# Efficacy of gaseous nitric oxide in the treatment of skin and soft tissue infections

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## ABSTRACT

Bacterial burden significantly interferes with the healing process in chronic ulcers. Nitric oxide (NO) plays a key role in regulating skin's response to infection and wound healing. In previous studies, we demonstrated that exogenous NO gas (gNO) at 200 parts per million (ppm) exhibits potent antimicrobial effects against a representative range of pathogens. The aim of the present study is to explore the antimicrobial properties of gNO *in vivo* and to determine skin cells' sensitivity to the cytotoxic effects of gNO. To test gNO's antimicrobial effects, full-thickness wounds were infected with *Staphylococcus aureus* on the dorsal skin surface of New Zealand White rabbit and treated with 200 ppm gNO for 8 hours/day for 3 consecutive days. Significant reduction in wound bacterial content was observed in the presence of gNO. In a separate experiment, primary cultures of human fibroblasts, keratinocytes, and endothelial cells were established to test gNO's cytotoxicity in the skin. Methyl thiazolyl tetrazolium proliferation assays demonstrated that human skin cells, unlike bacterial cells, exhibited significant resistance toward gNO cytotoxicity. *In vitro* migration studies on keratinocytes and endothelial cells revealed that gNO treatment does not seem to interfere with re-epithelialization and angiogenesis during the process of wound healing. Following 24 hours of gNO treatment, fibroblasts expressed significantly higher levels of procollagen and, to a lesser degree, a decrease in matrix metallo-proteinase -1 mRNA. In conclusion, the present study provides evidence for the potential application of high doses of gNO as an antimicrobial agent for the treatment of infection in chronic nonhealing ulcers or burn patients, without compromising the viability, and function of skin cells.

It is well known that wound bacterial burden significantly interferes with the normal process of healing in chronic cutaneous ulcers.[1] The emergence of antibiotic-resistant strains, the relatively avascular nature of nonhealing wounds, and the presence of a complex network of bacterial biofilm, hinder the effect of systemically administrated conventional antibiotics.[2,3] Therefore, research and development of novel antimicrobial agents have become extremely crucial in the fight against chronic wound infection.

The potential role of nitric oxide (NO) in nonspecific host defense mechanisms was first brought to light in the early 1990s.[4] In the past decade, it has become increasingly evident that this small gaseous molecule possesses antimicrobial effects on a wide range of microorganisms including bacteria, viruses, fungi, and yeast.[5–7]

NO is expressed and released by all major cell types in the skin such as keratinocytes, fibroblasts, endothelial cells, and melanocytes and plays a significant role in the skin's response to infection and injury.[8–10] In recent years, several investigators have studied the potential therapeutic role of topical NO donors in controlling skin infection and promoting repair in chronic nonhealing wounds.[7,11–13] To overcome some of the difficulties with predicting the parameters of NO release and the side effects of residual donor complexes, we recently investigated the application of exogenous medical-grade gaseous NO (gNO) as a potential topical antimicrobial agent in cutaneous infections. Pure gNO can easily be administered to cells and tissues without the need for a carrier vehicle, a time-release system, and optimizing reaction conditions such as pH and temperature. Our previous studies have demonstrated significant bactericidal action at 200 parts per million (ppm) gNO against a representative range of pathogens common in skin infection.[14–17]

At higher concentrations, it has been shown that NO can act as a cytostatic and cytotoxic effector molecule in various cells and tissues[18–20] and can also promote cell apoptosis.[21,22] Mammalian cells vary greatly, however, in their sensitivity toward the cytotoxic effects of NO. For example, human keratinocytes and fibroblasts demonstrate significantly higher resistance toward NO cytotoxicity in comparison with islet or neuron cells.[22]

We have previously demonstrated the potent cytotoxic effect of gNO against several skin pathogens in vitro.[15] However, an antimicrobial agent must demonstrate minimal side effect on the host cells, while retaining the ability to destroy the pathogens. Therefore, in the present study, we examined the cytotoxic effects of gNO on the viability, proliferation, migration, and differentiation of primary human fibroblasts, keratinocytes, and endothelial cells. Furthermore, in a pilot study, we investigated the

potential antimicrobial effects of gNO in an in vivo model of skin infection.

## METHODS AND MATERIALS

### NO exposure device

The design and validity of the continuous horizontal-flow gNO delivery device used in this study have been described in detail elsewhere.[14] 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, 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 NO/nitrogen dioxide/oxygen electrochemical analyzer (AeroNOx, Pulmonox Medical Inc., Tofield, AB, Canada) to detect the exact composition of the various gases in the mixture. Gases were supplied from pressurized cylinders at a constant pressure of 50 pounds per square inch (psi). These included 800 ppm medical-grade NO diluted in nitrogen (ViaNOx-H, Pulmonox Medical Inc.), medical air, oxygen, and carbon dioxide (Praxair, Mississauga, ON, Canada). These gases were then mixed together at predetermined concentrations using a dilution manifold and a digital mass flow meter (TSI Inc., Shoreview, MN). The gas mixture was delivered to the exposure chamber at 2.0 L /minute through two independent humidifiers (MR850, Fisher & Paykel Healthcare, Laguna Hills, CA) set at 90% relative humidity (RH%).

### Fibroblast, keratinocyte, and endothelial cell cultures

Rheinwald and Green's procedure[23] was utilized for cultivation of human foreskin keratinocytes using keratinocyte serum-free medium (KSFM) (Invitrogen, Burlington, ON, Canada) supplemented with bovine pituitary extract (BPE) and epidermal growth factor (EGF) at final concentrations of 50 and 5 μg/mL, respectively. Primary-cultured keratinocytes from passages 3–5 were used in this study. To establish fibroblast cultures, normal skin punch biopsies obtained from patients were established in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) as described previously.[24] Fibroblasts from passages 3–6 were used for this study. These cells were used for either monoculture or fibroblast-populated collagen gel (FPCG) experiments. Human umbilical vein endothelial cells (HUVECs) were isolated by collagenase digestion[25] and grown in a gelatin-coated flask in M199 medium (Invitrogen) supplemented with 20% FBS and endothelial cell growth supplement (ECGS) (VWRCanlab, Mississauga, ON, Canada) at a final concentration of 10 μg/mL.

### Cell proliferation assay

Colorimetric methyl thiazolyl tetrazolium (MTT) [3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay was used to evaluate the cytotoxic effects of gNO on proliferation. 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. Cells were plated at a density of $5 \times 10^4$/well in a 24-well plate (Corning, Lowell, MA). Cells were treated with 200 ppm gNO for 8 hours/day for 3 days. Control cultures were treated with medical air alone under identical conditions as the treated group. At the end of the treatment intervals, 100 μL of MTT solution (5 mg/mL; Sigma, Oakville, ON, Canada) in phosphate-buffered saline was added to the wells and incubated at 37 °C for 5 hours. After the MTT incubation, 500 μL of dimethyl sulfoxide (DMSO) was added to each well and incubated at 37 °C for 5 minutes. The resultant solution was read in a microplate reader at 570 nm. The optical density reflects the number of living cells present in the culture. Experiments were performed in triplicate and repeated twice.

### Fibroblasts populated in collagen gel (FPCG)

FPCG were prepared, using bovine type-I procollagen extracted in our laboratory by a procedure described by Volpin and Veis,[26] utilizing a modification by Bell et al.[27] Experiments were performed using six-well plates (Fisher, Nepean, ON, Canada) with a 35 mm diameter. Each well contained 350 μL 3×DMEM+Ab, 26 mL 0.4 N NaOH, and 440 μL cell suspension ($2 \times 10^6$ cells/mL) in 1× DMEM+10% FBS, 125 μL FBS (for a final concentration of 10%), and 870 μL of acid-extracted fetal bovine type-I procollagen (2.14 mg/mL). Each treatment group was prepared in triplicate and was immediately transferred to a humidified incubator at 37 °C in an atmosphere of 5% $CO_2$. To investigate the effects of gNO on fibroblast migration and their subsequent proliferation, a migration, and proliferation assay was performed.[28] In brief, following 8 hours/day treatment with 200 ppm gNO for 3 days, 6 mm punch biopsies were taken from treated and untreated FPCG. Punch biopsies were placed in triplicate onto six-well plates. Fibroblast migration from the collagen gel onto a plastic plate was monitored and photographed for 10 days. The cell population migrating out of the gel and their subsequent proliferation were measured in treated and untreated groups by removing the punch biopsies and performing an MTT proliferation assay, as described before.

### Keratinocyte migration

Primary human skin keratinocytes were seeded on 60 mm plates. After reaching 90% confluency, "wounds" were created by scratching a monolayer of cells with a sterile 1000 μL pipette tip. After washing away suspended cells, the media were changed with fresh KSFM. Cells were then exposed to 200 ppm gNO or medical air alone for 8 hours/day for 3 days. The progress of migration was photographed at the start or end of each 24-hour period post-wounding, with an inverted microscope equipped with a digital camera. Photographs were calibrated and distances were analyzed using microtome slide images at different magnifications in combination with a scientific image management system (Quartz PCI, Quartz Imaging Corp., Vancouver, BC, Canada).

## Matrigel tube formation assay

Twenty-four-well plates were coated with growth factor-reduced Matrigel (0.4 mL/well; Becton Dickinson Labware, Oakville, ON, Canada) and incubated at 37 °C for 30 minutes. HUVECs were treated with trypsin-ethylene diamine tetra acetic (EDTA) and suspended in the M199 medium. The cells were seeded at a density of $2 \times 10^4$ cells/well on polymerized Matrigel. The plates were then treated with 200 ppm gNO or medical air for 3, 8, or 24 hours. Photographs were taken under a phase contrast light microscope (Carl Zeiss, Toronto, ON, Canada) at each time point to monitor the tube formation of HUVECs.

## Northern blot analysis

Fibroblasts and keratinocytes were released from the culture plate by trypsinization and pelleted by centrifugation at 1,100 rpm for 10 minutes. Pellets were then lysed with 500 μL of 4 M guanidium isothiocyanate (GITC) solution, and the total ribonucleic acid (RNA) from each group was isolated using the GITC/CsCl procedure of Chirgwin et al.,[29] applying 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/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 hours at 80 °C under vacuum and prehybridized for 4 hours at 45 °C in a prehybridization solution. Hybridization was performed at 45 °C in the same solution, using cDNA probes for human matrix metalloproteinase-1 (MMP-1), type-I procollagen, involucrin, or tumor growth factor-β1 (TGF-β1). The cDNA probes were labeled with P-$\alpha^{32}$-dCTP by nick translation. Filters were initially washed at room temperature with 2× sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1 hour and finally washed for 20 minutes at 65 °C in 0.1× sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing a Kodak X-Omat film (Eastman Kodak Company, New Haven, CT) to nitrocellulose filters at −80 °C, in the presence of an intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry.

## In vivo wound infection model

Four New Zealand White adult rabbits, weighing 2–3 kg each, were utilized in this pilot study. All work conducted on these animals was consistent with the standards and protocols approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee. The rabbits were premedicated with buprenorphine (Buprenex™, Reckitt & Coleman, Richmond, VA) before each surgery. Surgical plane anesthesia was induced and maintained by facemask using Isoflurane™ (Biomeda-MTC, Cambridge, ON, Canada) and oxygen. The animals were shaved and the skin was examined to been sure that it was infection-free. The dorsal surface was prepared with 70% ethanol and 10% Betadine™ (Purdue Fredrick Inc., Toronto, ON, Canada) before manipulation. Eight full-thickness exci-sional wounds were made on each rabbit (four wounds on each side of the dorsal midline) using an 8 mm Accu-Punch™ disposable biopsy tool (Dormer Laboratories, Mississauga, ON, Canada). After bleeding had been controlled, each wound was inoculated with 100 μL of bacterial suspension containing $1.0 \times 10^6$ colony-forming units (cfu) of *Staphylococcus aureus*. The wounds were left untouched for 5–10 minutes to allow bacterial cells to settle down and adhere to the wound bed. The entire experimental area was then covered with a sterile nonadhering mesh gauze (Xeroform, Sherwood Medical, St. Louis, MO) and secured with self-adherent wrapping (Coban, 3 M, St. Paul, MN) for 24 hours. On day 1 postwounding, following visual examination and confirmation of the infection, the wound area was directly exposed to either 200 ppm gNO or medical air (control) continuously for 8 hours/day. The gas was securely delivered to the back of the animal by utilizing a specialized wound cover attached to a tether and swivel system. The tether and swivel system allowed the rabbits to be treated in their standard cages without impacting their ability to groom themselves or their easy access to food and water. The animals did not show any behavioral change or discomfort postoperation. Following 3 days of treatment, the animals were sacrificed and samples were collected from each wound, using a sterile 6 mm biopsy tool, and processed for histological hematoxylin & eosin (H&E) staining and wound bacterial counts. For bacterial counts, a serial dilution of full-thickness wound tissue homogenate was plated on tryptic soy agar (TSA) and incubated at 37 °C for 24 hours. The gNO and control groups were compared in terms of their cfu count/g of tissue at the appropriate serial dilutions. An arterial blood sample was collected at the beginning and end of each experiment to assess methemoglobin and nitrite levels in the serum. Methemoglobin level in the blood was measured with a blood gas analyzer (Radiometer ABL700, Diamond Diagnostics, Holliston, MA), and serum nitrite level was measured using Griess reagent.[31]

## 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 measurements.

# RESULTS

## Proliferation of dermal cells in the presence of exogenous gNO

Exogenous gNO exhibits potent antimicrobial properties at a concentration of 200 ppm following 8–12 hours of exposure.[14,15] In previous dose–response studies, the proliferation of primary culture of human dermal fibroblast was significantly reduced following exposure to 400 ppm of gNO after 24 hours.[15] In order to utilize this gas in treatment of skin infection, one must minimize its cytotoxic effect on skin resident cells. As a result, we decided to explore the cytotoxic effects of gNO at 200 ppm on various cells resident in the skin. As one of the markers of

cytotoxicity, we monitored the proliferation of dermal fibroblasts, keratinocytes, and HUVEC primary cells in the presence or absence of gNO. Cells were exposed to either air (control) or gNO for 8 hour each day, in order to minimize gNO's cytotoxic effects. In between treatments, all cells were incubated in a conventional $CO_2$ chamber at 37 °C. Proliferation data were expressed as a relative percentage of the control cells (Figure 1). All primary human skin cells demonstrated relatively strong resistance toward the cytotoxic and cytostatic effects of gNO at 200 ppm. Compared with other skin cells, keratinocytes expressed greater sensitivity toward the gNO treatment (Figure 1B), although this did not reach statistical significance (*t* test, *p*=0.0939).

## Cell migration was not compromised by exogenous gNO

Following an injury to the skin, migration ability of resident cells is crucial for wound closure, deposition of new granulation tissue, and tissue remodeling. To test the effect of gNO 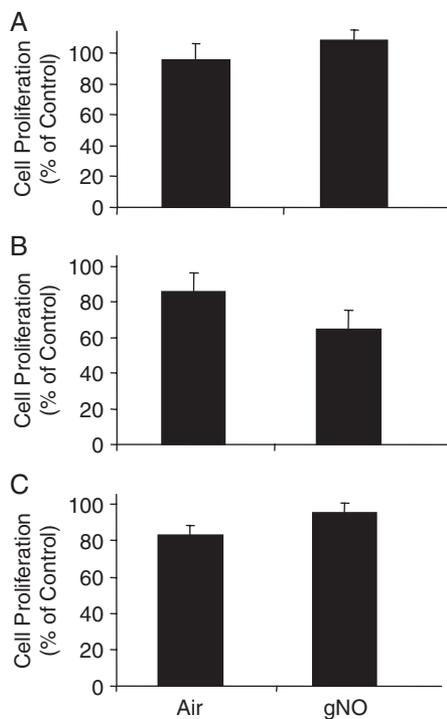exposure on cell migration, we established assays for fibroblasts, major cell type in skin dermis, keratinocytes, cells responsible for wound closure, and endothelial cells that form new blood vessels. The ability of fibroblasts to migrate out of a collagen gel lattice was studied in the presence and absence of gNO for up to 10 days. Cells were treated with gNO for 8 hour/day, followed by incubation in a conventional $CO_2$ chamber. In comparison with controls on day 2, 5, and 10, gNO-treated fibroblasts did not show any difference in their migration ability out of the collagen gel lattices (Figure 2A). In addition, proliferation assays of treated and untreated cells did not reveal any significant difference in the number of cells migrating out of gel and repopulating the culture dish (Figure 2B).

To assess keratinocyte migration in the presence of 200 ppm gNO, scratch migration assays were utilized. The "wound closure" in treated and untreated groups was monitored and photographed for 3 days postwounding (Figure 3A). Following calibration of digital images, distances migrated by keratinocytes were measured and expressed as a relative percentage of the original "wound" size. Exposure to gNO did not cause any delay, in comparison with the control, in the migration pattern or closure rate of keratinocytes (Figure 3B). Both gNO-treated
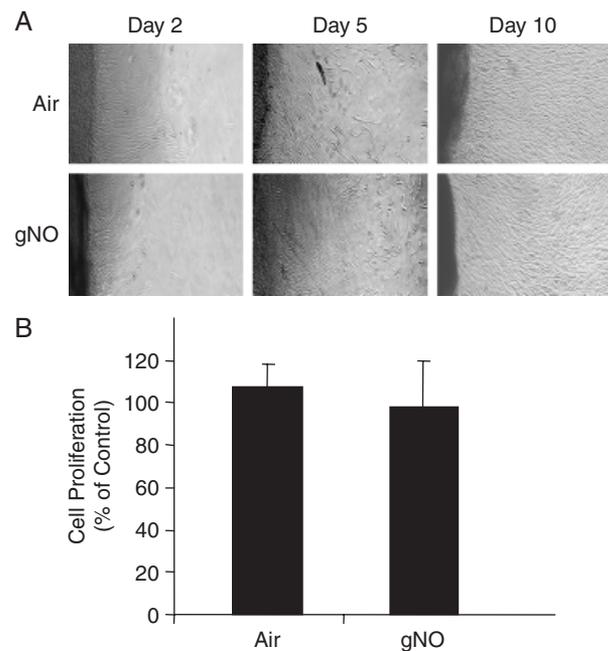
**Figure 1.** Effect of gaseous nitric oxide (gNO) on human skin cell proliferation. Fibroblast-populated collagen gel (A), keratinocytes (B), and endothelial cells (C) were exposed to 200 ppm gNO or medical air for 8 hours/day inside the gNO chamber. Culture media were replaced with fresh medium following each treatment. Methyl thiazolyl tetrazolium assay was performed following 3 consecutive days to monitor cell proliferation. The control group was incubated in a conventional $CO_2$ culture incubator. Data are presented as a relative percentage of proliferation compared with the control group. Data represent the mean ± standard deviation for three separate experiments.

**Figure 2.** Gaseous nitric oxide (gNO)-treated and non-treated fibroblasts have a similar pattern of cell migration and proliferation. Fibroblasts embedded in collagen gel were treated either with medical air or 200 ppm gNO for 3 days (8 hours/day). The control group was incubated in a conventional $CO_2$ culture incubator. Following the last treatment, three 6 mm punch biopsies were taken from each gel and placed onto six-well plates, and the pattern of migrating cells was microscopically photographed for up to 10 days (A). On day 10, punch biopsies were removed and methyl thiazolyl tetrazolium proliferation assay was performed and expressed as a percentage relative to the control group (B).

and nontreated keratinocytes achieved approximately 40% ''wound'' closure by day 3.

In a separate study, the influence of gNO on the ability of HUVECs to reorganize as capillary tubes, when cultured or embedded in Matrigel lattice, was evaluated. Pseudotube formation was observed as early as 3 hours postincubation both in the control and gNO-treated groups (Figures 4A and D). Extensive tube formation with numerous cellular extensions forming multicentric junctions did not appear until 16–24 hours postincubation (Figures 4C and F). Digital images from a phase-contrast microscope were analyzed for qualitative evaluation of pseudotube formation in ten randomly selected fields. Treatment with 200 ppm gNO for up to 24 hours produced
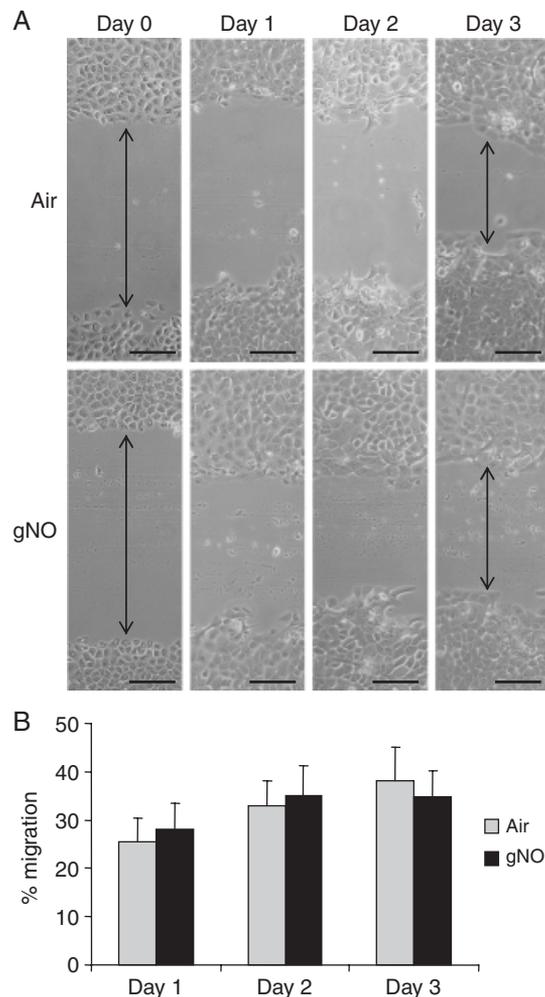
**Figure 3.** Keratinocyte migration pattern is not altered following gaseous nitric oxide (gNO) treatment. Scratch wounds were created in confluent primary keratinocyte cell cultures and artificial wound closure was photographed for 3 days (A). During this period, cells were treated with 200 gNO or medical air for periods of 8 hours/day. Distances between the ''wound'' margins were measured (shown by arrows) by an imaging software. The distance migrated by each leading edge was calculated and presented as a percentage of original wound size on day 0 (B). Solid bars at the bottom of images represent 150 μm.
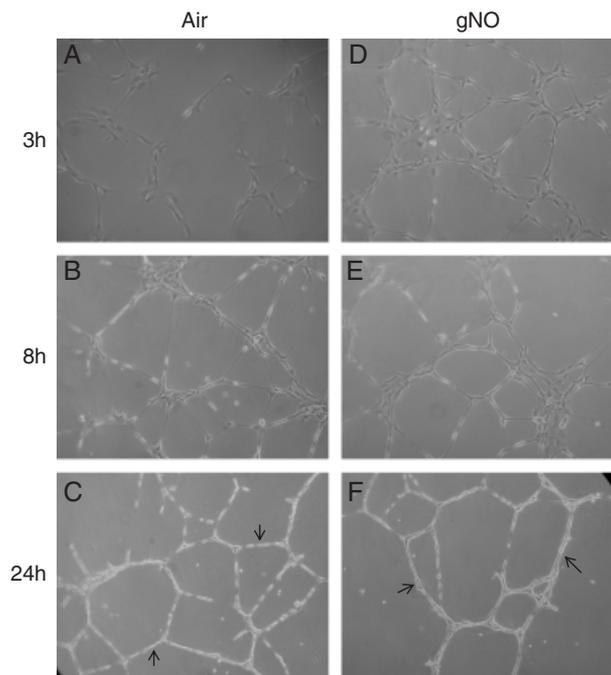
**Figure 4.** Gaseous nitric oxide (gNO)-treated and nontreated human umbilical vein endothelial cells (HUVECs) show a similar pattern of angiogenesis. Subconfluent endothelial cells were plated to Matrigel-coated six-well plates and incubated under 200 ppm gNO or medical air. Representative photomicrographs of capillary-like structure formations are shown at specified time points. Arrows in (C) and (F) show the capillary structures formed by HUVECs following 24 hours of treatment. Images were observed under ×100 magnifications.

no apparent inhibition in HUVECs' ability to migrate and reorganize on the Matrigel.

## Extracellular matrix expression in FPCG

Collagen is the most abundant protein in ECM of skin tissue. Collagen production and its degradation and rearrangement by MMP family are essential for proper healing of the injured tissue. Dermal fibroblasts are the major contributors in the production of both collagen and MMPs during wound healing.[32] To examine the effect of gNO on ECM expression in dermal FPCG, we analyzed the expression of type-I procollagen and interstitial collagenase (MMP-1) mRNA in the presence and absence of 200 ppm gNO. In order to mimic our in vivo study, FPCG were exposed to gNO for 8 hours/day for a total of 3 days. At the end of the last exposure, fibroblasts were dissociated from the gel by collagenase treatment, and Northern Blot analysis was carried out on the total RNA extracted from cells. Densitometry analysis was performed to adjust mRNA expression relative to ribosomal 18S mRNA expression. Collagen gene expression was up-regulated by almost 50% following gNO treatment (Figures 5A and B). The expression of MMP-1 in FPCG exposed to gNO appeared to be down-regulated compared with the control, although this difference did not become statistically significant in any of the three independent experiments (Figures 5A and C).
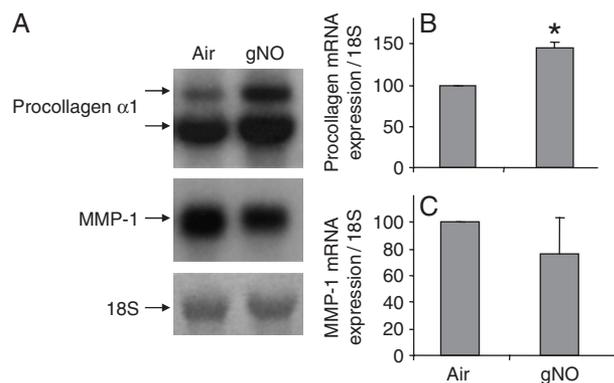
**Figure 5.** Matrix metalloproteinase (MMP-1) and type-I procollagen mRNA expression in fibroblast-populated collagen gel (FPCG). Fibroblasts were treated with either medical air or 200 ppm gaseous nitric oxide (gNO) for 8 hours/day. At the end of the 3rd day, total RNA was extracted and subjected to Northern Blot analysis. (A) shows the representative pattern of MMP-1 and procollagen-1 mRNA expression. Expression of 18S rRNA was used as the loading control. (B and C) show the combined densitometry results of three independent experiments showing the ratio of MMP-1 and procollagen-1 mRNA to 18S rRNA expression, respectively. For analysis, the densitometric values for the control (air) signals were set at 100% and other groups were calculated relative to this. (*$p < 0.05$ vs. air in [B]).

### Keratinocyte differentiation was not altered by gNO Treatment

Continuous differentiation of basal keratinocytes is vital for the formation and integrity of the epidermis barrier.[33] The in vitro effect of gNO treatment on the expression of involucrin, a strong marker for normal keratinocyte differentiation, was measured in human primary keratinocyte cultures. Confluent cultures of keratinocytes were exposed 8 hours/day for as long as 10 days to 200 ppm gNO or medical air (control). Involucrin mRNA expression was evaluated by Northern Blotting as shown in Figure 6A. In the control group, the transcript level of involucrin was significantly increased on day 5 posttreatment and heightened expression continued through day 10. The keratinoctyes exposed to gNO also demonstrated their ability to differentiate as indicated by the increase in the mRNA level of involucrin on both days 5 and 10. Densitometry analysis was performed to adjust for mRNA loading and to quantify the relative expression of involucrin (Figure 6B).

### Antimicrobial effect of gNO in vivo

Following an injury to the skin, the control of bacterial infection is vital in the early stages of wound healing as well as in chronic nonhealing wounds. *S. aureus* is one of the most common pathogens isolated in skin infections.[34] To examine the antimicrobial effect of gNO in vivo, full-thickness wounds on dorsal surface of New Zealand White rabbits were infected with a known concentration of *S. aureus* 24 hours before treatment. Following visual confirmation
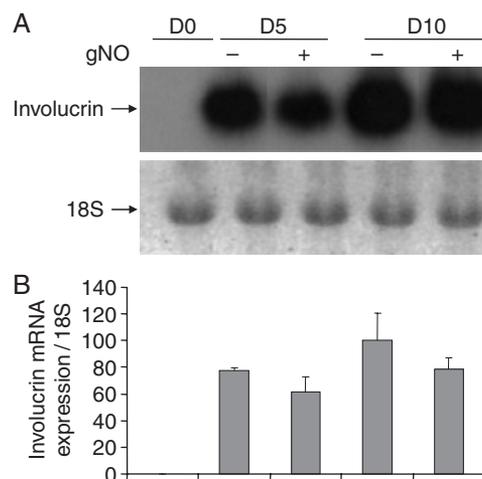


**Figure 6.** Basal keratinocytes retain the ability to differentiate following long-term gaseous nitric oxide (gNO) treatment. The mRNA expression of a differentiation marker, involucrin, in human skin keratinocyte cell cultures was analyzed in the presence and absence of 200 ppm gNO. Keratinocyte cell cultures were allowed to reach confluency before treatment with gNO. On days 0, 5, and 10, total RNA was extracted and subjected to Northern Blot analysis (A). Expression of 18S rRNA was used as the loading control. To quantify the involucrin expression, combined densitometry results of three separate experiments are used to show the ratio of involucrin mRNA to 18S rRNA expression (B). All signals are expressed as percentages relative to the highest signal (set to 100%) observed on day 10 in the absence of gNO.

of infection, wounds were treated with 200 ppm gNO for 8 hours/day for 3 consecutive days. Bacterial counts and histological samples were obtained from both gNO- and air-treated (control) wounds at the end of the third day. The number of surviving *S. aureus* in the wounds exposed to gNO was significantly reduced compared with the non-treated wounds (Figure 7A). Histological analysis of the paraffin sections stained with H&E showed a significant reduction of infiltrating immune cells throughout the wounds treated with gNO. A more severe immune response, possibly due to a higher bacterial burden, was observed in the wounds of all control animals. Interestingly, $NO_2$ and $NO_3$ levels ($NO_x$), the predominante end products of NO in systemic circulation, did not increase following topical administration of gNO. Blood serum NOx levels on day 3 of study were measured at $34.0 \pm 4.0$ and $30.2 \pm 2.0 \, \mu mol/L$ in control and gNO-treated animals, respectively. In addition, no significant differences were observed in the serum methemoglobin levels between the two groups (data not shown), indicating lack of systemic toxic effects following topical administration of gNO.

## DISCUSSION

Infection of chronic wounds and nonhealing ulcers can lead to serious complications including sepsis, amputation, and death. Bacteria impair repair processes by producing toxic byproducts and competing with cells for oxygen and
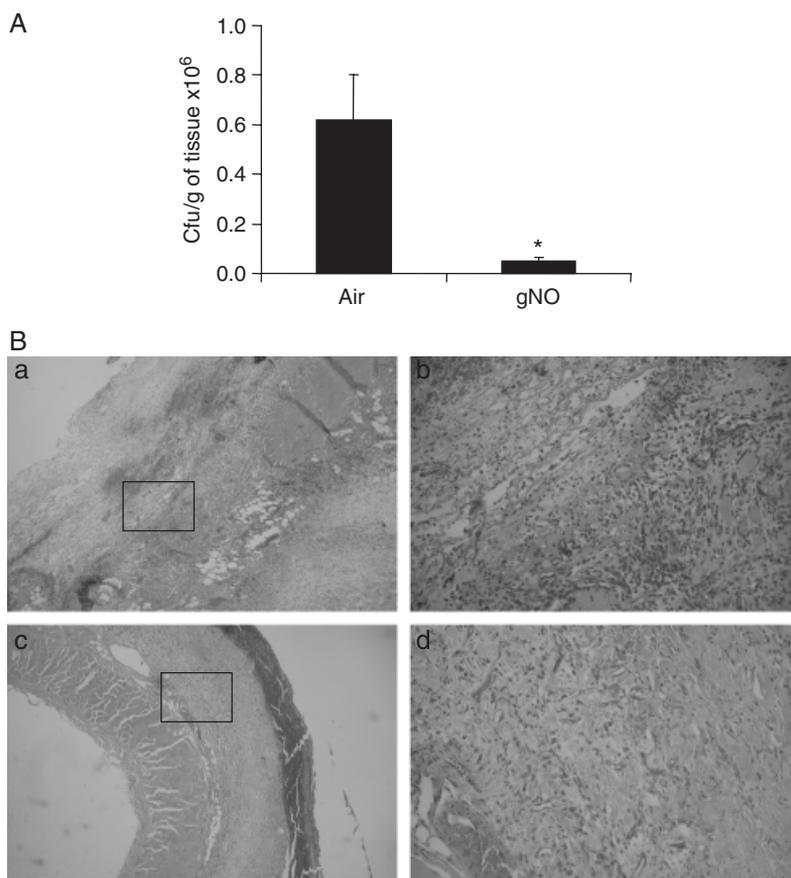
A



B



**Figure 7.** Antimicrobial properties of gaseous nitric oxide (gNO) in vivo. Full-thickness 6 mm punch biopsy wounds were infected with $1 \times 10^6$ cfu *Staphylococcus aureus* 1 day before treatment. Once the presence of infection had been confirmed, the animals were treated with 200 ppm gNO or medical air alone for a period of 8 hours/day. Following 3 days of treatment, a bacterial count (cfu/g of tissue) was obtained for each wound (A). Paraffin serial sections from day 3 wounds treated in the presence (C, D) or absence (A, B) of gNO were stained with hematoxylin and eosin in order to visualize tissue cellularity and immune cell infiltration. Areas outlined in boxes in (A) and (C) ($\times 100$) are magnified under (B) and (D) ($\times 400$).

nutrients.[35] However, it has been shown that wounds retain the ability to recluse after the bacterial burden is reduced.[36] During the past two decades, *S. aureus* and *enterococci* have gained prominence as causes of wound infection and in many countries *methicillin-resistant S. aureus* (MRSA) infections have been increasing in frequency since 1985.[37] Exogenous NO exhibits strong antimicrobial properties against a wide range of pathogens.[5,7,13] NO signaling also appears to play a vital role in wound healing.[38] Application of a variety of NO donors as topical agents has been used with some success in augmenting wound healing and reducing wound bacterial burden.[11,12,39,40] We have recently demonstrated the potent antimicrobial properties of gNO blended with air against a wide range of pathogens including clinical and MRSA, Group B *Streptococcus*, *Candida albicans*, *Escherichia coli*, and *Pseudomonas aeruginose*.[14–16] Although different strains demonstrate variable resistance, almost all bacteria exposed to 200 ppm gNO show significant reduction in growth after 5 hours of exposure.[15] In the present study, full-thickness wounds infected with *S. aureus* revealed a significant reduction in bacterial content following 3 days of intermittent (8 hours/day) exposure to 200 ppm gNO. Concurrently, skin cells exposed to the same level of gNO in vitro did not demonstrate any cytotoxic effect.

The skin, the largest organ of the body, is composed of the epidermis, the dermis, and a subdermal layer. All of the major cell types residing in these three layers, including keratinocytes, fibroblasts, melanocytes, and endothelial cells, express NO synthase (NOS) and are capable of releasing NO.[41] In addition to binding to heme groups such as hemoglobin and cytochrome c oxidase, NO may also bind with nucleophilic centers like sulfur, nitrogen, oxygen, and aromatic carbons. Therefore, NO has the ability to react with proteins and nucleic acids, its primary targets in the cell.[22] Interestingly, the cytotoxic effects of NO are not consistent across different cell types and seem to be dose dependent.[42] Studies carried out by Krischel, et al.,[43] revealed that both epidermal human keratinocytes and fibroblasts are resistant to the cytotoxic or apoptosis-inducing effect of exogenous NO. In their study, they exposed primary culture cells to a number of different NO donors such as nitroprusside, DETA/NO, and SNAP at concentrations ranging from 0.5 to 5.0 mM for up to 48 hours. Given that the diffusion and reactivity of gNO are very different in aqueous media in comparison with release of NO from donor complexes used in Krischel studies, we investigated whether 200 ppm exogenous gNO expresses the same level of cytotoxicity in human skin cells as it does in common pathogens in skin infection. The discontinuity of gNO treatment in our study was designed to minimize the cytotoxic effects of this molecule toward the host cells. The diffusion rate for 200 ppm gNO was calculated to be approximately 88.4 µM/hour in a cell culture medium such as DMEM.[14] At this rate, less than 1.0 mM gNO is taken

up cumulatively by the media in an 8-hour exposure. Although potent antimicrobial effects were observed at these levels of gNO in the first 8-hour exposure, our current investigation did not reveal any significant cytotoxic effect in FPCG, keratinocytes, or endothelial cells following 3 days of treatment. It is important to note that a significant reduction in fibroblast proliferation was observed on doubling the dose to 400 ppm in a similar delivery system.[15] This ruled out the possibility that gNO is not reaching the intracellular compartment of treated cells. Further support for diffusion capacity of gNO in an aqueous medium was provided by the significant sensitivity of bacterial cells exposed to the same level of gNO in previous studies.[14,15] Although the exact mechanism of tolerance to these levels of gNO is the subject of an ongoing study, it has been suggested that a more established S-nitrosoglutathione reductase pathway in mammalian cells may be responsible, in part, for this effect.[44,45]

The biphasic action of NO on the proliferation of keratinocytes has been reported previously where increasing keratinocyte proliferation was manifested at low NO concentrations, whereas the pattern changed at higher doses of NO. Skin fibroblasts, however, have exhibited a significant decrease in proliferation at low concentrations of NO.[43] Under intermittent exposures to 200 ppm gNO, we did not observe a different reaction in the proliferation of keratinocytes and FPCG. It should be noted that keratinocytes exhibited a slight decrease in their proliferation rate in the presence of gNO, but it did not reach statistical significance under our exposure conditions.

In addition to the role of cell proliferation in the post-injury phase, the ability of skin cells to migrate into the wound area is also extremely crucial during the process of wound repair.[46] Several hours following an injury to the skin, reepithelialization is initiated, with keratinoctye migration from the wound edge into the wound clot.[47] NO's important regulatory role is apparent from the severe delay in wound reepithelialization and wound closure in iNOS-deficient or selective iNOS inhibitor-treated animals.[48,49] Using the "scratch" techniques, a migration rate of approximately 1.0 mm in 48 hours was reported for cultured keratinocytes.[50,51] In our study, the migration pattern of "injured" keratinocyte layer did not reveal any significant difference in the presence or absence of gNO. Similarly, migration of endothelial cells to form tubular structures, as observed during the angiogenesis phase of wound healing, was not jeopardized following gNO treatment.

Simultaneously with and subsequent to reepithelialization, fibroblast proliferation, migration, and ECM deposition play a vital role in the granulation and remodeling phase of wound repair.[47] It is imperative that a topical antimicrobial agent not impede such functions in fibroblasts during wound healing. As the most abundant ECM protein in the skin, collagen production and its degradation by MMPs such as collagenase-1 (MMP-1) is critical in proper healing of the injured tissue.[52] To investigate the functional integrity of fibroblasts at the transcription level, we looked into the gene expression of MMP-1 and collagen following exposure to 200 ppm gNO. FPCG in the treated group demonstrated a significant increase in collagen type I mRNA expression. Although a slightly lower expression of MMP-1 was observed, this did not reach statistical sig-

nificance. Up-regulation of collagen production in fibroblasts treated by NO donors has been described previously by other groups as well.[11,53,54] As the main focus of the study was on the antimicrobial effect of gNO in clearing wound infection, the 3-day in vivo exposure was not designed to observe the effect of type-I collagen up-regulation on wound closure. Interestingly, in a recent case study of a 2-year-old nonhealing venous ulcer, exposure to gNO resulted in clearance of critical colonization and wound closure after 14 days of treatment.[16]

The predominant cell types in early wound healing are lymphocytes, granulocytes, and macrophages. Immune cells play a critical regulatory role in transition between wound inflammation and the next phase of granulation tissue deposition in wound healing.[55] Although the exact role of NO in immune regulation is still unclear, it is mainly considered to inhibit the expression of genes involved in cellular proliferation and growth.[56] Interestingly, NO has also been shown to express antiapoptotic effects.[57] Although the initial iNOS knockout animal experiment suggested a role in inhibition of $T_{h1}$ and promotion of $T_{h2}$ cells,[58] subsequent studies indicate that both $T_{h1}$ and $T_{h2}$ respond similarly to NO and both subsets produce comparable amounts of NO possibly as part of a negative feedback.[59] In treating nonhealing infected ulcers with gNO, as proposed in our study, it is unlikely that immunosuppressive effects of NO will lead to any significant impairment of the healing process. While in acute wounds the inflammatory phase is transient, in chronic wounds there is a prolonged and increased presence of T-cell infiltrate. In chronic venous and diabetic ulcers, the wound seems to be frozen in a chronic low-grade inflammatory state and transition into granulation deposition by fibroblasts and angiogenesis by endothelial cells does not take place.[55] It is likely that an immunosuppressive agent such as NO might shock the wound environment out of this frozen state and revive the healing process.[16] Furthermore, persistence of infection and bacterial antigens, as commonly seen in chronic ulcers, can induce anergy and apoptosis of T cells. Rapid accumulation of bacterial toxins can cause hyperactivation of the immune system and massive release of antiinflammatory cytokines, resulting in septic shock, acute respiratory distress syndrome, and death.[60] Chronic infection can further disrupt the normal process of healing by impairing recruitment and migration of appropriate cells (neutrophils, macrophages, fibroblasts, etc.) to the site of injury, leading to abnormal levels of cytokines and growth factors.[61] Therefore, the clearance of bacterial burden is of the highest priority in treating chronic and nonhealing ulcers. Based on clinical observations, epithelialization can proceed normally once the quality of wound bed has improved.[55] In addition, by inducing vascular permeability and vasodilation, gNO can potentially increase the blood supply and oxygenation in wound bed microenvironment.[62]

In conclusion, our results, along with our previous in vitro studies, provide further evidence of the effectiveness of gNO as an antimicrobial agent in vivo. We also demonstrated a differential in the cytotoxicity of gNO between bacterial and human skin cells. Recognizing that NO acts as a "double edged sword" in pathophysiology of the body, it is possible to identify a narrow range where the cytotoxic effects of this molecule are minimized without

jeopardizing its antimicrobial properties. Considering the recent increase of antibiotic-resistant strains, the topical application of gNO as an antimicrobial agent could prove to play a crucial role in the treatment of skin infection, particularly in burns and nonhealing chronic wounds.

## ACKNOWLEDGMENTS

## REFERENCES

1. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms. *Lancet* 2001; 358: 135–8.
2. Costerton JW, Stewart PS. Battling biofilms. *Sci Am* 2001; 285: 74–81.
3. Nagesha CN, Shenoy KJ, Chandrashekar MR. Study of burn sepsis with special reference to *Pseudomonas aeruginosa*. *J Ind Med Assoc* 1996; 94: 230–3.
4. Liew FY, Cox FE. Nonspecific defence mechanism: the role of nitric oxide. *Immunol Today* 1991; 12: A17–A21.
5. Fang FC. *Nitric oxide and infeciton*. New York: Kluwer Academic/Plenum Publishers, 1999.
6. Akerstrom S, Mousavi-Jazi M, Klingstrom J, Leijon M, Lundkvist A, Mirazimi A. Nitric oxide inhibits the replication cycle of severe acute respiratory syndrome coronavirus. *J Virol* 2005; 79: 1966–9.
7. Weller R, Price RJ, Ormerod AD, Benjamin N, Leifert C. Antimicrobial effect of acidified nitrite on dermatophyte fungi, Candida and bacterial skin pathogens. *J Appl Microbiol* 2001; 90: 648–52.
8. Jackson M, Frame F, Weller R, McKenzie RC. Expression of nitric oxide synthase III (eNOS) mRNA by human skin cells: melanocytes but not keratinocytes express eNOS mRNA. *Arch Dermatol Res* 1998; 290: 350–2.
9. Ivanova K, Le Poole IC, Gerzer R, Westerhof W, Das PK. Effect of nitric oxide on the adhesion of human melanocytes to extracellular matrix components. *J Pathol* 1997; 183: 469–76.
10. Wang R, Ghahary A, Shen YJ, Scott PG, Tredget EE. Human dermal fibroblasts produce nitric oxide and express both constitutive and inducible nitric oxide synthase isoforms. *J Invest Dermatol* 1996; 106: 419–27.
11. Witte MB, Kiyama T, Barbul A. Nitric oxide enhances experimental wound healing in diabetes. *Br J Surg* 2002; 89: 1594–601.
12. Shabani M, Pulfer SK, Bulgrin JP, Smith DJ. Enhancement of wound repair with a topically applied nitric oxide-releasing polymer. *Wound Rep Reg* 1996; 4: 353–62.
13. Weller R, Ormerod AD, Hobson RP, Benjamin NJ. A randomized trial of acidified nitrite cream in the treatment of tinea pedis. *J Am Acad Dermatol* 1998; 38: 559–63.
14. Ghaffari A, Neil DH, Ardakani A, Road J, Ghahary A, Miller CC. A direct nitric oxide gas delivery system for bacterial and mammalian cell cultures. *Nitric Oxide* 2005; 12: 129–40.
15. Ghaffari A, Miller CC, McMullin B, Ghahary A. Potential application of gaseous nitric oxide as a topical antimicrobial agent. *Nitric Oxide* 2006; 14: 21–9.
16. Miller CC, Miller MK, Ghaffari A, Kunimoto B. Treatment of chronic nonhealing leg ulceration with gaseous nitric oxide: a case study. *J Cutan Med Surg* 2004; 8: 233–8.
17. Miller CC. *The antibacterial role of exogenous nitric oxide gas*. Vancouver, Canada: University of British Columbia, 2004.
18. Kolb H, Kolb-Bachofen V. Nitric oxide: a pathogenetic factor in autoimmunity. *Immunol Today* 1992; 13: 157–60.
19. Ormerod AD, Weller R, Copeland P, Benjamin N, Ralston SH, Grabowksi P, Herriot R. Detection of nitric oxide and nitric oxide synthases in psoriasis. *Arch Dermatol Res* 1998; 290: 3–8.
20. Rowe A, Farrell AM, Bunker CB. Constitutive endothelial and inducible nitric oxide synthase in inflammatory dermatoses. *Br J Dermatol* 1997; 136: 18–23.
21. Lipton SA, Choi YB, Pan ZH, Lei SZ, Chen HS, Sucher NJ, Loscalzo J, Singel DJ, Stamler JS. A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds. *Nature* 1993; 364: 626–32.
22. Kroncke KD, Fehsel K, Kolb-Bachofen V. Nitric oxide: cytotoxicity versus cytoprotection—how, why, when, and where? *Nitric Oxide* 1997; 1: 107–20.
23. Rheinwald JG, Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975; 6: 331–43.
24. Ghahary A, Marcoux Y, Karimi-Busheri F, Tredget EE. Keratinocyte differentiation inversely regulates the expression of involucrin and transforming growth factor beta1. *J Cell Biochem* 2001; 83: 239–48.
25. Narumiya H, Zhang Y, Fernandez-Patron C, Guilbert LJ, Davidge ST. Matrix metalloproteinase-2 is elevated in the plasma of women with preeclampsia. *Hypertens Pregnancy* 2001; 20: 185–94.
26. Volpin D, Veis A. Isolation and characterization of the cyanogen bromide peptides from the 1 and 2 chains of acid-soluble bovine skin collagen. *Biochemistry* 1971; 10: 1751–5.
27. Bell E, Ivarsson B, Merrill C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential in vitro. *Proc Natl Acad Sci USA* 1979; 76: 1274–8.
28. Sarkhosh K, Tredget EE, Karami A, Uludag H, Iwashina T, Kilani RT, Ghahary A. Immune cell proliferation is suppressed by the interferon-gamma-induced indoleamine 2,3-dioxygenase expression of fibroblasts populated in collagen gel (FPCG). *J Cell Biochem* 2003; 90: 206–17.
29. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 1979; 18: 5294–9.
30. Rasouli-Nia A, Karimi-Busheri F, Weinfeld M. Stable downregulation of human polynucleotide kinase enhances spontaneous mutation frequency and sensitizes cells to genotoxic agents. *Proc Natl Acad Sci USA* 2004; 101: 6905–10.
31. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 1982; 126: 131–8.
32. Tredget EE, Nedelec B, Scott PG, Ghahary A. Hypertrophic scars, keloids, and contractures. The cellular and molecular basis for therapy. *Surg Clin North Am* 1997; 77: 701–30.

33. Morasso MI, Tomic-Canic M. Epidermal stem cells: the cradle of epidermal determination, differentiation and wound healing. *Biol Cell* 2005; 97: 173–83.

34. Lee SY, Kuti JL, Nicolau DP. Antimicrobial management of complicated skin and skin structure infections in the era of emerging resistance. *Surg Infect (Larchmt)* 2005; 6: 283–95.

35. Robson MC, Stenberg BD, Heggers JP. Wound healing alterations caused by infection. *Clin Plast Surg* 1990; 17: 485–92.

36. Robson MC, Shaw RC, Heggers JP. The reclosure of post-operative incisional abscesses based on bacterial quantification of the wound. *Ann Surg* 1970; 171: 279–82.

37. Devlin HR. Bacteria for the nineties. *Ostomy Wound Manage* 1998; 44: 32–40.

38. Rizk M, Witte MB, Barbul A. Nitric oxide and wound healing. *World J Surg* 2004; 28: 301–4.

39. Witte MB, Thornton FJ, Efron DT, Barbul A. Enhancement of fibroblast collagen synthesis by nitric oxide. *Nitric Oxide* 2000; 4: 572–82.

40. Hardwick JB, Tucker AT, Wilks M, Johnston A, Benjamin N. A novel method for the delivery of nitric oxide therapy to the skin of human subjects using a semi-permeable membrane. *Clin Sci (Lond)* 2001; 100: 395–400.

41. Cals-Grierson MM, Ormerod AD. Nitric oxide function in the skin. *Nitric Oxide* 2004; 10: 179–93.

42. Gansauge S, Gansauge F, Nussler AK, Rau B, Poch B, Schoenberg MH, Beger HG. Exogenous, but not endogenous, nitric oxide increases proliferation rates in senescent human fibroblasts. *FEBS Lett* 1997; 410: 160–4.

43. Krischel V, Bruch-Gerharz D, Suschek C, Kroncke KD, Ruzicka T, Kolb-Bachofen V. Biphasic effect of exogenous nitric oxide on proliferation and differentiation in skin derived keratinocytes but not fibroblasts. *J Invest Dermatol* 1998; 111: 286–91.

44. Padgett CM, Whorton AR. Cellular responses to nitric oxide: role of protein S-thiolation/dethiolation. *Arch Biochem Biophys* 1998; 358: 232–42.

45. Liu L, Hausladen A, Zeng M, Que L, Heitman J, Stamler JS. A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans. *Nature* 2001; 410: 490–4.

46. Santoro MM, Gaudino G. Cellular and molecular facets of keratinocyte reepithelization during wound healing. *Exp Cell Res* 2005; 304: 274–86.

47. Martin P. Wound healing—aiming for perfect skin regeneration. *Science* 1997; 276: 75–81.

48. Yamasaki K, Edington HD, McClosky C, Tzeng E, Lizonova A, Kovesdi I, Steed DL, Billiar TR. Reversal of impaired wound repair in iNOS-deficient mice by topical adenoviral-mediated iNOS gene transfer. *J Clin Invest* 1998; 101: 967–71.

49. Stallmeyer B, Kampfer H, Kolb N, Pfeilschifter J, Frank S. The function of nitric oxide in wound repair: inhibition of inducible nitric oxide-synthase severely impairs wound re-epithelialization. *J Invest Dermatol* 1999; 113: 1090–8.

50. Richards TS, Dunn CA, Carter WG, Usui ML, Olerud JE, Lampe PD. Protein kinase C spatially and temporally regulates gap junctional communication during human wound repair via phosphorylation of connexin43 on serine368. *J Cell Biol* 2004; 167: 555–62.

51. Tenaud I, Leroy S, Chebassier N, Dreno B. Zinc, copper and manganese enhanced keratinocyte migration through a functional modulation of keratinocyte integrins. *Exp Dermatol* 2000; 9: 407–16.

52. Ghahary A, Karimi-Busheri F, Marcoux Y, Li Y, Tredget EE, Taghi KR, Li L, Zheng J, Karami A, Keller BO, Weinfeld M. Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. *J Invest Dermatol* 2004; 122: 1188–97.

53. Masters KS, Leibovich SJ, Belem P, West JL, Poole-Warren LA. Effects of nitric oxide releasing poly(vinyl alcohol) hydrogel dressings on dermal wound healing in diabetic mice. *Wound Repair Regen* 2002; 10: 286–94.

54. Schaffer MR, Efron PA, Thornton FJ, Klingel K, Gross SS, Barbul A. Nitric oxide, an autocrine regulator of wound fibroblast synthetic function. *J Immunol* 1997; 158: 2375–81.

55. Loots MA, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD, Middelkoop E. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. *J Invest Dermatol* 1998; 111: 850–7.

56. Vig M, Srivastava S, Kandpal U, Sade H, Lewis V, Sarin A, George A, Bal V, Durdik JM, Rath S. Inducible nitric oxide synthase in T cells regulates T cell death and immune memory. *J Clin Invest* 2004; 113: 1734–42.

57. Kroncke KD, Fehsel K, Suschek C, Kolb-Bachofen V. Inducible nitric oxide synthase-derived nitric oxide in gene regulation, cell death and cell survival. *Int Immunopharmacol* 2001; 1: 1407–20.

58. Taylor-Robinson AW, Liew FY, Severn A, Xu D, McSorley SJ, Garside P, Padron J, Phillips RS. Regulation of the immune response by nitric oxide differentially produced by T helper type 1 and T helper type 2 cells. *Eur J Immunol* 1994; 24: 980–4.

59. Van DV. Nitric oxide and T helper cell immunity. *Int Immunopharmacol* 2001; 1: 1491–500.

60. Sakhno LV, Leplina OY, Norkin MN, Chernykh ER, Ostanin AA. Role of nitric oxide in activation of human T lymphocytes induced by bacterial superantigen. *Bull Exp Biol Med* 2000; 130: 957–60.

61. Falanga V. The chronic wound: failure to heal. In: Falanga V, editor. *Cutaneous wound healing*. London: Martin Dunitz Ltd, 2001: 155–64.

62. Ignarro LJ. Nitric oxide as a unique signaling molecule in the vascular system: a historical overview. *J Physiol Pharmacol* 2002; 53 (4 Pt 1): 503–14.