

# Gaseous nitric oxide exhibits minimal effect on skin fibroblast extracellular matrix gene expression and immune cell viability

Alireza Moeen Rezakhanlou<sup>\*†</sup>, Chris Miller<sup>‡</sup>, Bevin McMullin<sup>§</sup>, Abdi Ghaffari<sup>\*</sup>, Rosa Garcia<sup>||</sup> and Aziz Ghahary<sup>1\*</sup>

<sup>\*</sup> Department of Surgery, BC Professional Burn and Wound Healing Research Lab, Rm 350, Jack Bell Research Centre 2660 Oak Street, Vancouver, BC V6H 3Z6, Canada

<sup>†</sup> School of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran

<sup>‡</sup> Division of Respiratory, Department of Medicine, University of British Columbia, Vancouver, BC, Canada

<sup>§</sup> Respiratory Services, Vancouver Coastal Health at Vancouver General Hospital, Vancouver, BC, Canada

<sup>||</sup> Department of Surgery, Vancouver Coastal Health Research Institute University of British Columbia, Vancouver, BC, Canada

## Abstract

NO (nitric oxide) molecule is produced by various mammalian cell types and plays a significant role in inflammation, infection and wound healing processes. Recently, gNO (gaseous nitric oxide) therapy has been utilized for its potential clinical application as an antimicrobial agent, with special focus on skin infection. In a previous study, we demonstrated that 200 ppm gNO, 8 h/day for three consecutive days significantly reduced the number of bacteria in dermal wounds without compromising the viability and function of skin cells. To increase the feasibility and ease of its clinical use, we propose that different doses of gNO (5 to 10 K ppm) for 8 h and as short as 10 min be used, respectively. To achieve this, we set up *in vitro* experiments and asked whether (i) different doses of gNO have any toxic effect on immune cells and (ii) gNO has any modulating effect on key ECM (extracellular matrix) components in fibroblasts. To further investigate the effect of gNO, expression of more than 100 key ECM genes have been examined using gene array in human fibroblasts. As immune cells play an important role in wound healing, the effect of gNO on proliferation and viability of human and mouse lymphocytes was also examined. The findings showed that, the 5, 25, 75 and 200 ppm of gNO for 8 h slightly increased the expression of Col 5A3 (collagen type V alpha 3), and gNO at 5 ppm decreased the expression of MMP-1 (matrix metalloproteinase 1), while exposure of fibroblast to 10 K ppm of gNO for 10 min does not show any significant changes in ECM genes. Exposure to gNO resulted in inhibition of lymphocyte proliferation without affecting the cell viability. Taken together, our findings show that skin could be treated with gNO without compromising the role of ECM and immune cells in low concentrations with long time exposure or high concentrations for a shorter exposure time.

Keywords: extracellular matrix (ECM); fibroblast; gaseous nitric oxide; matrix metalloproteinase 1 (MMP-1); lymphocyte

## 1. Introduction

NO (nitric oxide) is produced and released by major cell types in skin such as fibroblasts, keratinocytes, melanocytes and endothelial cells (Wang et al., 1996, 1997; Ivanova et al., 1997; Jackson et al., 1998) and plays a significant role in the response to infection and injury in skin (Weller et al., 2001; Akerstrom et al., 2005). NO is also involved in skin cell growth and differentiation (Weller, 1999). The role of NO on immune cells has also been investigated by several groups. It was shown that cytokines such as IFN- $\gamma$  (interferon gamma) and TNF (tumour necrosis factor) induce (Rimbach et al., 2000) and interleukin-4 reduces induction of iNOS (inducible nitric oxide synthase) in murine macrophages by LPS (lipopolysaccharide) (Perretti et al., 1995). NO has dual roles in inflammation. It exhibits an anti-inflammatory effect under normal physiological conditions and a proinflammatory role due to overproduction of inflammatory cytokines, such as during infection or injury (reviewed by MacMicking et al., 1997a; Sharma et al., 2007; Tripathi, 2007). NO plays a significant role

in the non-specific innate immune defence system (Liew and Cox, 1991; MacMicking et al., 1997a). Production of NO has been directly correlated to the host's ability to suppress microbial proliferation (MacMicking et al., 1997b). Mice lacking the inducible form of iNOS are more susceptible to infection compared with their wild-type counterpart (MacLean et al., 1998). *In vitro* studies utilizing a number of different NO donors suggest that NO possesses antimicrobial activity against bacteria, viruses, helminths and parasites (Sager et al., 1997; Saura et al., 1999; Weller et al., 2001). Previous studies indicated that delivery of exogenously delivered gNO (gaseous nitric oxide) also possesses similar antimicrobial activity (Ghaffari et al., 2006). We have previously shown *in vivo* that exogenous gNO reduced wound bacterial load as well as concurrent inflammatory response (Ghaffari et al., 2007). This observation resulted in the successful use of gNO in treatment of a critically colonized non-healing ulcer in a human case study (Miller et al., 2004).

iNO (inhaled gaseous nitric oxide) is a commonly used drug for the treatment of persistent pulmonary hypertension in the full-term infant (Soll, 2009), and gNO is currently under clinical trials as an

<sup>1</sup> To whom correspondence should be addressed (email aghahary@interchange.ubc.ca).

**Abbreviations:** ConA, Concanavalin A; gNO, gaseous nitric oxide; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GC, guanylyl cyclase; iNOS, inducible nitric oxide synthase; ITGB1, integrin beta-1; NO, nitric oxide; PHA, phytohaemagglutinin; UBC, University of British Columbia.

antimicrobial treatment for non-healing wounds, fungal dermatophyte and parasitic infections, sinusitis, cerebral malaria and cystic fibrosis indications. Combined with air or oxygen, gNO can easily be administered topically to cells and tissues without the need for a carrier vehicle, a time-release system or optimizing reaction conditions such as pH and temperature. Therefore, exogenous medical-grade gNO has a therapeutic potential in a number of human and animal disorders (Ghaffari et al., 2006).

ECM (extracellular matrix) proteins, such as collagens, integrins and MMP-1 (matrix metalloproteinase-1) play a pivotal role in wound healing. Studies have shown that these proteins play a significant role in healing of injured skin as well as development of fibrotic conditions (reviewed in Kisseleva and Brenner, 2008). Modulation of ECM expression has also been linked to immune cell activities mainly through the paracrine effect of released cytokines. One subtype of immune cells that plays a critical role in inflammation and wound healing are the T-lymphocytes, which promote wound healing through the release of cytokines such as TGF- $\beta$  (transforming growth factor- $\beta$ ) (Wang et al., 2007). In addition, cytokines secreted from macrophages, monocytes and lymphocytes and skin fibroblasts and keratinocytes are increased after skin wound healing (Kong et al., 2007). We have previously demonstrated the microbial cytotoxicity effect of 200 ppm gNO for 8 h on several skin pathogens with minimum effect on ECM genes. The primary objective of this study is to test the effect of a very wide range of topically applied gNO concentration on the expression of ECM proteins to identify potential modulatory effects in wound healing. As high concentrations of gNO over a short time could potentially be used as a potent antimicrobial agent for infected wounds, the study also attempts to examine the effect of gNO on immune cells. Our findings indicate that low to moderate doses of gNO over a long period (8 h) has a minor modulatory effect on some ECM gene expression with the ability to reduce immune cell proliferation without toxic effect on human lymphocytes. Also, we demonstrate that very high concentration of gNO (10K ppm) for 10 min does not modulate any ECM genes but reduced the immune cell proliferation without toxic effect on human lymphocytes.

## 2. Materials and methods

### 2.1. Cell culture

Cultures of human foreskin fibroblasts were prepared as described previously (Sarkhosh et al., 2003). In brief, punch biopsy samples were obtained from human foreskins. The tissue was collected, minced into small pieces and distributed into Petri culture dishes (Corning Inc.) with DMEM (Dulbecco's modified Eagle's medium) containing antibiotics (penicillin G sodium 100 units/ml, streptomycin sulfate 100  $\mu$ g/ml and amphotericin B 0.25  $\mu$ g/ml; 3 ml, GIBCO) with 10% FBS (fetal bovine serum). The cells were incubated at 37°C in a water-jacketed humidified incubator in an atmosphere of 5% CO<sub>2</sub>. The procedure was done based on the approval of the Ethics Committee of the UBC (University of British Columbia). Fibroblasts from passages 4–7 were used for this study.

### 2.2. Human peripheral and mouse spleen lymphocyte isolation

A volume of 10 ml of human peripheral blood was collected from healthy donors in three 4 ml heparinized collection tubes (Vacutainer™, BD) and layered on Histopaque®.1077 (Sigma-Aldrich). The lymphocyte fraction was collected and incubated in RPMI-1640 (Hyclone), 0.1 units of penicillin/ml and 0.1 mg of streptomycin/ml supplemented with 10% FBS. Mouse spleen was kindly provided by Dr R. Jalili, UBC. The erythrocytes were lysed by 1 × RBC Lysis buffer (eBioscience), and mononuclear cells were centrifuged, washed in HBSS and re-suspended in complete RPMI-1640. The cells in RPMI-1640 were supplemented with 10% FBS incubated in PHA (phytohaemagglutinin) 1  $\mu$ g/ml and also 10  $\mu$ g/ml ConA (Concanavalin A) in separate experiments for 72 h to induce lymphocyte proliferation. Proliferation induced by PHA and ConA was monitored using the Trypan Blue staining method. Cells were subsequently harvested for exposure to different concentrations of gNO.

### 2.3. Gaseous NO exposure device

The design and validity of the continuous horizontal-flow gNO delivery device used in this study has been described in detail elsewhere (Ghaffari et al., 2005). 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), 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 analyser (AeroNOx, Pulmonox Medical Inc.) to detect the exact composition of the various gases in the mixture. Medical-grade NO diluted in nitrogen (Airgas), medical air, oxygen and carbon dioxide (Praxair) gases supplied from pressurized cylinders at a constant pressure were then mixed together at predetermined ratios using a dilution manifold and a digital mass flow meter (TSI Inc.). The different gas mixtures for the experiments were then delivered to the exposure chamber at 2.0 l/min through two independent humidifiers (MR850, Fisher & Paykel Healthcare) set at 60–90% relative humidity (RH%). These final concentrations included 5, 25, 75, 200 and 10000 ppm gNO.

### 2.4. Cell survival assay

The survival of lymphocytes was compared by 7-AAD (7-Aminoactinomycin D) staining. 7-AAD intercalates into double-stranded nucleic acids. It is excluded by viable cells, but can penetrate cell membranes of dying or dead cells. After each treatment, cells were harvested, washed in PBS, stained for 7-AAD and then examined using FACSCanto analysis as per the manufacturer's protocol (BD). The survival of human lymphocytes also was examined by Trypan Blue staining method. For this purpose, human peripheral lymphocytes were isolated and treated with 10  $\mu$ g/ml ConA and 1  $\mu$ g/ml PHA for 4 days. The

cells were then exposed to 10000 ppm (10 K) gNO and 10% O<sub>2</sub> for 10 min and kept in a culture incubator for another 16 h. The viability of the cells was examined by Trypan Blue staining.

## 2.5. *In vitro* immune cell proliferation in response to gNO

Lymphocyte proliferation was assayed by using [<sup>3</sup>H]-thymidine incorporation as an index for cell proliferative capacity of immune cells in response to different doses and times of gNO treatment. To achieve this, Jurkat cells (T immune cell line), primary human and mouse lymphocytes were treated with various concentrations of gNO and then kept in the same chamber without any gNO for another 16 h. The cells were then pulsed with [<sup>3</sup>H]-thymidine (1 μCi/ml, PerkinElmer) and harvested 16–20 h later, and cell lysate-associated tritium was counted by a β-scintillation counter. All proliferation experiments were performed in triplicate and reported as the total of c.p.m. (cell count per minute). For control, the same number of cells were kept in a CO<sub>2</sub> incubator without gNO.

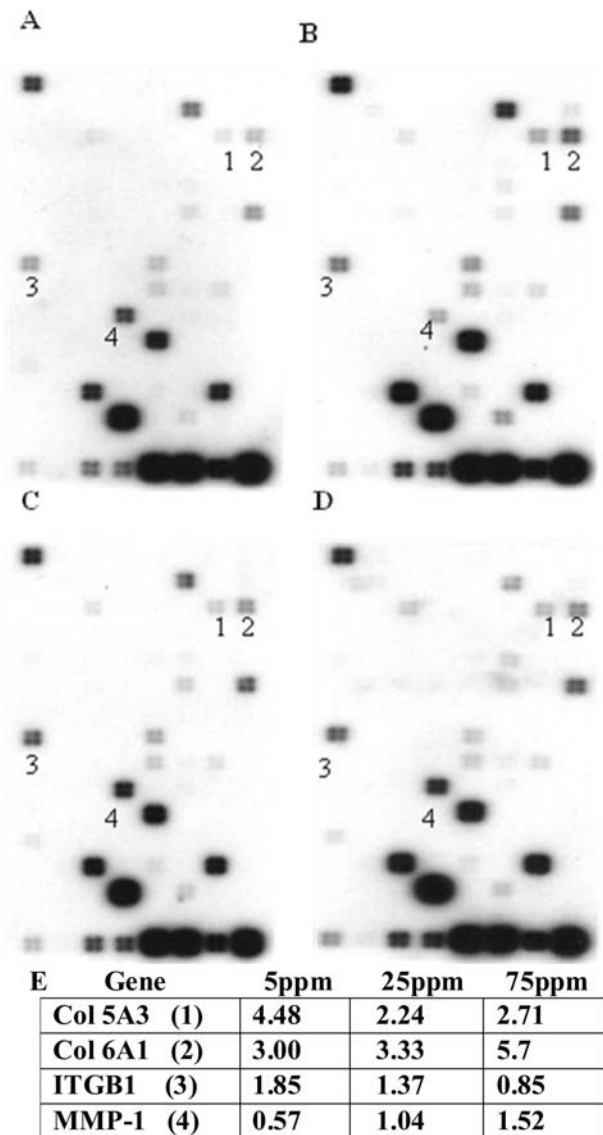
## 2.6. RNA isolation and analysis of gene expression by ECM-specific microarray

To examine whether gNO has any ECM modulatory effects in dermal fibroblasts, Oligo-GEArray<sup>®</sup> pathway-specific gene expression array systems were utilized (SuperArray Bioscience Corporation). Each human ECM and adhesion molecule gene array consists of 113 genes known to be involved in cell adhesion, ECM deposition and degradation as well as control sequences [PUC18 plasmid DNA as negative control, β-actin and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) for loading]. Using different arrays, the gene expressions of untreated dermal fibroblasts (negative control) and treated with 5, 25, 75, 200 ppm gNO (for 8 h), and 10000 ppm gNO (for 10 min) were evaluated and compared. At the end of the exposure, cells were kept in the same chamber without any gNO for another 16 h. Cells were then harvested, and total RNA was extracted from 1–2 × 10<sup>6</sup> cells by using RNeasy kit (Qiagen) and subjected to microarray and PCR. The integrity and purity of RNA was assessed by visualization of ethidium bromide-stained gels and 260/280 nm absorbance ratios. The microarrays were used according to the manufacturer's instructions. The results were analysed by GEArray Expression Analysis Suite software (SuperArray Bioscience Corporation).

## 2.7. Confirmation by RT-PCR

An aliquot of total RNAs used for the microarray was subjected to cDNA synthesis using SuperScript<sup>™</sup> first-strand synthesis system for RT-PCR (Invitrogen). Briefly, 2–5 μg of total RNA was reverse transcribed in a 20-μl reaction volume for 50 min at 42°C with Oligo dT15 primer according to the manufacture's instruction. Primers designed for analysis were, Col 5A3 (collagen type V alpha 3) (NM\_015719) sense 3'-CTGTGATCATGATGCCGTTCC-5' and anti-sense 3'-CTTCTGCTCCCTCCTCTCC-5', Col 6A1 (NM\_001848) sense 3'-AGTGACGAGGTGGAGACTCA-5' and anti-sense 3'-TCGGTACCACAATCAGGTA-5', ITGB1 (integrin

beta-1) (NM\_002211) sense 3'-GGCCTTGCATTACTGCTGAT-5' and anti-sense 3'-CAGTGTGTGGGATTGCAC-5' and matrix metalloproteinase-1 (interstitial collagenase) (NM\_002421) sense 3'-GATGTGGAGTGCCTGATGTG-5' and anti-sense 3'-TGCTTGACCCTCAGAGACCT-5'. The amount of each sample mRNA was evaluated relative to β-actin housekeeping gene.



**Figure 1** The gene expression profile of ECM in gNO-treated fibroblasts. Fibroblasts were exposed to various dosing regimens of gNO, and total RNA was extracted and subjected to microarray analysis. SuperArrays containing 123 ECM genes expressed in fibroblasts are shown. The blots shown are representative of three experiments. ECM gene expression was analysed in untreated fibroblasts (A) as well as cells exposed for 8 h to 5 (B), 25 (C) and 75 ppm gNO (D). The average change in the gene expression of four genes is summarized in the table below panel (E). To calculate the level of gene expression, the expression of individual gene in all panels were normalized against a housekeeping gene (GAPDH). Then, the expression of genes (B, C and D) were normalized against the gene expression (A) as an untreated control panel.

### 3. Results

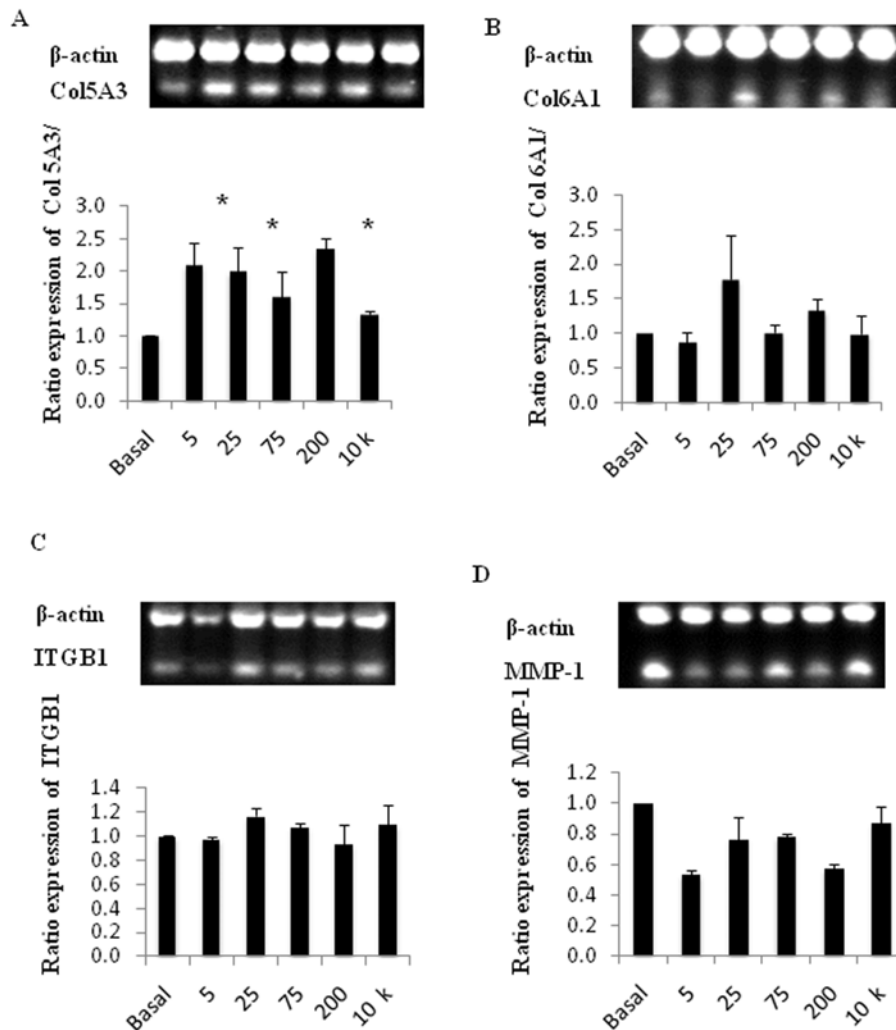
#### 3.1. Effect of gNO on ECM gene expression

To investigate the effect of a wide range of concentrations for use of gNO therapy on the wound healing process, we treated human dermal fibroblasts with different concentrations of gNO and analysed the ECM gene expression profile in an ECM-specific microarray. The results of gene expression in cells untreated or exposed to 5, 25 and 75 ppm gNO are shown (Figure 1). These findings revealed that the expression of four ECM genes was modulated slightly in various concentrations of gNO-treated fibroblasts. These genes are (i) Col 5A3, (ii) Col 6A1, (iii) ITGB1 and (iv) MMP-1 (matrix metalloproteinase 1). Following 8-h exposure to 5, 25 and 75 ppm gNO, Col 5A3 and Col 6A1

expression were increased, whereas ITGB1 doubled under 5 ppm gNO and slowly went down as gNO concentration was increased (Figure 1, table). MMP-1 gene expression decreased at 5 ppm but increased as gNO concentration increased. Majority of 120 ECM genes tested in our microarray remained unchanged following gNO treatment.

#### 3.2. Confirmation of selected genes by RT-PCR analysis

To confirm the results of the microarray, the level of the mRNA expression of genes in response to 5, 25, 75, 200 or 10 K ppm gNO treatment were analysed by RT-PCR. The effect of 5, 25, 75, 200 ppm and also 10 K ppm of gNO on the expression of Col 5A3, Col 6A1, ITGB1 and MMP-1 genes in fibroblasts are shown (Figure 2). From the four different genes whose expression was



**Figure 2** Confirmation of array results with RT-PCR fibroblasts were treated with various concentrations of gNO

Total RNA was extracted, cDNA synthesized and subjected to PCR with the specific primers defined in the Materials and methods section. The genes investigated were Col 5A3, Col 6A1, ITGB1 and MMP-1. Expression of  $\beta$ -actin was examined as a housekeeping gene. The result in each graph displays the ratio of gene expression in treated cells compared with basal levels (untreated) and are adjusted based on the  $\beta$ -actin expression. The asterisks indicate a  $P$ -value lower than 0.05 calculated by performing ANOVA (analysis of variance) Tukey–Kramer multiple comparisons test.

modulated in our Superarray, the expression level of Col 5A3 and MMP-1 evaluated by RT-PCR were consistent. However, the levels of Col 6A1 and ITGB1 remained unchanged.

### 3.3. Effect of gNO on proliferation and viability of human peripheral lymphocytes

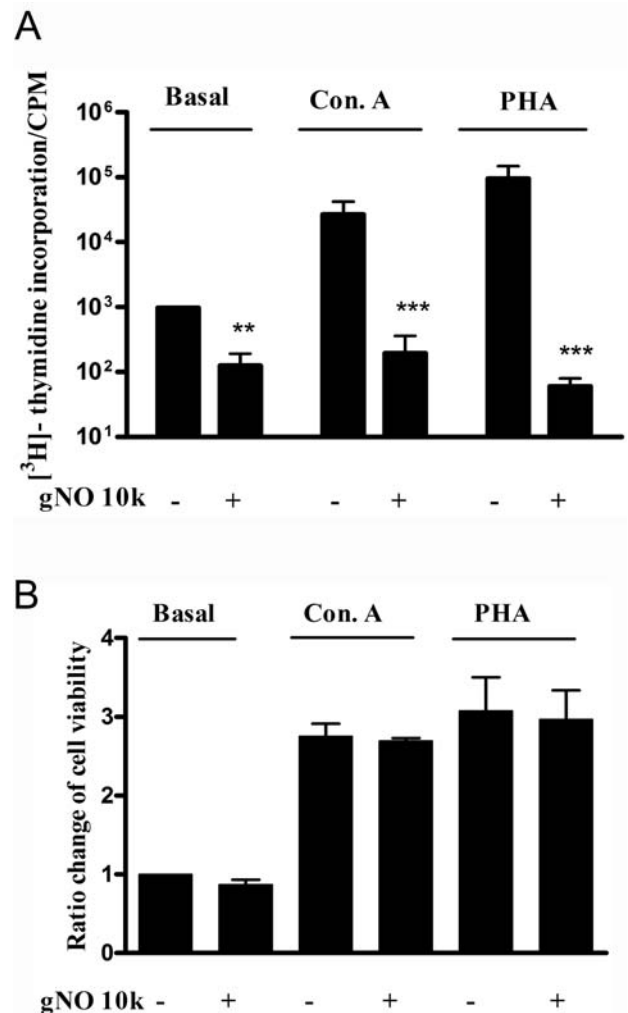
Immune cells such as lymphocytes play a major role in the wound healing process. To examine whether lymphocyte proliferation is influenced by gNO, human primary lymphocytes were isolated and remained either inactivated or activated with ConA and PHA. These cells were then exposed to a maximum dose of gNO 10 K ppm gNO with 10% O<sub>2</sub> for 10 min. The proliferation rate was examined by using [<sup>3</sup>H]-thymidine incorporation. The finding shows that gNO significantly decreased (10- to 100-fold) [<sup>3</sup>H]-thymidine incorporation into DNA of either inactivated or ConA (Figure 3A). and PHA-activated lymphocytes. This finding suggests that gNO has an anti-inflammatory effect on immune cells at this concentration and exposure time. To confirm the viability of the lymphocytes treated with 10 K ppm gNO (10 min), Trypan Blue staining was carried out. As demonstrated, exposure to high doses of gNO for 10 min did not have a significant toxic effect on these cells (Figure 3B).

### 3.4. gNO is not toxic for primary lymphocytes

To further evaluate the toxicity of gNO treatment, mouse spleen lymphocytes were isolated and analysed. Cells remained either unstimulated or stimulated with ConA and were exposed to 25 or 200 ppm gNO for 8 h or 10 K ppm for 10 min. Cells were incubated with 7-AAD, and the level of 7-AAD dye incorporation in the genome was evaluated by FACS analysis. The effects of 25, 200 and 10 K ppm of gNO on mouse spleen lymphocytes are demonstrated (Figures 5A and 5B). Based on the results shown, lymphocytes exposed to gNO at low (5 ppm) or high dose (10 K ppm) did not demonstrate any significant effect on cell viability.

### 3.5. Effect of gNO on the proliferation and viability of Jurkat cells

To further evaluate the potential toxic effects of gNO on immune and non-immune cells, a different type of cells was examined in the presence of gNO at different exposures times and concentrations. The rate of proliferation of Jurkat cells, a CD<sup>+</sup> cell line, was determined by detection of [<sup>3</sup>H]-thymidine incorporated. Furthermore, the viability of the cells was determined by Trypan Blue staining method. The numbers of the dead cells were unchanged in untreated, treated with gNO, and ConA (data not shown). To evaluate the effect of gNO on Jurkat cells, these cells were treated with ConA and then exposed with 5, 25, 75 and 200 ppm for 8 h or 10 K ppm gNO for 10 min. The proliferation rate was examined by using [<sup>3</sup>H]-thymidine incorporation into cellular DNA. Exposure of Jurkat cells to various concentrations of gNO did not significantly alter the proliferation capacity of these cells (Figure 4A). This finding indicates that Jurkat cells are likely to be resistant to this treatment. To identify the effect of gNO on viability of Jurkat cells, the cells were treated with 25 and 200 for 8 h or 10 K ppm for 10 min of



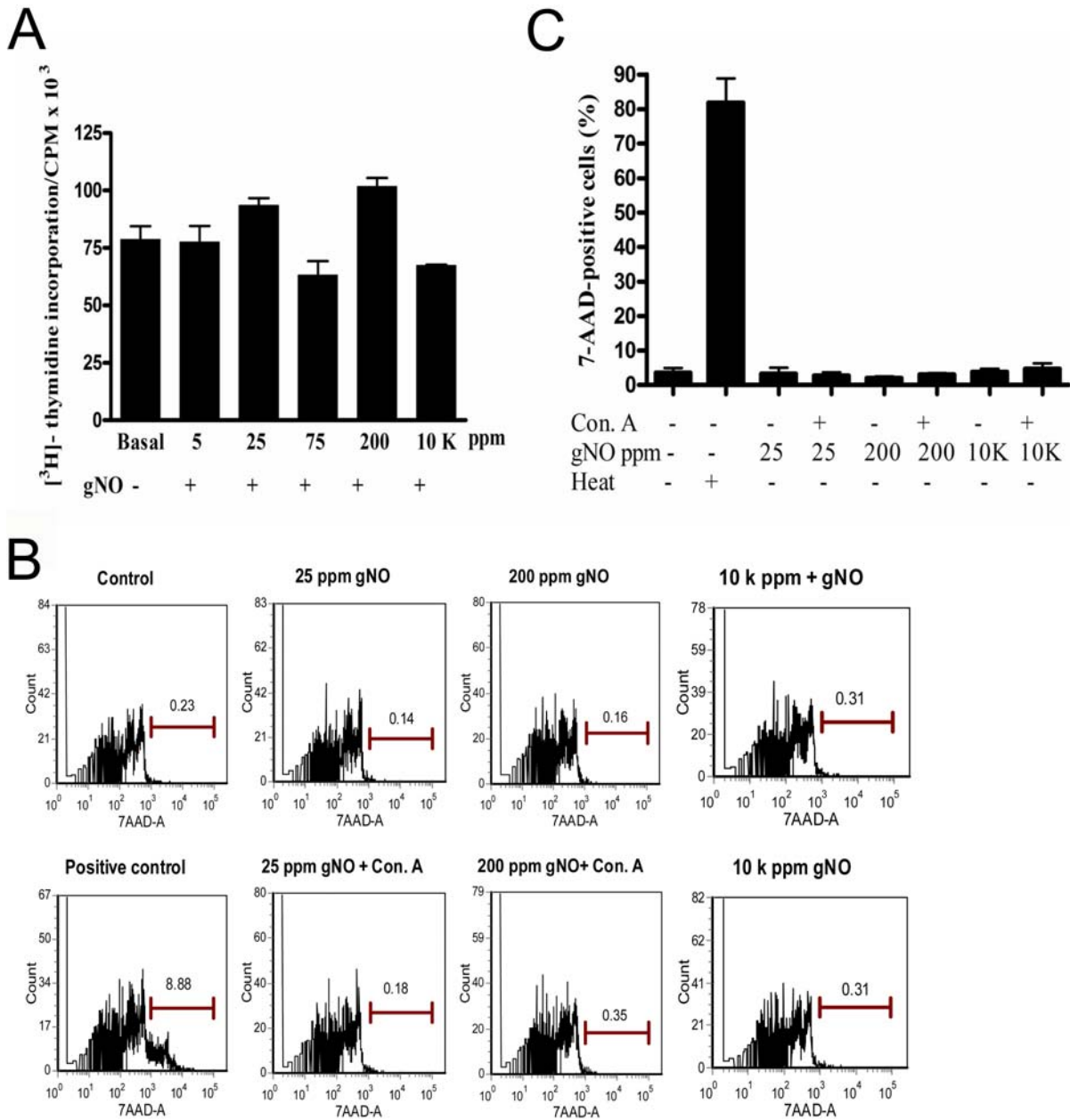
**Figure 3** Proliferation and viability of human peripheral lymphocytes in response to gNO

To evaluate the inflammatory effect of gNO, human peripheral lymphocytes were treated with 10 μg/ml of ConA or 1 μg/ml of PHA for 4 days. Then, cells were then exposed with 10 K ppm gNO with 10% O<sub>2</sub> in 10 min. Cells were then incubated at 37°C for 16 h. (A) [<sup>3</sup>H]-thymidine incorporation used as an index for cell proliferation rate. (B) The cell viability ratio compared with that of basal level examined by Trypan Blue staining. ConA, Concanavalin A; PHA, phytohaemagglutinin. Asterisk represents a *P*-value less than 0.05.

gNO followed by 10 μg/ml ConA for 16 h. Cells were then incubated with 7-AAD, and the level of 7-AAD dye incorporation in cell genome was evaluated by FACS analysis (Ikewaki et al., 2007; Wang et al., 2005). Based on the FACS analysis, gNO did not significantly influence the cell viability (Figure 4B). The effect of 25, 200 and 10 K ppm gNO on Jurkat cells in the presence and absence with ConA is demonstrated (Figure 4C).

## 4. Discussion

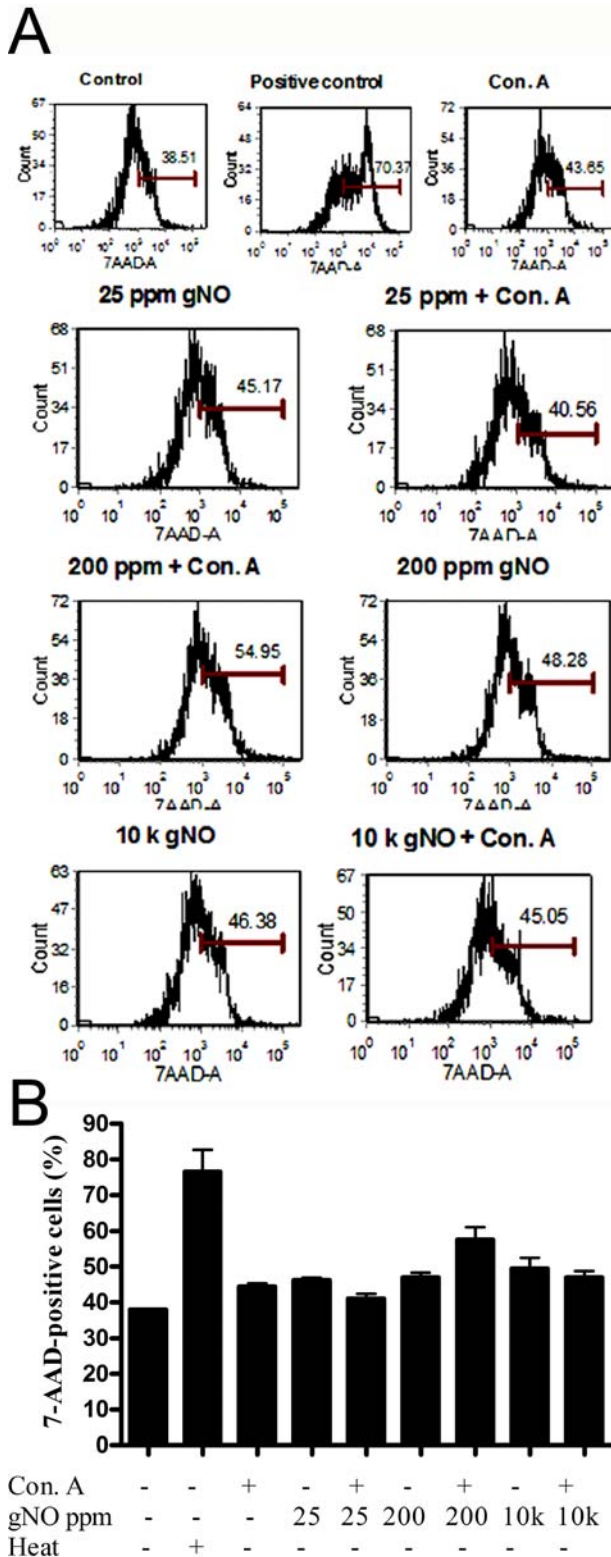
NO is a gaseous molecule produced by a iNOS enzyme that catalyses the oxidation of the amino acid L-arginine in mammalian cells (Marletta et al., 1998). NO displays anti-inflammatory and



**Figure 4** Effect of different concentrations of gNO on proliferation and viability of Jurkat cells  
 To evaluate the effect of gNO on proliferation and viability, Jurkat cells were treated with 10 µg/ml ConA for 16 h and then exposed to various gNO concentrations. The proliferation rate was examined by using [<sup>3</sup>H]-thymidine incorporation. The viability of Jurkat cells was also examined with FACS analysis using 7-AAD staining. (A) The result of the proliferation tests. The cells were treated with 5, 25, 75, 200 ppm gNO for 8 h and 10 K ppm for 10 min and incorporation of [<sup>3</sup>H] was measured by scintillation counter. (B) The results of the viability tests in which Jurkat cells were treated with 25 and 200 ppm of gNO for 8 h and 10 K ppm for 10 min, in the presence and absence of 10 µg/ml ConA for 16 h. The viability of cells was determined by FACS analysis using 7-AAD staining. The results from FACS analysis are quantified and demonstrated (C). Negative control are untreated cells, and positive control are cells killed by heat.

antimicrobial properties and plays a significant role in wound healing (Marletta et al., 1998; Shabani et al., 1996; Witte and Barbul, 2002; Witte et al., 2002; Zhu et al., 2008) and infection (Ghaffari et al., 2006). In previous studies (Ghaffari et al., 2006, 2007), we used 200 ppm to examine the gNO effect on both bacteria and human cells. In those studies, we demonstrated that 200 ppm gNO for 8 h/day for 3 consecutive days significantly

reduced wound bacterial cells without compromising the viability and function of skin cells. To increase the clinical therapeutic practicality, different doses of gNO (5 to 10 K ppm) for 8 h and 10 min were used, respectively. We treated human lymphocytes and fibroblasts with various concentrations of gaseous nitric oxide (5, 25, 75, 200 and 10 K ppm) to evaluate its effect on more than 113 ECM mRNA modulation and also lymphocyte proliferation and



**Figure 5** The effect of gNO on the viability of the mouse lymphocytes. Mouse lymphocytes were isolated from spleen according to the procedure described in the Materials and methods section. Lymphocytes were left either untreated or treated with 25 and 200 ppm of gNO with 10% O<sub>2</sub> for 8 h and 10 K ppm for 10 min in the presence or absence of 10 μg/ml ConA for 16 h. The cell viability was determined by FACS analysis using 7-AAD staining. (A) The FACS analysis data and (B) the FACS analysis results with a bar graph. Negative controls are untreated cells, and positive controls are cells killed by heat. ConA, Concanavalin A.

viability functions. Based on our results, exogenous gNO has no significant effect on the majority of ECM gene expression in fibroblasts over 10 min to 8 h. The slightly modulated level of Col 5A3, Col 6A1 and ITGB1 and MMP-1 gene expression in microarray results were confirmed only for Col 5 A3 and MMP-1 with RT-PCR results. The Col 5A3 mRNA expression level is in line with previously reported studies (Schaffer et al., 1999; Masters et al., 2002; Witte et al., 2002). However, some reports demonstrated that NO increased collagens in the skin, but alternatively, it has been reported by Witte and Barbul (2002), where a stimulatory effect of NO observed on collagen synthesis at low concentrations while abrogated at higher doses. The biphasic action of NO and its metabolites on cell proliferation of keratinocyte has been studied. The result showed that NO increased in keratinocyte proliferation at low concentrations, whereas the pattern changed at higher doses of NO (Krischel et al., 1998). Interestingly, regarding this article, the biphasic effect of gNO is on keratinocytes not on fibroblast. This phenomenon might be related to cell differentiation status. Therefore, this result supported our finding. We further evaluated a very wide range of concentration in various time points of gNO, and we did not observe toxic effect on fibroblasts and immune cells. The gNO has broad-spectrum antimicrobial effect (Saura et al., 1999; Weller et al., 2001; Miller et al., 2004; Ghaffari et al., 2007; Soll, 2009). The role of gNO has been investigated by delivery of free medical grade of gNO (Ghaffari et al., 2006) or by a probiotic patch system (Jones et al., 2010). Therefore, the effect of different concentrations of gNO on modification of genes was a major goal of this investigation. Based on our finding, there is no significant variation of gene expression level at the ECM genes. However, the effect on genes, such as the genes which are involved in cell cycles and growth factors, should be investigated.

Recent publications have revealed mechanisms that may account for gNO effects on membrane and intracellular targets, which might be significant in expression of ECM genes. For instance, Miersch et al. (2008) showed that NO increases intracellular cGMP level, and cGMP signalling pathway is involved in collagen expression in response to gNO (Hsu et al., 2007). In the classical signal transduction pathway, NO markedly increases the catalytic activity of GC (guanylyl cyclase). Stimulation of GC by NO increases intracellular cGMP level and then activation of several effector molecules such as cGMP-dependent protein kinases, cGMP-regulated phosphodiesterase and cGMP-gated ion channels.

In this study, we tested the effect of 10 K ppm gNO for a 10-min exposure time on proliferation and viability of Jurkat cells, a CD4 positive T cell line as well as primary human lymphocytes. Our results showed that gNO inhibits primary lymphocyte proliferation at a concentration of 10 K ppm. These findings suggest that gNO might have an anti-inflammatory effect. It also appears that the anti-inflammatory effect of gNO is more pronounced in primary lymphocyte cells than immortal cell lines such as CD4+ Jurkat cells. However, gNO does not compromise the viability of Jurkat cells and primary human lymphocytes. Previous studies have demonstrated that NO inhibits proliferation of different cell types such as vascular smooth muscle cells, mesangial cells and also reduces lymphocyte proliferation in high concentration (Garg and Hassid, 1989a, 1989b; Liew, 1995). Sun

et al. (2005) previously revealed that an NO donor, SNAP (S-nitroso-*N*-acetyl penicillamine) did not affect Jurkat cell viability, but reduced the cell adhesion. The anti-inflammatory effect of NO has also been demonstrated by Young et al. (2001). They have shown that caveolin-1, a cell membrane protein, reduces PMN (polymorphonuclear neutrophil) infiltration by maintaining NO release from cardiac endothelium. The results showed that gNO treatment suppresses the proliferation of immune cells, indicating an anti-inflammatory effect without compromising cell viability. The mechanism of antiproliferation effect of gNO is unclear. However, it has been suggested that NO uses MAPK pathway to decrease cell proliferation (Maeda et al., 2000).

In summary, we have shown that gNO modulates the expression of some of ECM genes slightly, whereas the majority of the genes that are playing a key role in wound healing process appear to be unaffected. This indicates that gNO therapy could be evaluated as a novel antimicrobial agent in infected wounds without impairing the wound healing process. Results presented also revealed that gNO treatment should have minimal effect on host's immune response, as exposure to gNO appears to reduce primary lymphocyte proliferation without compromising their viability.

#### Author contribution

Alireza Moeen Rezakhanlou was in charge of conducting the study and carried out cell culture, SuperArray, RT-PCR and FACS. Chris Miller was involved in developing the idea and use of different doses of gaseous NO for treatment of cells. Bevin McMullin contributed in setting up and controlling the gaseous NO chamber. Abdi Ghaffari was involved in analysing SuperArray results and writing the manuscript. Rose Garcia arranged FACS experiments and analysed the data generated from FACS. Aziz Ghahary supervised the project.

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