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Original Article

A phase I clinical study of inhaled nitric oxide in healthy adults

Chris Miller[a, b,*], Minna Miller[b], Bevin McMullin[a, c], Gilly Regev[a], Lena Serghides[d], Kevin Kain[d], Jeremy Road[a], Yossef Av-Gay[b]

[a] *Respiratory Division, Department of Medicine, University of British Columbia, Vancouver, Canada*
[b] *Division of Infectious Diseases, Department of Medicine, University of British Columbia, Vancouver, Canada*
[c] *Respiratory Services, Vancouver, Canada*
[d] *SA Rotman Laboratories, McLaughlin-Rotman Centre for Global Health, Tropical Disease Unit, Division of Infectious Diseases, Department of Medicine, University Health Network-Toronto General Hospital and University of Toronto, Canada*

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Abstract

Background: Nitric oxide (NO) is an approved pulmonary vasodilator for neonates and full term infants up to a dose of 80 ppm. At 100 ppm to 200 ppm, NO has potent antimicrobial activities *in vitro* and in animal studies which suggest its therapeutic use for infectious diseases in humans. However, whether inhaled NO is safe at 160 ppm in healthy human adults is unknown. The aim of the phase I study was to assess the safety of delivery and the physiologic effects of intermittent 160 ppm NO in healthy human adults.
Methods: Ten healthy adult volunteers (5 males, 5 females; 20–62 years) were recruited and inhaled 163.3 ppm (SD: 4.0) NO for 30 min, 5 times daily, for 5 consecutive days. Lung function and blood levels of methemoglobin, nitrites/nitrates, prothrombin, pro-inflammatory cytokines and chemokines were determined before and during treatment.
Results: All individuals tolerated the NO treatment courses well. No significant adverse events occurred and three minor adverse events, not attributable to NO, were reported. Forced expiratory volume in 1 sec % predicted and other lung function parameters, serum nitrites/nitrates, prothrombin, pro-inflammatory cytokine and chemokine levels did not differ between baseline and day 5, while methemoglobin increased significantly during the study period to a level of 0.9% (SD: 0.08) ($p<0.001$).
Conclusion: These data suggest that inhalation of 160 ppm NO for 30 min, 5 times daily, for 5 consecutive days, is safe and well tolerated in healthy individuals.

Keywords: Phase I; Nitric oxide; Antimicrobial; Safety; Methemoglobin; Lung function

1. Introduction

In patients with cystic fibrosis (CF), life-threatening chronic microbial lung infections are the leading cause of morbidity and mortality [1]. The high prevalence of these infections is linked to mutations in the epithelial chloride channel, CF transmembrane conductance regulator (CFTR) which impair various mechanisms of innate immunity [2]. Early antibiotic eradication treatment of CF patients for the most prevalent bacterial pathogen, *Pseudomonas aeruginosa*, has considerably increased the life expectancy in CF [3], however still the vast majority of adult CF patients suffer from chronic *P. aeruginosa* lung infections which are difficult to treat due to biofilm formation and the development of antibiotic resistant clones [3]. Also among other species, found in CF airways, antibiotic resistant strains are present including methicillin-resistant *S. aureus* (MRSA), members of the *Burkholderia cepacia* complex, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, non-tuberculous *mycobacteria* (NTM) species and various strict anaerobic bacteria [4,5]. In the light of a

* Corresponding author at: Division of Infectious Disease, University of British Columbia, Rm D433, HP East, Vancouver Hospital, 2733 Heather Street, Vancouver, BC, Canada V5Z-3J5. Tel.: +1 778 899 0607; fax: +1 604 875 4013.
E-mail address: miller42@mail.ubc.ca (C. Miller).

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onerous development of potent chemotherapeutic agents [6] and the unavailability of vaccines against the major CF pathogens in the last decade [7], it becomes more and more difficult to prevent or treat these and other infections with the existing classes of antimicrobial drugs. Thus, there is an unmet need to develop alternative treatment strategies for patients with CF and other patient groups at increased risk for microbial infections. The use of gaseous nitric oxide (NO) might be such an alternative.

NO is an essential part of the innate defense mechanism of the human immune system [8,9] which becomes up-regulated by the inducible NO synthase (iNOS) during various inflammatory conditions and microbial infections [10]. In chronically inflamed and infected CF patients, however, exhaled NO levels remain low [11], a phenomenon which has led to nebulized L-arginine supplementation therapy [12]. Inhalation of L-arginine transiently improved pulmonary function in CF through NO formation [12], while the inhalation of NO containing gas in concentrations of 100 ppb to 40 ppm had no immediate effect on lung function [13].

At 160–200 ppm, NO has also been shown *in vitro*, *ex vivo* and in animal models [14–20] to exert potent antimicrobial effects against a broad range of microbes, suggesting its use in appropriate concentrations in CF patients. At present, however, inhalation of NO as a selective, short acting vasodilator is approved only at 80 ppm for use in full term infants with hypoxic respiratory failure associated with pulmonary hypertension [21,22]. Thus, higher antimicrobial doses of NO for treating CF patients requires phase I safety studies in healthy human individuals.

Potential side effects of high dose NO treatment include the binding of NO to hemoglobin, forming methemoglobin, which could lead to decreased oxygen transport and the capacity of NO to act as a nitrosylating agent on proteins and other cell constituents [23–25]. Thus, concerns have been raised regarding the potential use of NO as a microbicidal agent in various clinical scenarios [26].

Here we report on a prospective phase I open label safety study in 10 healthy adults, who inhaled 160 ppm gNO for 30 min, five times a day, for 5 consecutive days. Neither significant adverse events nor adverse events attributable to NO occurred and all individuals tolerated the NO treatment courses well. Forced expiratory volume in 1 sec (FEV1) % predicted and other lung function parameters and serum nitrites/nitrates, prothrombin, pro-inflammatory cytokine and chemokine levels did not differ between baseline and day 5, while methemoglobin levels increased during the study period to a level of 0.9%. We conclude that inhalation of 160 ppm NO for 30 min, 5 times daily, for 5 consecutive days, is safe and well tolerated in healthy individuals.

2. Individuals, materials and methods

2.1. Study design

Ten healthy adult volunteer subjects (5 males, 5 females), aged 20 to 62 years, were enrolled in the study after screening their medical history, a physical examination, pulmonary function tests and blood values. Exclusion criteria included individuals less than 19 years of age, pregnant females and unwilling to practice birth control during the study, diagnosed with pulmonary disease, epistaxis, hemoptysis, methemoglobinemia, organ transplant recipient or receiving antibiotic therapy. After obtaining informed consent, treatment was initiated within 5 days of enrollment. Subjects were housed in a hospital ward and received 160 ppm NO for 30 min every 4 h, five times a day, for 5 consecutive days by inhalation. Subjects were administered NO through a modified disposable mouthpiece to maximize mixing. Inspiration was spontaneously initiated by the subject from a conventional intermittent positive pressure breathing respirator (Mark-7,Carefusion, USA) in fixed flow mode delivering 48 l per minute (LPM). Flows of gas were verified with a calibrated mass flow meter (TSI, USA). Nitric oxide (INOmax, Ikaria, USA) of 800 ppm nitric oxide at 12 LPM was titrated into a distal delivery port on the mouth piece connected to the respirator during inspiratory phase only (pressure switch). The Mark 7 respirator was supplied by an air/oxygen blender (Bird Sentry, Carefusion, USA) set to deliver 26% oxygen. Subjects returned for follow-up evaluations 3, 7 and 21 days after the final NO administration. Subject safety was determined by monitoring vital signs, methemoglobin levels, lung function, blood chemistry, hematology, prothrombin time, inflammatory cytokine/chemo-kines levels and endothelial activation. These parameters were compared to baseline and at various timepoints during and after NO administration. The protocol was approved by the University of British Columbia clinical research ethics board, Vancouver Coastal Health Research Institute and the Therapeutic Products Directorate of Health Canada (TPD, CTA Number 129958). In addition, all components of the NO delivery system were approved by the Therapeutic Product Directorate of Health Canada.

2.2. Monitoring of NO, NO_2, O_2 and methemoglobin levels

The target gas mixture was 160 ppm gNO with a nitrogen dioxide (NO_2) less than 5 ppm and an oxygen (O_2) level between 21% and 25%. Inspiratory NO, NO_2 and O_2 levels were continuously monitored by sampling from the mouthpiece sample port located about 6 millimeters from the mouth of the subject with an AeroNOx (Pulmonox, AB, Canada) NO, NO_2 and O_2 electrochemical analyzer. Delivery safety was determined by the number of occasions that NO_2 exceeded 5 ppm, NO exceeding 10% variation and O_2 dropping below 20% during NO treatments. A commercially available noninvasive pulse oximeter (Rad 57, Masimo Corporation, USA) was used to measure methemoglobin saturation (SpMet). These parameters were measured continuously during every NO treatment course and for 3.5 h after the first treatment of the day. Daily serum samples were collected and frozen at −80 °C and the serum nitrite/nitrite level was measured using the Griess reagent [27].

2.3. Pulmonary function tests

Subjects had full pulmonary function tests (PFT) performed including lung diffusing capacity (DLCO) by a trained technician utilizing a calibrated pulmonary function system (Jaeger MasterScreen, VIASYS Healthcare, USA) on screening and

days 2, 8, 12 and 26. Bedside spirometry (Microloop by Micro Medical, England) was performed on days 1, 3 and 4. Effect of NO on lung function and DLCO was determined by changes between baseline, treatment days and follow up days.

2.4. Oxygenation and vital sign measurements

Full physicals were performed by a pulmonary physician on screening and on days 8, 12 and 26. Abbreviated physical examination by a registered nurse was done each day prior to initiation of treatments on days 1–5. Oxygenation was measured with a pulse oximeter (Rad 57, Masimo Corporation, USA) which was used according to manufacturer's guidelines to measure functional oxygen saturation of arterial hemoglobin (SpO_2) and heart rate. These parameters were measured continuously during every NO treatment and for 3.5 h after the first treatment of the day. Cardiovascular status was determined by monitoring heart rate, blood pressure, respiratory rate and temperature. Values were recorded prior to the start of each treatment, following a 5-min rest. During treatments, vital signs (except temperature) were also performed 15 min after the start of the treatment and at the end of NO treatment and recorded. After the first treatment each day, vital signs were recorded at every 30 min until the start of the second treatment of the day.

2.5. Blood chemistry, hematology and inflammation measurements

Hematological assessment included a complete blood count and differential (hemoglobin, hematocrit, red blood cell count, white blood cell count, white blood cell differential, and platelet count). The blood chemistry profile included serum creatinine, and liver function tests: aspartate aminotransferase (AST) serum glutamic oxaloacetic transaminase (SGOT), alkaline phosphatase, and gamma-glutamyl transferase (GGT). The effect of NO on coagulation was determined by the prothrombin time (PT) and its derived measures of prothrombin ratio (PR) and international normalized ratio (INR). Heparinized plasma was collected at baseline and on days 1, 2, 4, and 5 of NO treatment, and on follow-up days 3, 7 and 21 and frozen at −80 °C. Plasma cytokine levels were assessed using the human inflammation cytokine bead array kit (BD Bioscience, Canada). Plasma levels of angiopoietin (Ang)-1 and Ang-2 were determined by ELISA (R&D Systems, USA).

2.6. Statistical analysis

Descriptive characteristics of the subjects prior to, during, and at the end of the study were tabulated and expressed as mean± standard deviation (SD). Differences in continuous variables (methemoglobin, serum nitrate and SpO_2 levels) over the course of the study were analyzed utilizing repeated measures analysis of variance. Categorical events (number of subjects with a particular adverse event) were determined by constructing 95% confidence limits for their incidence. Differences between important continuous variables at two specific times were evaluated with the paired *t*-test. Important categorical events such as clinical pulmonary function and lung diffusion changes, changes in serum inflammatory markers, hematology, clinical chemistry and incidence of adverse events were analyzed by constructing 95% confidence limits for their incidence. The data were analyzed using the unpaired Mann–Whitney test for comparison between any two groups and ANOVA for repeated measures of variance. Baseline comparisons were analyzed by repeated measures ANOVA with Bonferroni post test for parametric data, or Friedman test with Dunn's post test for non-parametric data. Data analysis and graphical presentation were done using a commercial statistics package (Graphpad-Prism V 3.0, GraphPad Software Inc., USA). Unless otherwise specified, $p<0.05$ indicated statistical significance. Results were represented by mean±SD from at least three independent measurements.

3. Results

3.1. Adverse effects

We investigated the safety to deliver repeatedly a concentration of 160 ppm NO into airways of 10 healthy adult individuals without unacceptable adverse effects including excessive NO_2 levels, while maintaining acceptable arterial hemoglobin oxygen saturation (SpO_2). A total of 250 NO treatments were administered to the 10 subjects during the study period. All treatments were well tolerated and no significant adverse events were observed. Three minor adverse events were reported: One subject reported bruising of the arm from multiple attempts to successfully draw blood, while two other subjects reported a numbing sensation of the tongue during NO treatment. This was resolved by instructing the subject to relax and reposition the mouth piece.

3.2. NO, NO_2 and O_2 levels

A total of 750 measurements of NO were recorded during the study. The average inspired NO was 163.3 ppm (SD=4.0). The highest NO concentration recorded was 177 ppm. The highest NO_2 level recorded during the treatments was 2.8 ppm (mean: 2.32; 95% confidence level: 2.17–2.47 ppm) and none of the subjects experienced a NO_2 level>5 ppm. This was consistent with the performance specifications provided by the manufacturer of the apparatus of 1.56 ppm (SD=0.3). Of the 300 recorded oxygen values, the average oxygen % was 22.0% (SD=0.22).

3.3. Vital signs and clinical safety

During and after the NO exposure, all vital signs remained within normal limits for age and with respect to baseline values. Specifically, there was no drop in blood pressure (potentially due to the vasodilator effect of NO) during or after NO treatments. No sudden incidences of hypoxemia (<85% SpO_2) were observed during or after NO administration. The lowest observed SpO_2 was 93%. SpO_2 levels over time decreased slightly between the pretreatment and post treatment but neither differed significantly statistically nor clinically. ANOVA analysis ruled out that this

decrease was associated with the five repeated exposures to NO over the course of the same day. All 930 recorded methemoglobin percent levels (SpMet) remained below 5%. The initial baseline SpMet was 0.16% (SD=0.10). The highest SpMet was observed at the end of the 30 min treatment and was 2.5% with an average increase of 0.9% (SD=0.08). SpMet increased as predicted by ~1% between pretreatment and post treatment ($p<0.001$) and returned to baseline after 3.5 h prior to the next NO treatment (Fig. 1A). ANOVA analysis ruled out that this increase was associated with repeated treatments on the same day. There was no accumulative effect on SpMet after five daily treatments for 5 consecutive days (Fig. 1B). Follow-up SpMet measurements on 3, 7 and 21 days after the final NO exposure on day 5 did not demonstrate any residual increase in SpMet. Methemoglobin is reduced by an enzymatic reductase resulting theoretically in an increase in blood nitrite/nitrate levels. However, no significant differences in serum nitrite/nitrate levels from baseline were observed during the trial. One subject had significantly higher peak nitrite and nitrate values ($p<0.001$) which was also slightly different at baseline ($p=0.038$) compared to the other subjects.

There were no statistically, nor clinically significant changes in blood coagulation parameters, clinical chemistry and hematological parameters from baseline to completion of day 5. Although eosinophil cell numbers decreased during the study (baseline 0.15 giga/L; SD=0.12; end of study: 0.19 giga/L (SD=0.19), this difference was not significant ($p=0.104$). A 1% increase in neutrophil cell numbers from a baseline value of zero to 0.01 giga/L at the end of study was found, which also did not reach statistical nor clinical significance ($p=0.169$).

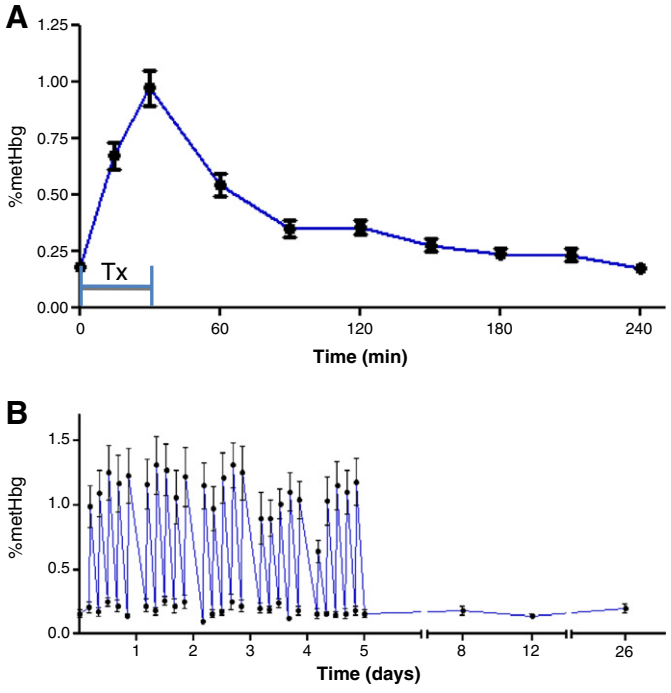

Fig. 1. Methemoglobin levels (%) before, during and after inhalation of 160 ppm nitric oxide in healthy human individuals. The 10 individuals performed 5 NO treatment courses daily, lasting 30 min each, for 5 consecutive days. Methemoglobin levels were measured using a pulse oximeter. A, Values represent pooled values of 250 individual 30 min NO treatment courses from 10 subjects and pooled values obtained at 120 min, 180 min and 240 min after NO treatment was discontinued. B, Values represent pooled values of 10 individuals at the beginning and end of 30 min NO treatment courses and pooled values obtained 8, 12 and 26 days after NO treatment was discontinued.

3.4. Pulmonary function, inflammatory cytokines and endothelial activation factors

Pulmonary function tests did not reveal any abnormalities for any subjects during and after NO treatments. Specifically, airflow as measured by FEV_1 and maximum mid-expiratory flow (MMEF) did not differ from baseline during the course of the study. Other lung function measurements such as DLCO, forced vital capacity (FVC), total lung capacity (TLC) and residual volume (RV) also did not change from baseline measurement (Fig. 2).

To assess whether NO treatment resulted in inflammation or endothelial activation we quantified cytokines and the vascular endothelium activation factors Ang-1 and Ang-2 in peripheral plasma at baseline and various time points thereafter. Cytokine levels of TNF, IL-6, IL-8, IL-10, IL-1b and IL-12p70 were unaffected by inhalation of NO as compared to baseline. Comparisons between baseline cytokine levels and levels at each of the sampling time points for all 10 participants resulted in no significant differences (compared by repeated measures ANOVA with Bonferroni post test for parametric data, or Friedman test with Dunn's post test for non-parametric data (Fig. 3). Furthermore, Ang-2 and Ang-2/Ang-1 ratios were not affected in this study (Fig. 4). Outlier data in Fig. 4 did not show any correlation with changes in any of the other parameters, and thus appears to be an isolated finding of unknown significance.

4. Discussion

Here we provide evidence that 160 ppm NO, a concentration previously identified as an effective antimicrobial dose against various microorganisms *in vitro*, *ex vivo* and in animal models [16,17,19], can be safely delivered to healthy human lungs in a pulsed manner for 5 consecutive days. No significant adverse events occurred and only three minor adverse events, not attributable to NO, were reported. Furthermore all vital signs remained well within acceptable clinical margins.

Our findings are similar and even slightly better compared to continuous inhalation of 80 ppm NO—the approved NO dose for inhalational use in full term infants—with regards to methemoglobin and NO_2 levels, most likely due to the intermittent dosing strategy utilized here. While in our study all 930 recorded SpMet levels remained below 5%, continuous delivery of 80 ppm NO was reported to rise to at least 5% with 35% of the subjects exceeding 7% [28]. Formation of methemoglobin has been also observed in previous studies of NO inhalation by healthy human individuals [23–25]. NO was inhaled for 3 h up to 128 ppm and 55 min at 512 ppm before methemoglobin reached 5% [25]. The rise in methemoglobin and its decay after cessation of NO inhalation in that study followed a first order pharmacokinetics model and clearance time-

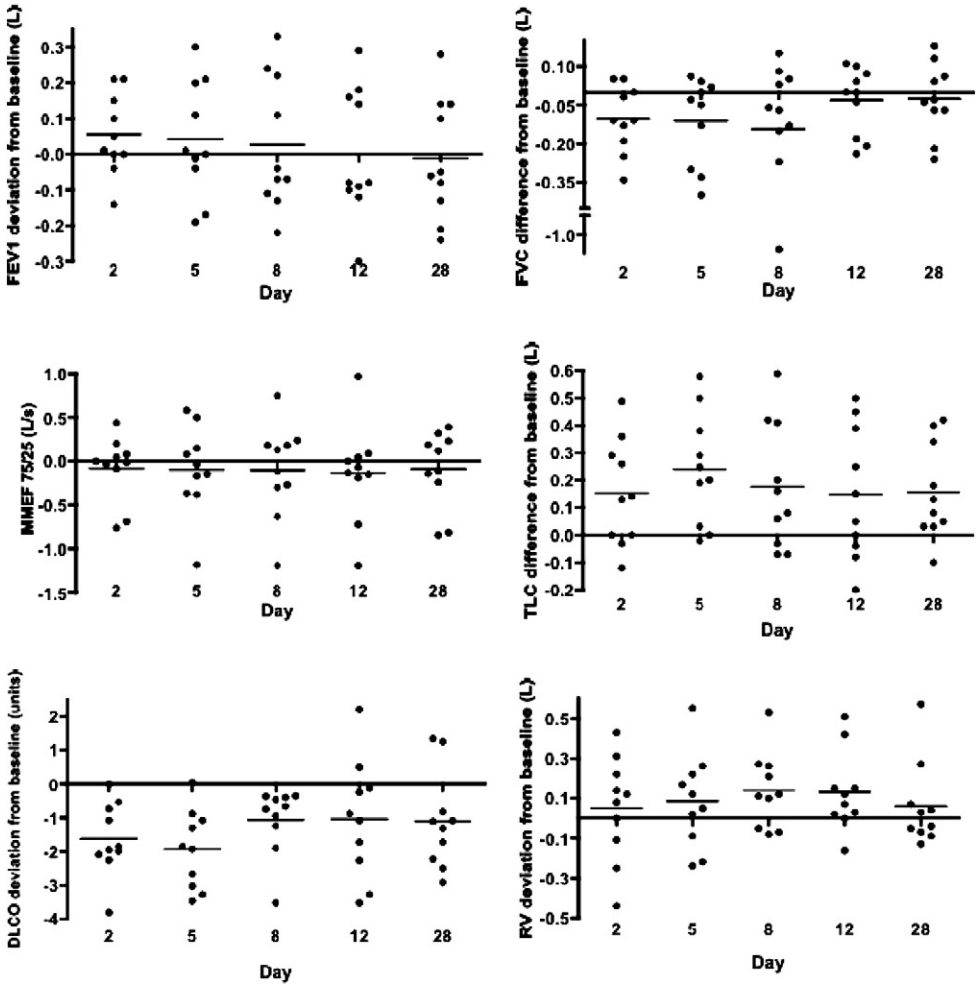

Fig. 2. Pulmonary function before, during and after inhalation of 160 ppm nitric oxide in healthy human individuals. Baseline values of pulmonary function tests were obtained within 7 days prior to NO therapy. Values during NO treatment were obtained on day 2. Further values were determined after the final NO treatment on day 5 and on days 8, 12 and 26. FEV_1: forced expiratory volume in 1 second; MMEF: maximum mid-expiratory flow; DLCO: carbon monoxide diffusing capacity; FVC: forced vital capacity TLC: total lung capacity; RV: residual volume. Data are presented as means of all ten subjects and absolute differences compared to baseline prior to NO treatment. Statistical differences were assessed by Mann–Whitney test.

constants lay between 39 and 91 min. The predicted half-life of methemoglobin in humans is approximately 1 h.

We hypothesized that administering high dose NO for 30 min followed by a 3.5 h break would allow methemoglobin levels to return to baseline between treatment courses [19]. The anticipated rise in methemoglobin during one treatment course was calculated to be 1%. The actual average rise in methemoglobin percent for the ten individuals for a single treatment course was consistent with these kinetic estimates at 0.9% considering the ± 1% absolute accuracy of the pulse oximeter. Our study also confirmed that the predicted 3.5 h interim period allowed the methemoglobin concentration to return to baseline, allowing five cycles daily for 5 days without a significant clinical increase in methemoglobin concentrations. Taken together, we have shown that our intermittent NO dosing strategy is safe with regard of methemoglobin production and metabolic burden.

Similar to methemoglobin levels, also the mean peak concentrations of NO_2 level in our study (2.8 ppm) was comparable with that observed during continuous delivery of 80 ppm (2.6 ppm) [28]. The limitations of this and other studies with regard to NO delivery are that the NO and NO_2 levels are only known at the entry point into the subjects' respiratory tract and the actual resulting levels of oxides of nitrogen in the lung are unknown. To date, studies indicate that acute pulmonary injury, pulmonary edema, hemorrhage, changes in surface tension of surfactant, reduced alveolar numbers and airway responsiveness may be caused by high airway levels of NO, NO_2 and other oxides of nitrogen [26]. It has been reported that human cells have significantly elevated thiol levels and can cope with high concentrations of NO better than prokaryotes [29]. We have reported that the same concentration level of NO that is cidal to microbes may be tolerated by the host cells [30]. This may not be surprising, considering that the body's innate defense mechanism relies on NO released from macrophage and neutrophils to act as one of the key non-specific antimicrobial agents [9,10]. Despite this resilience to nitrosative stress, it may well be prudent in future studies to screen subjects for thiol and methemoglobin reductase deficiencies.

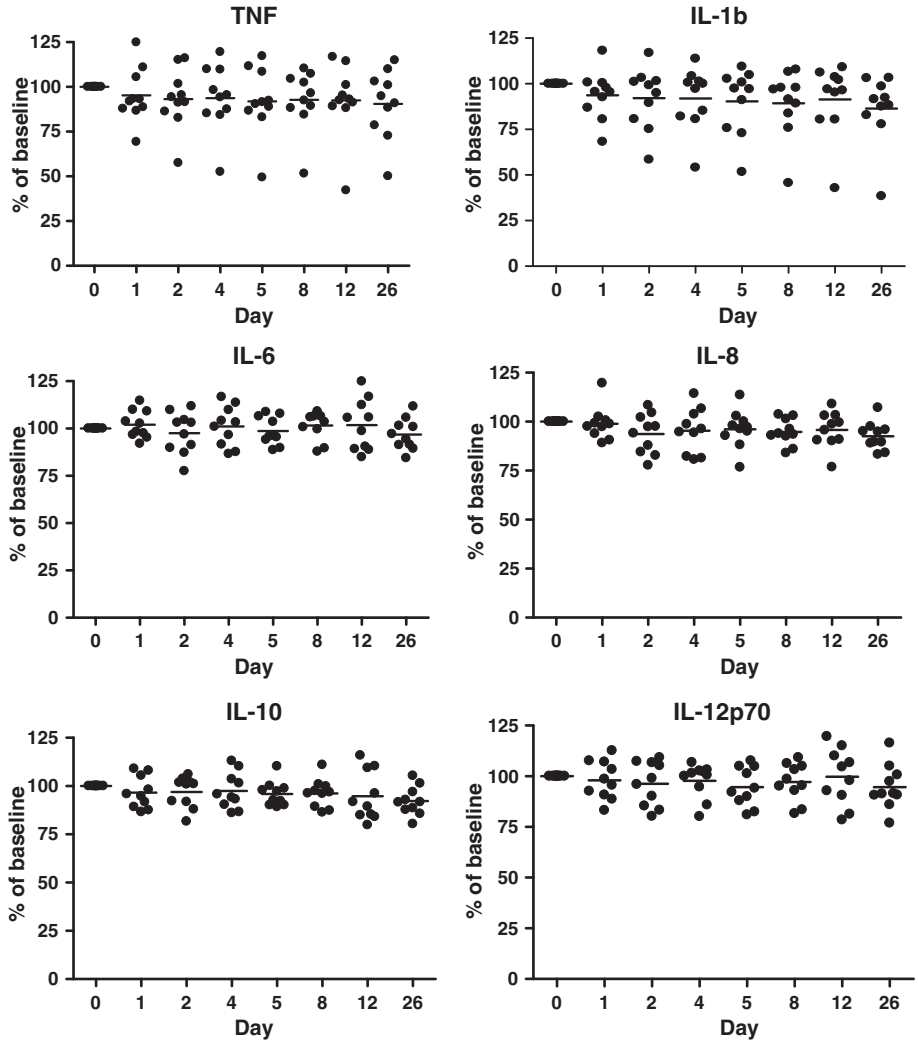

Fig. 3. Cytokine blood levels before and after inhalation of 160 ppm nitric oxide in healthy human individuals. Blood was collected within 7 days prior to NO therapy. Further blood samples were taken each day during NO treatment and on days 8, 12 and 26. Plasma levels of tumour necrosis factor (TNF)α, interleukin (IL)-1ß, IL-6, IL-8, IL-10 and IL-12p70 were determined by a cytometric bead array. Statistical differences were compared by repeated measures ANOVA with Bonferroni post test for parametric data (IL-6, IL-8, IL-10, IL-12p70), or Friedman test with Dunn's post test for non-parametric data (TNF and IL-1b).

Our study also demonstrates that 160 ppm NO, delivered as outlined, minimally impacts lung function. Specifically, acute airway inflammation, measured by determining flow rates, was not detectable. Possibly, potential deleterious airway reactivity could be masked by the ameliorative smooth muscle relaxation that can be exerted by NO [31]. In patients with pulmonary infection, high NO delivery might cause an increase in airway reactivity. However, the vasodilatory activity of NO may benefit the patient in addition to the antimicrobial activity of NO. The delivery of 160 ppm NO did not cause lung parenchymal injury, as measured by different lung function parameters. Likewise, plasma inflammatory cytokine levels, the earliest host responses to lung injury [32] and levels of eosinophils and neutrophils remained constant. In addition, the vascular endothelial activation factors Ang-1, -2 and the Ang-2/Ang-1 ratio [33,34] were unaffected by the NO treatment courses in our study.

Importantly, pulmonary function mechanics and inflammatory markers remained unchanged compared to baseline values in measurements 3 days and 28 days post treatment. While we cannot exclude the possibility that some longer term change may occur in lung function, the absence of any sign of inflammation in the post treatment period makes this unlikely. Serum inflammatory markers may possibly have not been sensitive enough to measure acute or even chronic changes in the lungs. Ideally, inflammatory markers from bronchoalveolar lavage (BAL) fluids would have been sampled but in our study it was not deemed ethical to perform BAL in the healthy volunteers enrolled.

The present results are the prerequisite for further NO inhalation studies in patients with lung infections such as patients with CF or COPD. For such studies, the currently approved dose of 80 ppm NO is presumably too low to exert bactericidal effects as shown by previous studies [35–37]. On the contrary, studies in animals reveal that 160 ppm NO is effective to reduce the pulmonary bioburden and leukocyte infiltration in a rat model of *Pseudomonas aeruginosa* pneumonia [38], and to

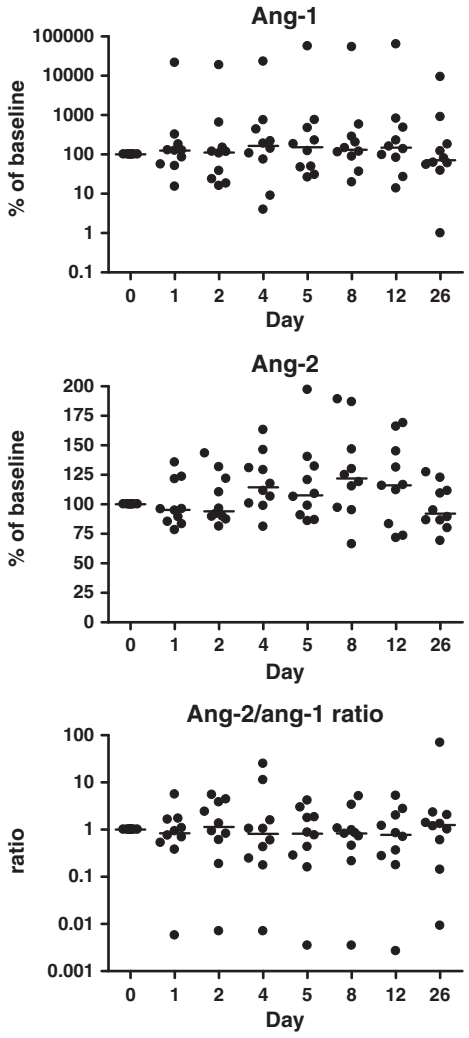

Fig. 4. Plasma levels of angiopoietin (Ang)-1 and Ang-2 before and after inhalation of 160 ppm nitric oxide in healthy human individuals. Blood was collected within 7 days prior to NO therapy. Further blood samples were taken each day during NO treatment and on days 8, 12 and 26. Plasma levels of Ang-1, Ang-2, and Ang-2/Ang-1 ratios were determined using a cytometric bead array. Statistical differences were assessed compared by Friedman test with Dunn's post test.

decrease the clinical symptoms of bovine respiratory disease in cattle [39]. Whether even doses higher than 160 ppm NO are needed to be bactericidal in atelectatic or consolidated areas of the lung in CF patients, has to be shown in further studies.

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