and placed in an inverted position inside the exposure chamber (37 °C) with four identical plates in both the gNO and control group. Following the indicated incubation period, a visual count of colony forming units (cfu) was obtained. Plates were then transferred to a conventional incubator and grown for an additional 24 h to ascertain any further difference in colony size and number.

To further study the antibacterial effect of gNO in the liquid phase, a 0.5 McFarland standard with 10^8 cfu/ml was prepared and further diluted 1:1000 with sterile saline to 10^5 cfu/ml to a volume of 20 ml. The concentration of 10^5 cfu was chosen, as that is an accepted threshold for determining infection [38]. Three milliliter aliquots were then pipetted into each well of a six-well cell culture plate (Corning 3516, Corning, NY) at a surface area to volume ratio of 3.2 cm²/ml. Triplicate wells were prepared for each bacteria, for both the treatment and control. Two sets of organisms per six-well plate were prepared.

When the temperature and gNO concentration reached a steady state in the chamber, the control plates were placed in the air chamber and the treatment plates were placed in the gNO chamber. To measure the effect of gNO, each plate was sampled at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, and 24 h. Samples were taken in three volumes (0.1, 0.01, and 0.001 ml) and were plated onto blood agar plates (Columbia agar with 5% sheep's blood, PML Microbiologicals, Willsonville, OR). The blood agar plates were then placed in a conventional incubator (35 °C). After 24 h, the blood agar plates were removed from the incubator and the colony forming units were visually counted by a microbiology technician blinded to the experiment.

Dermal fibroblast cell culture

Following informed consent, skin punch biopsies were obtained from patients undergoing elective reconstructive surgery, under local anaesthesia, according to a protocol approved by the University of Alberta Hospitals Human Ethics Committee and described previously in detail by Ghahary et al. [39]. In brief, the tissue was collected in sterile Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FBS) (GIBCO, Grand Island, NY), minced into small pieces of less than 0.5 mm in any dimension, washed with sterile medium six times, and distributed into 60×15 mm Petri culture dishes (Corning, Corning, NY), four pieces per dish. A sterile glass cover-slip was attached to the dish with a drop of sterile silicone grease to immobilize the tissue fragment. DMEM + Ab (penicillin G sodium 100 U/ml, streptomycin sulfate 100 µg/ml, and amphotericin B 0.25 µg/ml) (3 ml) with 10% FBS was added to each dish and incubated at 37 °C in a water-jacket humidified incubator in an atmosphere of 5% CO_2. The medium was replaced twice weekly. After 4 weeks of incubation, cells were released from dishes by brief (5 min) treatment with 0.1% trypsin (Life technologies, Gaithersburg, MD) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (pH 7.4) and transferred to 75 cm² culture flasks (Corning, Corning, NY) for incubation. Upon reaching confluency, the cells were released by trypsinization, split for subculture at a ratio of 1:5, and reseeded into 25-cm² flasks for exposure. Prior to each exposure, the old media (DMEM + 10% FBS) was removed, the cell monolayer was washed with PBS, and fresh DMEM medium with only 2% FBS was added to the cells. Four flasks were prepared for both the control and treated group. Fibroblasts from passages 3–6 were used for this study.

Nitric oxide exposure

Prior to treating cells with gNO, growth conditions and the viability of bacteria and human fibroblasts were established in a gNO exposure chamber [36]. A conventional CO_2 incubator (Forma Scientific, Marietta, OH, USA) was used as a standard for validating the conditions in our custom-designed NO chamber. Cells were exposed to various doses of NO (diluted with medical air—21% O_2) at a 2.0 L/min flow rate inside the gNO chamber for up to 48 h. The control group was exposed only to sterile medical air at the same flow rate. In dermal fibroblasts studies, 5% CO_2 was also added to the gas mixture for both the control and treatment groups.

Northern blot analysis

Fibroblasts were released from the culture plate by trypsinization and pelleted by centrifugation at 1100 rpm for

10 min. Pellets were then lysed with 500 μl of 4 M guanidium isothiocyanate (GITC) solution and the total RNA from each group was isolated by the guanidium isothiocyanante/CsCl procedure of Chirgwin et al. [40], using phenol:chloroform (1:1), followed by chloroform:isoamyl alcohol (49:1). Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 μg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and blotted onto nitrocellulose paper. To control the loading, quantities of 18S ribosomal RNA were compared visually by ethidium bromide fluorescence. The blots were baked for 2 h at 80 °C under vacuum and prehybridized for 4 h at 45 °C in a prehybridization solution. Hybridization was performed at 45 °C in the same solution, using cDNA probes for human MMP-1 or type I procollagen. The cDNA probes were labeled with P-α^{32}-dCTP by nick translation. Filters were initially washed at room temperature with $2x$ sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1 h and finally washed for 20 min at 65 °C in $0.1x$ sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at −80 °C, in presence of an intensifying screen. Quantitative analysis of the autoradiograph was accomplished by densitometry.

^{3}H-thymidine proliferation assay

To measure the rate of fibroblast proliferation in response to exposure to 20, 200, and 400 ppm gNO, a ^{3}H-thymidine proliferation assay was carried out on the cultured fibroblasts. In brief, ^{3}H-thymidine (Perkin–Elmer Life Sciences, Boston, MA) was added to the conditioned medium of each sample, following gNO exposure for 24 and 48 h, for a final concentration of 2.0 μCi/ml, and then incubated for 16 h in a conventional incubator. Fibroblasts were then harvested, washed three times with PBS, dissolved in GITC, and added to the scintillation fluid (Amersham, Oakville, ON, Canada). Radiation counts were performed using a scintillation counter (Beckman, Fullerton, CA, USA) and measured in counts per min (cpm).

Statistical analysis

The results were analyzed using the unpaired Student's t-test for comparison between any two groups. Unless otherwise specified, $p < 0.05$ indicated statistical significance. Results were represented by mean ± standard deviation from at least three independent studies.

Results

Nitric oxide uptake in culture media

The continuous horizontal-flow of gNO over the culture media provided controlled NO uptake over a prolonged period, as shown in Fig. 1. The NO transfer profile was
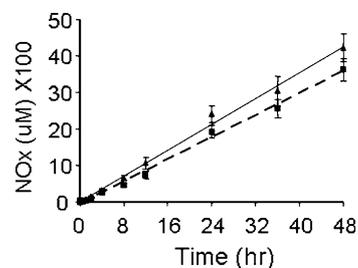

Fig. 1. Transfer profile of NO in culture media. The uptake of 200 ppm gNO into a cell-free media was estimated by cumulative NO_2/NO_3 concentration (μM × 100) in each sample, measured by Griess reagent. The uptake rate of gNO in DMEM (–▲–) and Saline solution (–■–) was calculated by the slopes of the best-fit linear regression lines in 48 h. Data points are presented as mean ± standard deviation, from three independent experiments.

obtained by continuous exposure of DMEM and Saline solution to 200 ppm of gNO at 37 °C and a pH range of 7.2 ± 0.1 to 7.5 ± 0.25 for 48 h. Samples were taken at various times and analyzed for cumulative NO_x (NO_2 and NO_3) levels by Griess reagent assay. No significant increase in NO_x level of the control group (exposed to air only) was observed. The volume-adjusted NO uptake rate was 226.2 ± 6.0 and 260.0 ± 7.0 nmol/h for saline and DMEM media, respectively. The rates were calculated from the slopes of the best-fit linear regression lines. The deviation from the best-fit is represented by the standard error of the slopes. The coefficient of determination (R^2) for both slopes was 0.998, indicating the linearity of the data points and consequently consistent uptake of gNO by the culture media over our exposure period.

Antibacterial effect of gNO

In a dose-response study on _S. aureus_ and _P. aeruginosa_, NO exhibited potent antimicrobial properties at concentrations above 160 ppm. Twenty-four hour continuous exposure to 160 ppm gNO reduced bacterial growth by 90%. At lower concentrations, partial bactericidal or bacteriostatic activity was observed, which seems to be dose-dependent (Fig. 2). Based on these preliminary findings, 200 ppm gNO was selected as the minimum dose of gNO for the remainder of the bactericidal studies. _S. aureus_ (ATCC), _S. aureus_
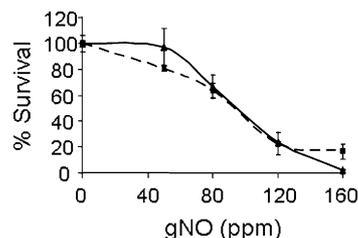

Fig. 2. Dose response effect of gNO on bacterial survival on solid media. Percent survival relative to the control of _S. aureus_ (–▲–) and _P. aeruginosa_ (–■–) following continuous exposure to various doses of gNO for 24 hr. Bacterial survival at each time point is presented as a percentage relative to the control group (air alone). Data are shown as mean ± standard deviation ($n = 3$).

(clinical), MRSA, GBS, *C. albicans*, *E. coli*, and a multi-drug resistant *P. aeruginosa* from a cystic fibrosis patient, were suspended in saline at a concentration of 10^5 cfu/ml and exposed continuously to 203 ($\pm$10.5) ppm gNO at a temperature of 37 °C ($\pm$1.2). A duplicate set of plates exposed to medical air alone, acted as the control group. In all experiments, the bacteria concentration of the controls remained the same or increased slightly when exposed to medical air only. As shown in Fig. 3, all the bacteria exhibited a 4- to 6-log reduction in concentration, indicating that 200 ppm gNO had a potent bacteriocidal effect. The survival curve for each organism was analyzed to determine the 100% lethal dose of gNO (LD_{100}). LD_{100} was defined as the time at which no further growth was observed. At 200 ppm gNO, the average exposure time required to achieve 100% killing was 4.1 ± 1.1 h. With the exception of *GBS*, there was no significant difference between organisms with regard to the LD_{100}. These experiments demonstrated that continuous exposure to 200 ppm gNO for a short period of time resulted in microbial killing. The bactericidal effect had a 1–4 h latency period, followed by rapid cell death.

Antibacterial effect of NO₂

In a horizontal-flow delivery system, gNO can react with oxygen to form nitrogen dioxide (NO_2). High concentrations of NO_2 have been reported to have toxic effects on host cells [41]. NO_2 levels, continuously monitored in the gas mixture during gNO exposure, did not exceed 15 ppm at a total gas flow rate of 2.0 L/min. To ensure that the bacteriocidal effect observed in these studies was due to gNO and not NO_2, bacteria were exposed continuously to 20 ppm NO_2, in the absence of gNO, for 8 h, and bacterial survival was monitored as previously described in materials and methods. As shown in Fig. 4, exposure to 20 ppm NO_2 did not have any bacteriostatic effect on *S. aureus* (A) or *P. aeruginosa* (B), whereas the bacteria exposed to gNO, reached LD_{100} in under 5 h. This demonstrates that the bacteriocidal effect observed using 200 ppm gNO was not due to concomitant delivery of NO_2.

Effect of gNO on fibroblast proliferation

The effect of gNO exposure in a continuous-flow system on the growth of human dermal fibroblasts was investigated by ^{3}H-thymidine incorporation assay. Exposure of fibroblasts to 20 and 200 ppm gNO for 24 and 48 h did not affect cell growth, as shown in Fig. 5. The proliferation rate, directly proportional to the number of radioactive thymidine nucleotides incorporated into the newly synthesized DNA in the cells, is represented as a percent count per minute relative to the control (exposed to air alone). No significant difference was observed between 24 and 48 h exposures, indicating that fibroblasts can safely be exposed
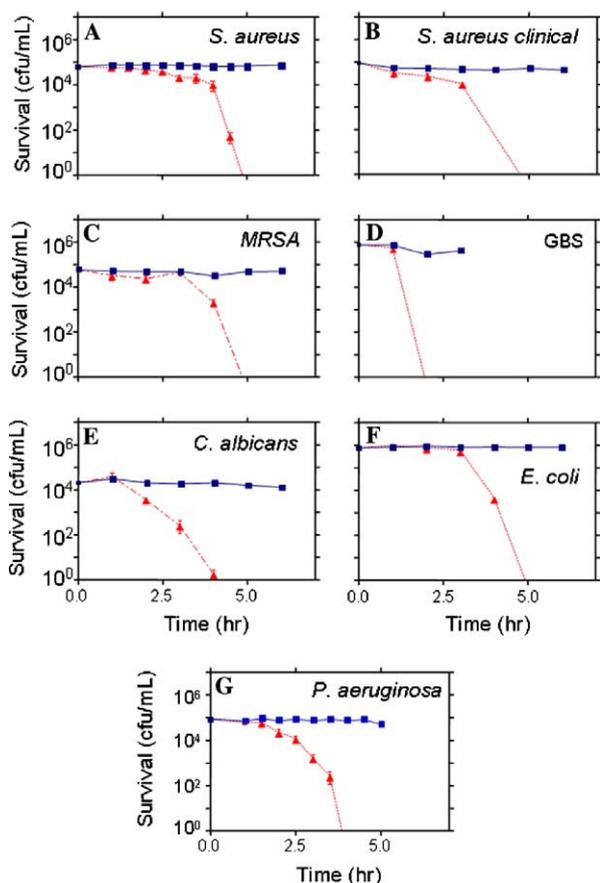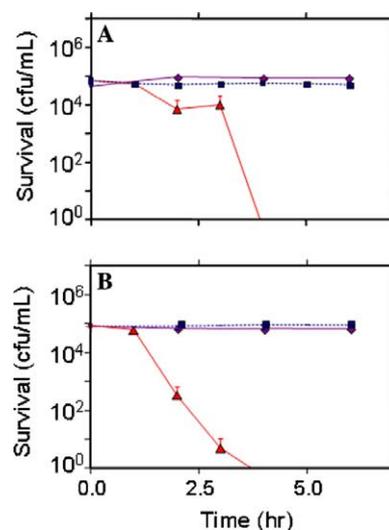

Fig. 3. Antimicrobial effect of 200 ppm gNO. Survival curves following continuous exposure to 200 ppm gNO (—▲—) or medical air alone (—■—) for a representative group of microorganisms. (A–G), respectively, represent survival curves for *S. aureus* (ATCC), *S. aureus* (clinical), MRSA, GBS, *C. albicans*, *E.coli*, and *P. aeruginosa* (ATCC) in a liquid media of 0.9% saline. Data points represent the mean ± standard deviation of at least three independent measurements.

Fig. 4. Antibacterial effect from NO_2 exposure. Survival curve for *S. aureus* (A) and *P. aeruginosa* (B) exposed to 20 ppm NO_2 (—◆—) or air alone (–■–). Results are superimposed over survival curve for bacteria exposed to 200 ppm gNO (—▲—). Data are presented as mean ± standard deviation ($n = 3$).
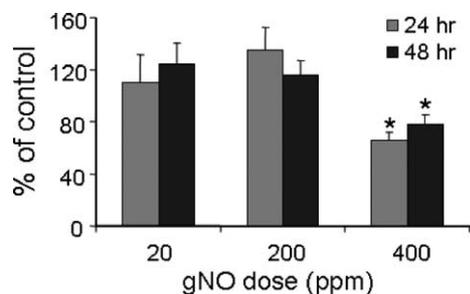
Fig. 5. Effect of gNO on human dermal fibroblast proliferation. Fibroblasts in culture flasks were continuously exposed to 20, 200, and 400 ppm of gNO or medical air (control) in the presence of 5% CO_2 for 24 and 48 h. After this period, ^{3}H-thymidine incorporation into the cellular DNA was determined for each group. The data for proliferation rate is expressed as a percentage of the control cells activity. Data represent the mean ± standard deviation for three separate experiments. The asterisk ($^*p < 0.001$) denotes a significant difference in proliferation of fibroblasts exposed to 400 ppm gNO, compared to those at 20 and 200 ppm for both 24 and 48 h exposures.

to 200 ppm gNO for up to at least 48 h. to determine the safe range of gNO for cells, fibroblast proliferation was investigated in the presence of 400 ppm gNO. In contrast to the data shown for 20 and 200 ppm, both 24 and 48 h exposures to 400 ppm gNO led to a significant reduction in the proliferation rate of fibroblasts.

Effect of gNO on extracellular matrix

To examine the effect of gNO on ECM expression in dermal fibroblasts, we analyzed the expression of MMP-1 and type I procollagen mRNA in the presence of 200 ppm gNO. Following continuous exposure to 200 ppm gNO, Northern blot analysis of the total RNA extracted 24 and 48 h after exposure was performed. Total RNA from the

dermal fibroblasts exposed to air plus 5% CO_2 inside the same chamber was used as the control. As shown in Fig. 6A, MMP-1 mRNA expression was significantly increased, relative to the control, in fibroblasts continuously exposed for either 24 or 48 h. In contrast, expression of type I procollagen mRNA was significantly reduced in response to same dose of gNO. Densitometry analysis was performed for MMP-1 and type I procollagen in order to adjust mRNA expression relative to the ribosomal 18S mRNA expression. Results of three separate experiments were combined in Figs. 6B and C for MMP-1 and collagen mRNA, respectively. Loading-adjusted mRNA expressions also demonstrated the same significant increase in MMP-1 as well as the reduction in procollagen post-24 and 48 h exposure to 200 ppm gNO ($p < 0.01$, $n = 3$).

To determine the minimum exposure time required to induce MMP-1 or collagen gene transcription, a time dependent experiment was carried out by exposing the same strains of dermal fibroblasts to 200 ppm of gNO continuously for 1, 2, 4, and 8 h. As shown in Fig. 7A, a marked increase in MMP-1 mRNA expression was observed as early as 2 h following gNO treatment. The reduction in procollagen mRNA expression showed a 2 h delay compared to the MMP-1 response. No further decrease in procollagen mRNA expression was observed by further exposure (up to 8 h) to gNO. Densitometry results of three separate experiments were combined and are presented in Figs. 7B and C, for MMP-1 and collagen, respectively.

Discussion

The present study investigated the effectiveness of the direct delivery of gaseous NO (diluted with air) as a potential topical antimicrobial agent. The delivery system was
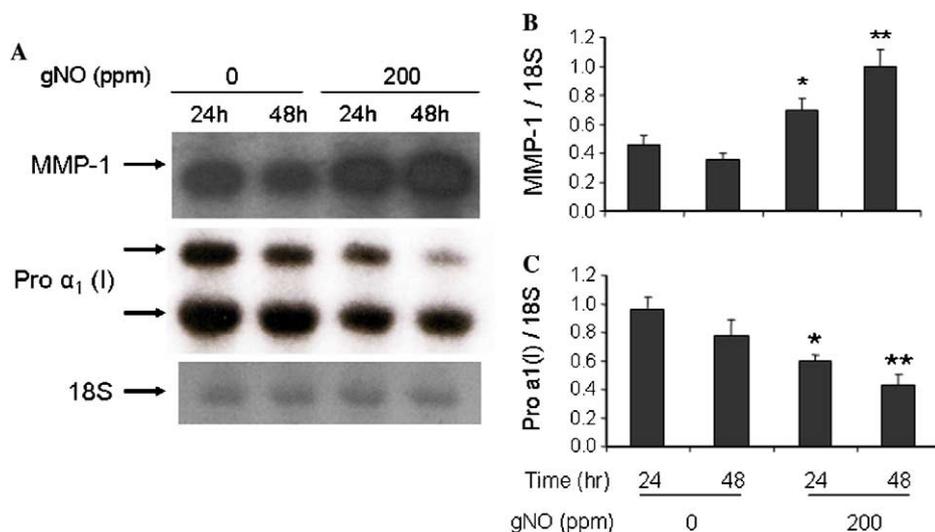

Fig. 6. Effect of gNO on MMP-1 and type I procollagen gene expression in human dermal fibroblasts in vitro. Cells were treated with either 0 (control) or 200 ppm of gNO for periods of 24 and 48 h. Following this period, total RNA was extracted and subjected to Northern blot analysis. (A) The representative pattern of MMP-1 and procollagen-1 mRNA expression is shown. Expression of 18S rRNA was used as the loading control. (B) and (C) are the combined densitometry results of three separate experiments showing the ratio of MMP-1 and procollagen-1 mRNA/18S rRNA expression, respectively. For analysis, the densitometric values for the strongest intensity signals were set at 1.0 and other groups were calculated relative to this ($^*p < 0.05$ versus 24 h control, $^{**}p < 0.01$ versus 48 h control in both (B) and (C)).
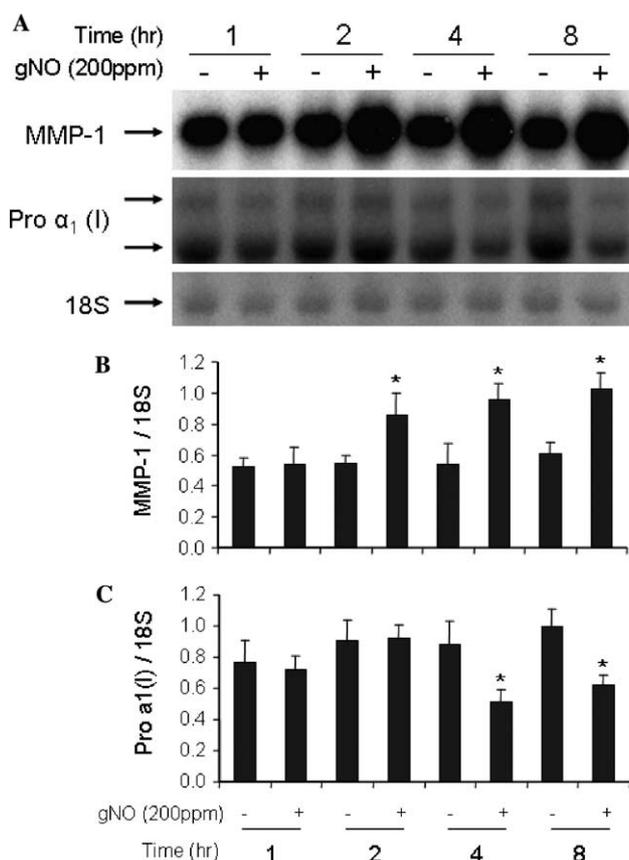
Fig. 7. Time-dependent expression of ECM in human dermal fibroblasts exposed to 200 ppm gNO. Following exposure to either medical air (control) or 200 ppm gNO for 1, 2, 4, and 8 h, expression of MMP-1 and pro-collagen-1 mRNA was evaluated by Northern blot analysis performed on the extracted total RNA. (A) Depicts the representative Northern blots for each gene with 18S rRNA used as a loading control. (B) and (C) are the combined densitometry results of three separate experiments showing the ratio of MMP-1 mRNA/18S rRNA and procollagen-1 mRNA/18S rRNA, respectively. For analysis, the densitometric values for the strongest intensity signals were set at 1.0 and other groups were calculated relative to this. The asterisk (*$p < 0.05$) denotes a significant difference in mRNA expression in cells treated with 200 ppm gNO compared to the corresponding controls.

capable of circulating sufficient gNO at a consistent rate even for long exposures. The dose response study against *S. aureus* and *P. aeruginosa* demonstrated over 90% kill at about 160 ppm gNO. Based on these findings, a dose of 200 ppm was selected to further explore the antimicrobial effects of gNO. Exposure to 200 ppm gNO produced a significant bacteriocidal effect on a wide range of bacteria, including antibiotic resistant and opportunistic pathogens. The presence of NO_2 in the gas mixture did not contribute to this bacteriocidal effect, as exposure to 20 ppm pure NO_2 had no apparent effect on bacterial growth compared to the control (air). The results from direct delivery of gNO, without utilizing a donor or carrier molecule, support previous reports on the bacteriocidal or bacteriostatic action of NO against *S. aureus* [6], *P. aeruginosa* [42], *GBS* [43], *E.coli* [44], and *C. albicans* [45]. Although chemically a very simple diatomic molecule, NO exhibits a broad range of functional activities. The high-output NO pathway in macrophages,

through upregulation of inducible nitric oxide synthase (iNOS), has evolved as a non-specific defense mechanism to protect the host from foreign pathogens [17]. The mechanism behind this bacteriocidal action is thought to involve, in part, the interaction of NO with oxygen, resulting in the generation of reactive oxygen intermediates with antimicrobial properties. Several lines of evidence indicate that the antimicrobial action of NO could also be attributed to deamination of DNA or inhibition of respiratory chain metalloenzymes [23]. However, the exact mechanism of how NO exerts this microbicidal effect is still not fully understood.

Although the host cells are not immune to high concentrations of NO, it appears that the majority of cells have evolved detoxification mechanisms against NO cytotoxicity. It has been proposed that the evolutionarily conserved *S*-nitrosoglutathione (GSNO) reductase pathway is partly responsible for tolerance against nitrosative damage [46]. This detoxification pathway could explain the presence of a latent period when no bactericidal effect is observed during gNO exposure. With continuous delivery of gNO, all bacterial protection pathways become saturated leading to a rapid cell death observed for all bacterial strains tested here. The steep killing curve is consistent with the lethal action of unrestricted levels of reactive nitrogen intermediates in the cytosol [10]. Interestingly, this study detected a significantly shorter latent period for GBS compared to other organisms exposed to gNO. This suggests that GBS has a less active protection mechanism against gNO cytotoxicity. Although the exact mechanisms are not known to us at this time, it is possible that the GSNO pathway is under-developed in GBS leading to rapid breakdown of cellular respiratory and replicating machinery [47].

Fibroblasts are the most common cells in the skin dermis and their proliferation, migration and extracellular matrix deposition is critical during process of cutaneous wound repair. The present study provides evidence that eukaryotic cells can cope with exposure to 200 ppm gNO for up to six-fold longer than bacteria since no deleterious effects were observed in human skin fibroblasts exposed to gNO for 48 h. Witte et al. [48] and Masters et al. [49] have also reported a lack of cytotoxicity to low levels of NO in dermal fibroblasts [48,49]. However, cytotoxic effects of NO appear to be dose dependent, with 400 ppm gNO significantly reducing fibroblast proliferation. In contrast to the results reported in a number of previous findings, exposure to low doses of gNO had no stimulatory effect on cell proliferation in our study [50–52]. Nevertheless, our results demonstrated the lack of cytotoxicity in the dermal fibroblasts at a dose which lead to a significant bacteriocidal effect in all of the pathogens tested here.

A tight balance between synthesis and degradation of ECM is essential for the integrity of dermal architecture as well as the wound healing process. Dermal fibroblasts have a critical role in ECM expression and production during the granulation and remodeling phases of wound healing [53]. To investigate the functional integrity of fibroblasts at

the transcription level, we looked into the gene expression of MMP-1 and procollagen type I following exposure to 200 ppm gNO. Several studies have shown that NO can regulate ECM gene expression in various cell types, including dermal fibroblasts [48,54–58]. In our experiment, exposing dermal fibroblasts to 200 ppm gNO led to a significant increase in MMP-1 mRNA expression as early as 2 h post-treatment. Since collagen metabolism is tightly regulated through the activity of MMPs and tissue inhibitors of metalloproteinases (TIMP) [48], we expected to observe an inhibitory effect on collagen mRNA expression. Exposing human dermal fibroblasts in culture to gNO did not increase collagen gene expression as in earlier studies on the effect of NO donors on wound healing [30,48,49,55,57]. Other investigators have also reported this inconsistency. In one of these studies, topical application of an NO donor (SNP) in a rat full thickness wound model significantly decreased wound collagen content [56]. Similarly, Kolpakov et al. [58] observed a dose-dependant inhibition of collagen synthesis in rabbit aortic smooth muscle cells, when treated with SNAP and SNP (NO donors). The stimulatory effect of NO in matrix metalloproteinase activity has been demonstrated in a rat incisional wound model as well as in human cervical fibroblasts in vitro [30,54]. It is possible that the effect of NO on ECM expression is cell-type and/or dose specific. Witte et al. have reported that the stimulatory effect of NO observed on collagen synthesis at low concentrations was abrogated at higher doses [48]. It is interesting to note that NOS expression and NO production are significantly reduced in hypertrophic scarring (fibrosis of the dermis) [59], and the prolonged blockage of NO synthesis leads to the development of renal, cardiac, and liver fibrosis [60]. Since the majority of fibrotic disorders, such as hypertrophic scarring post burn injuries, are characterized by excessive accumulation, disorganization, and crosslinkage of collagen fibers, it is possible that the induction of MMP-1 and MMP-2 gene expression plays a role in the remodeling of wound or post-burn scar.

In summary, consistent and controllable delivery of gNO against a wide range of pathogens, including antibiotic-resistant strains, demonstrated potent bacteriocidal activity without any apparent side effects on human dermal fibroblast proliferation or ECM expression. Therefore, the development of a delivery system for gNO may prove valuable in the control of skin infections in burn injuries and chronic ulcers, both of which commonly show resistance to conventional therapies. Further studies on the effects of gNO on more sensitive cells in the skin, such as basal keratinocytes or endothelial cells, as well as effect on collagen production in a three dimensional fibroblast culture model are under our investigation.

Acknowledgments

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