A novel method for the delivery of nitric oxide therapy to the skin of human subjects using a semi-permeable membrane

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Abstract

Nitric oxide (NO) is a mediator of essential biological processes, including vasodilation, antimicrobial activity and wound healing. A chemical system using sodium nitrite and ascorbic acid has been developed which generates significant amounts of NO. The originally described system was messy and impractical, and the high acidity may cause pain and further tissue damage in ulcerated skin. To overcome this, a selectively permeable, hydrophilic polyester co-polymer membrane system (Sympatex™) has been identified that can be placed between the NO-generating chemicals and the skin. The aim of the present study was to determine whether NO derived from this chemical system was able to diffuse through the membrane and have a measurable vasodilatory effect on forearm skin in healthy volunteers. The Sympatex™ 10 μm membrane was found to be highly permeable to NO, while preventing passage of the constituents of the NO-generation gel to the skin. The transmembrane NO-generation system had a vasodilatory effect comparable with that resulting from direct topical application. Additionally, the NO generated was effective in killing Staphylococcus aureus and Escherichia coli at doses lower than those required to increase skin blood flow. The vasodilatory and anti-microbial effects of this system may be useful as a patch-based topical therapy for skin ulceration, particularly when there is concomitant ischaemia and infection.

Introduction

Nitric oxide (NO) is generated from L-arginine via NO synthase enzymes and performs a variety of functions, including vasodilatation and host defence [1]. We have shown that NO is also manufactured on epithelial surfaces (such as in the mouth and stomach, and on the skin surface) in humans by sequential reduction of nitrate and nitrite [2]. This relies on the synthesis of nitrite by the bacterial reduction of inorganic nitrate present in saliva or sweat. Nitrite is further reduced to NO in an acidic environment, a reaction which is particularly enhanced in the presence of ascorbic acid:

\[
\text{NO}_2^- + \text{H}^+ \rightarrow \text{HNO}_2 \\
2\text{HNO}_2 \rightarrow \text{N}_2\text{O}_3 + \text{H}_2 \rightarrow \text{NO} + \text{NO}_2 \\
3\text{HNO}_2 \rightarrow 2\text{NO} + \text{NO}_3^- + \text{H}^+ + \text{H}_2\text{O}
\]

The combination of acid and nitrite is effective in killing a wide variety of pathogens [3,4], presumably by the generation of nitrogen oxides. It is likely that NO generated in this way has a significant role in host defence.

Key words: anti-microbial, blood flow, diffusion, infection, laser Doppler, nitric oxide, skin.
Abbreviations: LDF, laser Doppler fluxmetry.
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against microbial pathogens, many of which are known to be susceptible to this agent [5].

We have devised a system which mimics this endogenous mechanism of NO generation, using inorganic nitrite and an organic acid to produce NO on the skin surface. This method relies on keeping the components separate until applied directly to the skin. When used in this way, the system is effective in treating infectious skin disease [4] and increasing skin blood flow [6]. Individually, these components elicit no significant effects.

However, the previously described direct contact application was messy and impractical. In addition, we have noted previously in three patients with digital ulceration that the acidic nature of this NO-generating system causes pain and may cause further damage. To resolve these adverse aspects, we have devised a system intended to protect the skin from the acid contained in the nitrite/acid mixture, but which still allows the known actions of the NO generated, such as anti-microbial effects and vasodilatation. This involves the use of a selectively permeable, hydrophilic, polyester co-polymer membrane (Sympatex™ 10 µm membrane; Akzo Nobel, Wuppertal, Germany) to separate the acidic mixture from the tissue; this membrane still allows NO to pass freely into the skin.

Studies were designed to determine the efficacy of this novel NO delivery system using in vivo studies of human forearm to measure skin blood flow, and in vitro studies of the anti-microbial action of NO delivered through a gas-permeable membrane system. The topical application of such a system may be a useful therapy for skin ulceration or chronic wound infection, as it has been shown to be important in promoting effective wound healing [7–9] and exhibits potent anti-microbial properties [3,5].

**METHODS**

**Measurement of NO diffusion across gas-permeable membranes**

Nitric oxide was generated using sodium nitrite and ascorbic acid (eqn 1). The experimental arrangement is shown in Figure 1.

The constitutive ingredients were made up separately in 0.8% (w/v) agar. The Sympatex™ 10 µm membrane was placed over the sampling area of 1.33 cm², on to which 1 ml of each mixture was placed. The two agents were combined gently using a sterile swab stick, and the rate of NO diffusion was measured by passing air, free of NO and nitrogen dioxide, over the other side of the Sympatex™ and into the chemiluminescence NO meter.

The rate of NO generation was measured by a sensitive chemiluminescence meter (model 42C; Thermo Environmental Instruments Inc., MA, U.S.A.) connected to a data acquisition system and IBM computer, over a 20 h period. The gas phase in the lower collection chamber was sampled every 60 s.

The rate of release of NO from the gel surface (measured as nmol s⁻¹ cm⁻²) for 330 mM concentrations of sodium nitrite and ascorbic acid was measured, and compared with the rate of passage of NO through the Sympatex™ membrane.

**Skin blood flow experiment**

The protocol was approved by the East London and City Health Authority Ethics Committee, and subjects gave written informed consent prior to participation in the study, which was carried out according to the Declaration of Helsinki. The study group consisted of nine healthy male volunteers aged between 20 and 30 years. The subjects’ demographic data are summarized in Table 1. Subjects were non-smokers, normotensive, normo-cholesterolaeimic and normoglycaemic, with no evidence or history of auto-immune or vascular disease, and were not taking any medication.

All examinations were performed in a draught-free, temperature- and humidity-controlled laboratory (24 ± 1°C; relative humidity 30–40%) over a 2-week period. All subjects had a light breakfast, avoiding fatty foods, tobacco consumption and caffeine, and had abstained from vigorous exercise since the previous evening. The microvascular measurement techniques were non-invasive and the investigation was consistently reported to be painless. The volunteer was required to sit, lightly clad, with their arms supinated, slightly bent and supported by a foam stand at an angle of 30° to the horizontal.

Assessment of microvascular peripheral blood flow in the arm used laser Doppler fluxmetry (LDF) (model
DRT-4 laser Doppler fluxmeter; Moor Instruments Ltd, Axminster, Devon, U.K.). LDF is a technique that uses the Doppler shift of laser light of wavelength 780–810 nm to measure the flux (velocity × number) of red blood cells. IR light generated by the laser is directed to the tissue via an optical fibre; back-scattered light reflected from moving whole blood particles is then measured by one or more optical fibres [10].

The surface of one forearm was covered with Sympatex®; the other arm was in direct contact with the NO-generating gel. Selection of the arm that was to be in direct contact with the gel was determined by a sequence of random numbers. An integrated LDF probe was placed on the centre of the flexor surface of each forearm, with the centre equidistant from the lateral and medial borders, and marked on the skin to ensure accurate repositioning. Baseline measurements were taken for 3 min, after which 1 ml each of sodium nitrite (330 mM) and ascorbic acid (330 mM) was added to the surface of each arm (either directly on to the skin or on to the Sympatex®) and mixed to initiate NO generation (see eqn 1). Measurements of skin laser Doppler flux were then taken continuously for a further 50 min.

Preliminary studies have shown that KY jelly® containing sodium nitrite alone causes vasodilatation in the forearm skin of healthy volunteers; however, this is not a statistically significant increase and is not sustained. Additionally, KY jelly® containing ascorbic acid has no effect on skin microcirculation.

Statistical comparison between treatments was carried out using ANOVA.

Assessment of transmembrane antimicrobial activity
Nitric oxide was generated by the system described in the Introduction. The anti-microbial effects of NO were tested using Staphylococcus aureus NCTC9353 and Escherichia coli NCTC10148 using a range of concentrations of sodium nitrite and ascorbic acid (1000, 500, 330, 100, 50, 10, 5 and 1 mM).

A sample of 1 ml of an overnight culture of E. coli or S. aureus (mean counts of $8.8 \times 10^4$ and $4.95 \times 10^4$ colony-forming units respectively) was inoculated into 24 ml of 5% (w/v) agar in 1% (w/v) NaCl at 45 °C, which was then allowed to set. Discs of Sympatex® membrane (9 cm in diameter) were sterilized in 70% (v/v) ethanol and left to dry by evaporation in a laminar flow cabinet. Samples of 5 ml of 0.8% agar in 1% NaCl, containing sodium nitrite or ascorbic acid at concentrations of 1000, 500, 330, 100, 50, 10, 5 or 1 mM, were prepared.

In the centre of inverted Petri dish lids (sterile), 1 ml samples of each concentration of sodium nitrite and ascorbic acid were added and mixed. Disinfected Sympatex® membrane was placed over the top of this immediately using sterilized forceps, ensuring that it overhung the lid in all directions. The base of the Petri dish, containing the set inoculated agar, was placed upside down on top of the lid/mixture/Sympatex® membrane arrangement, ensuring that a 2–3 mm gap was left between the Sympatex® membrane and the inverted inoculated agar (Figure 2).

The apparatus was incubated overnight at 37 °C. The base of the Petri dish was then removed and the central area of agar was sampled by cutting a circle using a sterile plastic measuring cup. The agar was then homogenized by vortexing with sterile glass beads (1 mm diameter) for 4 min. Next, 10-fold serial dilutions were carried out, and the samples of each dilution were plated on to blood/agar plates. After incubation for 24 h at 37 °C, the resulting colony-forming units were then counted and percentage survival calculated.

RESULTS
Permeability of Sympatex® to NO
The Sympatex® 10 μm membrane was found to be highly permeable to NO (diffusion coefficient = 0.005 × 10⁻⁶ cm s⁻¹), while preventing passage of the
Figure 3  Permeability of the Sympatex\textsuperscript{TM} 10 \(\mu\)m membrane to NO
The concentration of sodium nitrite and ascorbic acid was 330 mM. PPM, parts per million.

Figure 4  Forearm microvascular flux responses to application of the NO generation gel in the presence and absence of a Sympatex\textsuperscript{TM} 10 \(\mu\)m membrane

Figure 5  Survival of E. coli and S. aureus after 24 h of exposure to NO diffused through a Sympatex\textsuperscript{TM} 10 \(\mu\)m membrane
\(\vartriangle\), E. coli (NCTC10148); \(\bigcirc\), S. aureus (NCTC9353). Values are medians (\(n = 3\)).

Transmembrane anti-microbial effect
Potent anti-microbial properties of NO were seen at concentrations of nitrite above 50 mM, resulting in complete killing of bacteria to below detectable levels. At lower concentrations, partial or no anti-microbial activity was seen, which was dose-dependent (Figure 5).

DISCUSSION
Chronic leg ulceration and wound infections are common disorders, which are placing an increasing strain on health service providers [11]. Chronicity is due primarily to the nature of the underlying causes of colonization with infective organisms and of local tissue ischaemia seen in diabetic subjects and in patients with peripheral vascular disease.

Current treatment regimens used for wound infection and leg ulceration include the administration of anti-
biotics, which may in time lead to the evolution of antibiotic-resistant strains of the organism, and the application of compression bandages as appropriate. Pathological changes in the microcirculation associated with ulceration are not addressed by current available treatments, which tend to rely on stimulating granulation of the wound [12].

With respect to this current problem in the management of wound infections and skin ulceration, and in view of the knowledge that NO has been shown to (1) increase microcirculatory blood flow [8], (2) kill a range of infective organisms [4,5,13], and (3) have a significant effect in promoting effective wound healing [7–9], we propose that the NO delivery system described in the present paper may be used in the treatment of such disorders.

The present study demonstrates an effective method for the delivery of NO to the skin surface using acidified nitrite. The system delivers sufficient NO to cause significant vasodilatation in vivo and to kill S. aureus and E. coli in vitro. An additional advantage of the system is that the potentially damaging acidic solution is prevented from coming into contact with the skin surface by the use of a Sympatex™ membrane, which may be developed into a self-contained delivery patch system.

The increase in microcirculatory blood flow seen in the arms covered with the Sympatex™ membrane confirms previous findings that NO has a vasodilatory effect [6]. The suggested mechanism of vasodilatation is that NO is able to diffuse 1–3 mm through the skin to the pre-capillary sphincters without being oxidized [10].

The baseline microcirculatory blood flow measurements in the two forearms were not significantly different. However, an increase in skin blood flow was noticeable almost immediately after the gels were applied, was maintained throughout the experiment and was statistically significant. The arm to which the NO-generating system was incorporated with the Sympatex™ membrane showed less of an increase in microcirculatory blood flow. These results are due to the membrane resisting the passage of the generated NO, therefore increasing the amount escaping to the atmosphere. Additionally, the Sympatex™ membrane may have altered the optical properties of the laser Doppler fluxmeter. It is evident from Figure 2 that the initial rate of increase was greater following the direct application of the NO-generating system. This finding demonstrates that the Sympatex™ membrane shows some resistance to the passage of NO.

The amount of NO released from this system is capable of initiating complete killing of two important pathogens, S. aureus and E. coli. Previous studies using acidified nitrite have shown that this combination is effective in killing a wide range of human pathogens, including Tinea pedis [4] and E. coli [5]. Reactive nitrogen intermediates are involved in the killing of S. aureus in human blood neutrophils [14]. The mechanism behind this NO-mediated killing is thought to involve oxygen and/or oxygen free radicals, which the NO encounters, leading to the production of nitrogen radicals [12]. The antimicrobial action of NO could also be attributed to the ease with which NO crosses cell membranes and inhibits respiratory chain enzymes via activation of sulphur–iron complexes [5].

These findings suggest that development of this NO-generating system may be particularly useful in the management of skin infections where there is ulceration and when exposure to acid should be avoided. The obvious clinical problem which may benefit from both increased skin blood flow and anti-microbial action is that of leg and foot ulcers, which are commonly resistant to conventional therapies.

Inhibition of NO synthase, the enzyme that mediates the production of NO from the amino acid l-arginine [1], impairs wound healing. NO may have benefits in the promotion of wound healing post-operatively, as it promotes angiogenesis, cell proliferation and migration, and anti-platelet activity [7]. Additionally, urinary nitrate excretion is elevated after incisional wounding [15] and in burns [16]. The topical application of the NO-generating gel and membrane may be of benefit to patients with leg ulceration and wound infection. It may also promote post-operative wound healing, and help to prevent wound infection in patients with burns and those with skin grafts.

Further planned studies involve obtaining mixed microbiological cultures from infected wounds and leg ulcers and exposing them to NO generated by the improved system. Additionally, the increase in microcirculatory blood flow may not necessarily equate to increased tissue oxygenation, particularly in areas of ulceration [17,18]. This is an area needing further investigation, with the use of a transcutaneous O₂ and CO₂ meter. The development of a patch system, allowing the ascorbic acid and sodium nitrite gels to mix when applied, would facilitate usage in a clinical environment and give patients the ability to self-administer. Another advantage of such a patch system would be to maximize the concentration of NO delivered locally, both prolonging and increasing anti-microbial and vasodilatory activities.

In summary, we have described a novel NO generation and delivery system. The significant development of this system over previous reports has been the identification and inclusion of a selectively permeable membrane, which allows the diffusion of the NO generated towards the skin. This delivery of NO to the skin significantly increases skin blood flow in healthy volunteers and completely lyses S. aureus and E. coli in vivo. The system may therefore be beneficial in the promotion of healing in skin ulceration, wound infection, burn injuries and skin grafts.
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REFERENCES


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