Antimicrobial efficacy and wound-healing property of a topical ointment containing nitric-oxide-loaded zeolites

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Topical delivery of nitric oxide (NO) through a wound dressing has the potential to reduce wound infections and improve healing of acute and chronic wounds. This study characterized the antibacterial efficacy of an ointment containing NO-loaded, zinc-exchanged zeolite A that releases NO upon contact with water. The release rate of NO from the ointment was measured using a chemiluminescence detection system. Minimum bactericidal concentration assays were performed using five common wound pathogens, including Gram-negative bacteria (*Escherichia coli* and *Acinetobacter baumannii*), Gram-positive bacteria (*Staphylococcus epidermidis* and meticillin-resistant *Staphylococcus aureus*) and a fungus (*Candida albicans*). The time dependence of antimicrobial activity was characterized by performing log-reduction assays at four time points after 1–8 h ointment exposure. The cytotoxicity of the ointment after 24 h was assessed using cultured 3T3 fibroblast cells. Minimum microbicidal concentrations (MMCs) for bacterial organisms (5×10^7 c.f.u.) ranged from 50 to 100 mg ointment (ml media)$^{-1}$; the MMC for *C. albicans* (5×10^4 c.f.u.) was 50 mg ointment (ml media)$^{-1}$. Five to eight log reductions in bacterial viability and three log reductions in fungal viability were observed after 8 h exposure to NO–zeolite ointment compared with untreated organisms. Fibroblasts remained viable after 24 h exposure to the same concentration of NO–zeolite ointment as was used in antimicrobial tests. In parallel studies, full-thickness cutaneous wounds on Zucker obese rats healed faster than wounds treated with a control ointment. These data indicate that ointment containing NO-loaded zeolites could potentially be used as a broad-spectrum antimicrobial wound-healing dressing.

INTRODUCTION

Nitric oxide (NO) is naturally produced by the immune system to combat a broad spectrum of pathogens (Jones *et al.*, 2010). Impaired wound healing has been associated with decreased levels of endogenous NO production (Rizk *et al.*, 2004), which suggests that exogenous NO has the potential to accelerate healing in such wounds. However, systemic delivery of NO is complicated by the short half-life of NO *in vivo* (Thomas *et al.*, 2001) and potential unwanted effects on non-wound physiology (Kröncke & Suschek, 2008). Controlled, localized delivery of NO through a topical wound dressing has the potential to reduce wound infections and improve healing of both acute and chronic wounds.

Several technologies have been proposed as controlled delivery vehicles for NO, including compressed gas cylinders that deliver NO directly to a wound (Ghaffari *et al.*, 2006, 2007; Miller *et al.*, 2004), nitrite creams that release a burst of NO in acidic environments (Ormerod *et al.*, 1999; Weller & Finnen, 2006), glassy matrix nanoparticles containing nitrites that undergo sugar-mediated thermal reduction to release NO in neutral environments (Friedman *et al.*, 2008), molecules such as diazeniumdiolates (Keefer, 2005; Shin *et al.*, 2007) and nitrosothiols (Li & Lee, 2010) that give off NO spontaneously and nanoporous materials such as zeolites and metal organic frameworks that adsorb NO gas and release it upon contact with moisture (Morris & Wheatley, 2008). Here, we report on a topical ointment that contains NO-loaded zeolites.

Zeolites are negatively charged nanoporous aluminosilicate structures that can be balanced using a variety of transition metal cations; these metal-exchanged zeolites are capable of storing more than 1 mmol NO (g zeolite)$^{-1}$ under anhydrous conditions (Wheatley *et al.*, 2006). Anhydrous zeolites have a high affinity for water, which causes NO to be displaced and released from the zeolite structure upon contact with moisture (Wheatley *et al.*, 2006). Previously published research has demonstrated that topically applied NO-loaded, manganese-exchanged zeolites in an aqueous cream base are capable of delivering NO to the dermis without the inflammatory effects that can be caused by acidified nitrite creams (Mowbray *et al.*, 2008). NO-loaded, cobalt-exchanged zeolites in electrospun polylactic acid polymer fibres are capable of releasing NO over a period of several hours (Liu & Balkus, 2009). The antibacterial efficacy of solid discs made from NO-loaded, zinc-exchanged zeolites mixed with a polytetrafluoroethylene polymer that release NO over periods of less than 1 h has been demonstrated (Fox *et al.*, 2010). The present study characterized the microbicidal efficacy of a topical ointment containing NO-loaded, zinc-exchanged zeolites that releases NO over several hours.

METHODS

Ointment formulation and storage. Zinc-exchanged zeolite A was loaded with NO gas according to the procedure described by Wheatley *et al.* (2006). NO-loaded zeolite powder (1.6 g) was combined with emulsifying ointment BP (3.2 g), resulting in a 33 % (w/w) NO–zeolite ointment. NO–zeolite ointment samples were vacuum sealed under inert gas in 3 ml nylon/poly bags to prevent the release of NO until the start of experiments. Stability under these storage conditions was observed for 4 months (data not shown). All samples used in these experiments were stored for less than 1 week.

Control zeolite ointment was prepared by mixing sodium zeolite A without zinc exchange or NO loading into emulsifying ointment BP to a final concentration of 33 % (w/w).

Measurement of NO release. The release profile of NO from NO–zeolite ointment was measured using a Sievers Nitric Oxide Analyser (NOA 280i; GE Analytical Instruments). Nitrogen gas was flowed through a sintered glass frit into a glass bubbler (Ace Glass) containing 15 ml 1 × PBS. Ointment samples weighing 25 mg were evenly spread over a 1 × 1 cm square of laboratory tissue (Kimwipe; Kimberly-Clark), forming a layer with less than 0.5 mm thickness. This ointment geometry is representative of a thin layer of ointment spread over skin or a wound. The ointment and tissue were placed directly into the PBS. Nitrogen gas flowed through the PBS and carried NO to the analyser as it was released from the ointment. The system was calibrated using standard volumes of PBS that had been equilibrated with 2000 p.p.m. NO gas in nitrogen gas (Airgas USA); the concentration of NO in each volume of PBS was calculated from the solubility of NO in PBS (1.75×10^{-6} mol ml^{-1} atm^{-1}) (Zacharia & Deen, 2005).

Concentration dependence of antimicrobial activity. Experiments were conducted to determine the minimum microbicidal concentration (MMC) of NO–zeolite ointment for micro-organisms incubated in growth media over a 24 h period. *Escherichia coli* (American Type Culture Collection [ATCC] 25922), *Acinetobacter baumannii* (ATCC 19606), *Staphylococcus epidermidis* (ATCC 12228) and meticillin-resistant

Staphylococcus aureus (MRSA, BAA1680) were cultured in tryptic soy broth to a concentration of 10^9 c.f.u. ml^{-1}. The concentration was adjusted to 10^7 c.f.u. ml^{-1} and 5 ml aliquots were placed in 50 ml conical tubes. Samples of NO–zeolite ointment and control zeolite ointment ranging in mass from 125 to 1000 mg [25–200 mg ointment (ml media)$^{-1}$] were added to each tube. All tubes were sealed with Parafilm, vortexed for 30 s and incubated for 24 h under gentle agitation at 37 °C. Samples of 100 µl from each tube were plated onto Trypticase soy agar and incubated overnight. Plates were inspected for colonies. The minimum amount of NO–zeolite ointment that yielded a plate with no colonies was considered the MMC. Because only 100 µl of each original sample was plated, a plate with no colonies indicated that the concentration of microbes was less than 10 c.f.u. ml^{-1}.

This procedure was repeated for *Candida albicans* (a gift from Dr Thomas Edlind, Drexel University College of Medicine, Philadelphia, PA, USA), except that yeast peptone glucose broth and agar were used instead of tryptic soy broth and Trypticase soy agar, and the initial concentration of organisms was 10^4 c.f.u. ml^{-1} instead of 10^7 c.f.u. ml^{-1}. All experiments were conducted in triplicate.

Time dependence of antimicrobial activity. Experiments were conducted to quantify the number of micro-organisms inactivated by NO–zeolite ointment across a range of exposure durations. *E. coli*, *A. baumannii*, *S. epidermidis* and MRSA were cultured in tryptic soy broth to a concentration of 10^9 c.f.u. ml^{-1}. The concentration was adjusted to 10^7 c.f.u. ml^{-1} and 5 ml aliquots were placed in three 50 ml conical tubes. Tubes were assigned to one of three treatment groups: (1) NO–zeolite ointment (0.5 g), (2) control zeolite ointment (0.5 g) or (3) no treatment (control). All tubes were sealed with Parafilm, vortexed for 30 s and incubated under gentle agitation at 37 °C. After 0, 2, 4, 6 and 8 h, 100 µl samples were removed from each tube and serially diluted five times in 100-fold increments. Samples of 100 µl of each dilution and undiluted media were plated onto Trypticase soy agar, and c.f.u.s were counted after overnight incubation. Because only 100 µl of each undiluted sample was plated, the minimum detectable concentration of organisms was 10 c.f.u. ml^{-1}.

This procedure was repeated for *C. albicans*, except that yeast peptone glucose broth and agar were used instead of tryptic soy broth and Trypticase soy agar, the initial concentration of organisms was 10^4 c.f.u. ml^{-1} instead of 10^7 c.f.u. ml^{-1}, and samples were plated at 0, 1, 2, 3 and 4 h. All experiments were conducted in triplicate.

Cytotoxicity testing. 3T3 fibroblast cells (ATCC) were seeded on 12-well Corning CellBIND culture plates at a density of 5000 cells cm^{-2}. After 72 h incubation at 37 °C and 5 % CO$_2$, 200 mg ointment was spread onto the polyester membranes of Corning Transwell inserts with 0.4 µm pores. Three inserts were treated with NO–zeolite ointment, three were treated with control zeolite ointment and three were left untreated. The inserts were placed into the 12-well plates, which each contained 1.5 ml growth medium (Eagle's minimal essential medium with 10 % horse serum; ATCC), and 0.5 ml growth medium was added to each Transwell insert. After 24 h incubation, the Transwell inserts were removed and the viability of the cells in each well was quantified using the AlamarBlue cell viability assay (Life Technologies). AlamarBlue is a trademark for resazurin (7-hydroxy-3*H*-phenoxazin-3-one 10-oxide), a blue and almost non-fluorescent dye that is converted by cellular respiration (thus, the viable cells) into resorufin (reduced form), a red and highly fluorescent product that can be measured fluorometrically. Briefly, 150 µl AlamarBlue reagent was added to each well and incubated for 2.5 h. A total of 100 µl medium from each well was transferred to a 96-well plate and fluorescence was measured using a Tecan Infinite 200 plate reader with excitation and emission wavelengths of 570 and 610 nm, respectively. This procedure was conducted five times.

Wound closure testing. The effect of NO on the wound closure rate was tested by applying NO–zeolite ointment to full-thickness cutaneous wounds in obese rats. This protocol was approved by the Institutional Animal Care & Use Committee at the Drexel University College of Medicine. Ten male obese Zucker rats were purchased from Charles River Laboratories. One full-thickness cutaneous wound was made on the left dorsal region of each rat using a scalpel and circular template (surface area 4.6 cm^2). All wounds were covered with a sterile transparent wound dressing (Tegaderm; 3M) for 24 h. Treatments were applied three times per week starting the day after wound surgery. Five rats received NO–zeolite ointment and five received control zeolite ointment; the amount of ointment applied was enough to cover the wound with a layer approximately 0.5–1.0 mm thick. All ointments were covered with a sterile Tegaderm dressing and the animals were wrapped with elastic bandaging (Vetrap; 3M) to prevent them from removing their dressings. Animals were killed 20 days after the surgery.

During the course of the study, all animals were supplied with food and water *ad libitum* and were housed in individual cages. During each dressing change, wounds were digitally photographed using a Fujifilm FinePix s700 digital camera with cross-polarizing filters to reduce surface reflection. A ruler was held in the imaging plane of each photograph to allow calculation of an absolute value for the wound area. The boundaries of each wound were manually traced using a computer mouse and Microsoft Paint software, and wound areas were calculated from the traced photographs using an image analysis code developed with MATLAB (MathWorks) software. This image analysis code counted the number of pixels within the traced wound boundary, and then calculated the wound area by approximating the size of each pixel from the image of the ruler in the imaging plane of each wound.

RESULTS

The purpose of this study was to understand the *in vitro* responses of various bacterial and fungal pathogens against NO–zeolite, and to evaluate antimicrobial efficacy, NO release over time and relative cytotoxicity.

NO release

The mean release profile of NO from three emulsifying ointment samples is shown in Fig. 1. The NO release rate peaked at 4.4 ± 1.2 nmol min^{-1} (mg zeolite)$^{-1}$ (mean $\pm$ SD) after 1 min exposure to water and fell to 0.06 ± 0.05 nmol min^{-1} (mg zeolite)$^{-1}$ after 3 h. Cumulative NO released after 3 h was 104 ± 14 nmol (mg zeolite)$^{-1}$.

## Concentration dependence of antimicrobial activity

MMCs [mg ointment (ml medium)$^{-1}$] for each organism after 24 h incubation with NO–zeolite ointment were as follows: *E. coli*, 100; *A. baumannii*, 100; *S. epidermidis*, 50; MRSA, 100; and *C. albicans*, 50. None of the control zeolite samples was microbicidal. Based on the cumulative NO release curve of NO–zeolite ointment (Fig. 1b), and assuming that the release of NO after 3 h was negligible, MMC values of 100 and 50 mg ointment (ml medium)$^{-1}$ corresponded to cumulative concentrations of 3.5 and 1.7 µmol NO (ml medium)$^{-1}$, respectively.

## Time dependence of antimicrobial activity

The results of the time-dependency experiments are shown in Fig. 2. Log reductions in the concentrations of each organism exposed to NO–zeolite ointment for 4 or 8 h are summarized in Table 1. The growth of organisms exposed to control ointment was similar to that of untreated organisms, indicating that the zeolite ointment itself does not inactivate microbes.

Cytotoxicity testing

Percentage viability of cells treated with NO–zeolite ointment and control zeolite ointment was calculated by dividing the
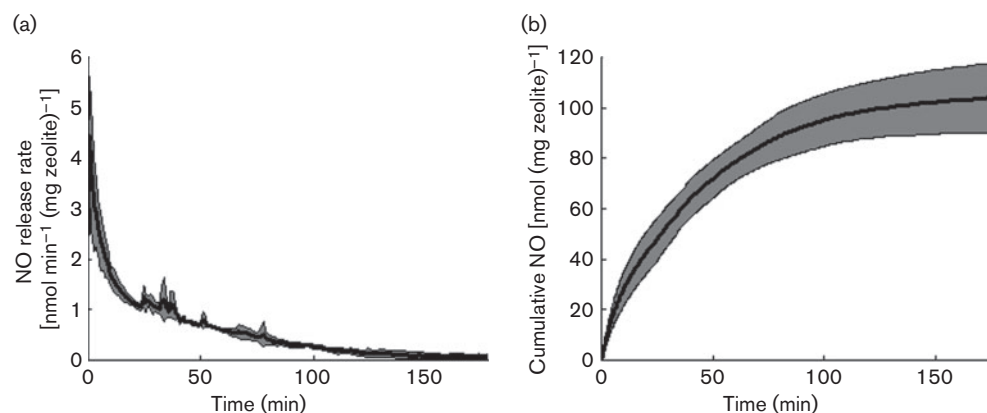
Fig. 1. NO release profiles from samples of emulsifying ointment containing 33 % (w/w) NO-loaded zeolites. (a) NO released per minute. (b) Cumulative NO release. Shaded areas represent the SD. *n*=3. All samples were stored for 1−7 days before measurement.
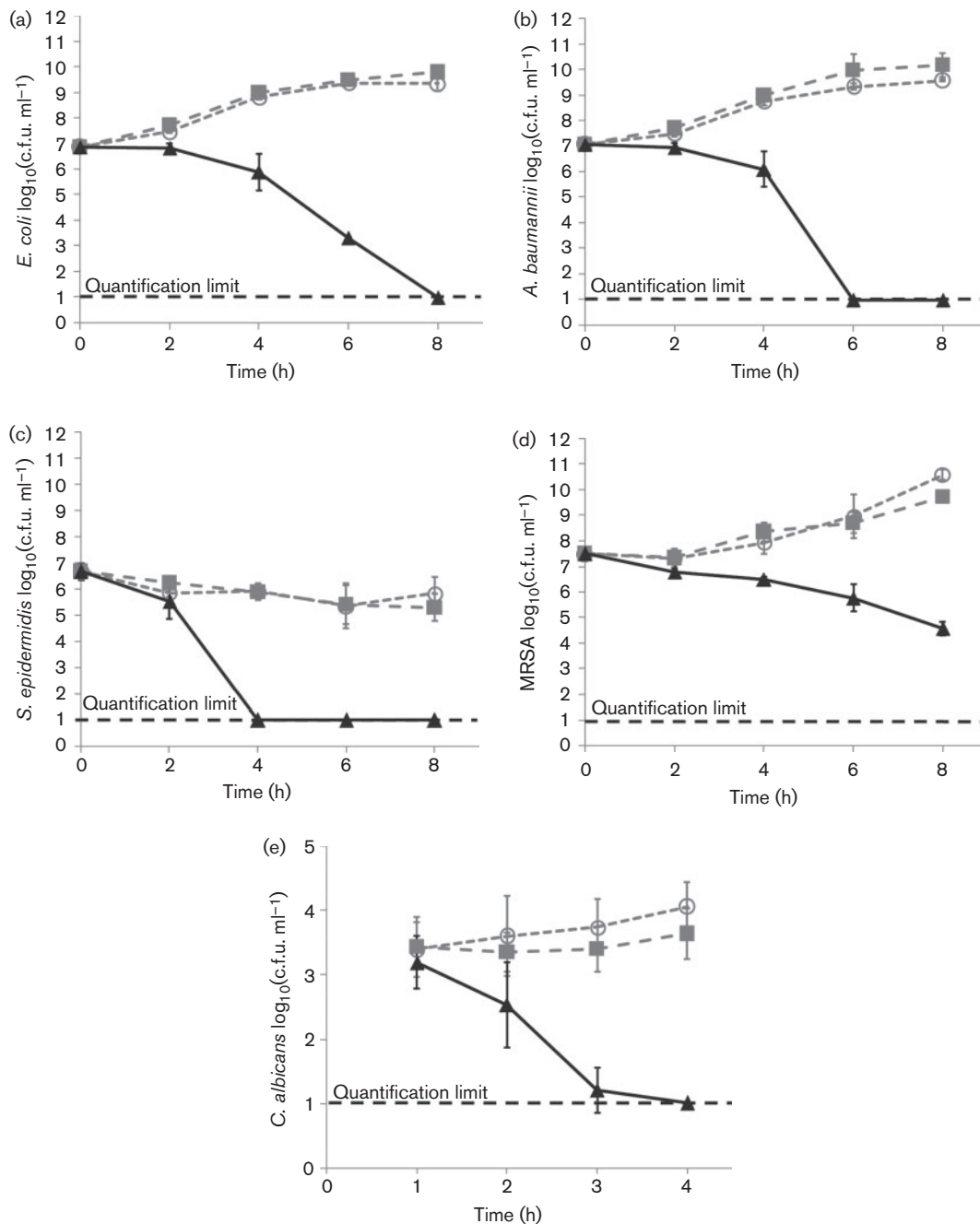
Fig. 2. Results of time-dependency experiments for (a) *E. coli*, (b) *A. baumannii*, (c) *S. epidermidis*, (d) MRSA and (e) *C. albicans*. ▲, Organisms treated with NO–zeolite ointment [100 mg (ml medium)$^{-1}$]; ■, organisms treated with control zeolite ointment [100 mg (ml medium)$^{-1}$]; ○, untreated organisms. Each point represents the mean of three trials. Error bars represent SDs.

mean fluorescence intensity of medium in treated wells by the mean fluorescence intensity of medium in untreated wells. The percentage viability values (mean ± SD) for cells treated with NO–zeolite and control zeolite ointment were 34 ± 8 and 91 ± 21 %, respectively. Assuming that NO was able to diffuse

freely across the Transwell membrane, the concentration of NO–zeolite ointment in growth medium was equal to the ointment concentration used in time-dependent antibacterial tests [100 mg ointment (ml medium)$^{-1}$, corresponding to 3.5 μmol NO (ml medium)$^{-1}$].

Table 1. Log reductions in viable microbe colonies after exposure to NO–zeolite ointment for 8 h (*E. coli*, *A. baumannii*, *S. epidermidis* and MRSA) or 4 h (*C. albicans*)

Log reduction (NO–zeolite versus initial) = log(initial microbe concentration) – log(final microbe concentration after NO–zeolite exposure). Log reduction (NO–zeolite versus untreated) = log(final untreated microbe concentration) – log(final microbe concentration after NO–zeolite exposure).

Organism	Log reduction (NO–zeolite versus initial)	Log reduction (NO–zeolite versus untreated)
E. coli	5.9	8.4
A. baumannii	6.1	8.6
S. epidermidis	5.7	5.1
MRSA	2.9	6.0
C. albicans	3.0	3.1

In vivo obese rat studies

Representative images of wounds from each treatment group are shown in Fig. 3(a). Mean wound sizes at each measurement time point are shown in Fig. 3(b). Wound-healing trajectories are often represented as exponential functions (Steed, 2003). The rate of wound healing was calculated by fitting the wound size versus time data from each animal to an exponential function, $S(t) = S_0 e^{-\theta t}$, where $S(t)$ is the wound size at time t as a percentage of the initial wound size, S_0 is the estimated initial wound size and θ is the wound-healing rate in units of per cent per day. The mean$\pm$SD healing rates of wounds treated with NO–zeolite ointment and control zeolite ointment were 15.1 ± 1.6 and $11.7\pm1.7\%$ day^{-1}, respectively. This difference was significant ($P=0.01$, two-tailed Student t-test). These data demonstrate the beneficial effect of NO-loaded zeolite ointment on wound healing.
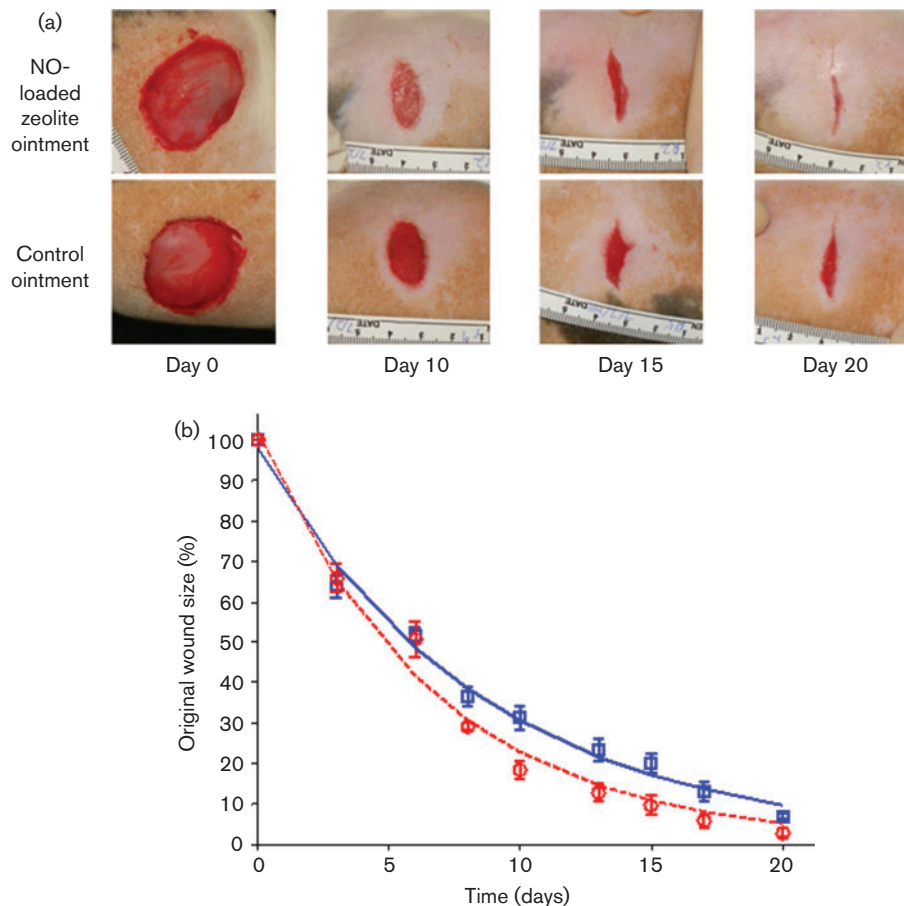
Fig. 3. (a) Images of treated and control wounds on Zucker obese rats on the day of wound surgery (day 0), and at three different time points after wound surgery. (b) Mean wound size in each treatment group as a percentage of each wound's original size. ○, Rats treated with NO–zeolite ointment; □, rats treated with control zeolite ointment. Error bars represent SEs. Lines represent exponential curves of best fit.

DISCUSSION

The present study demonstrates the antimicrobial efficacy of a topical ointment containing an NO-loaded zeolite powder that releases NO upon contact with moisture. The ointment formulation released measurable quantities of NO over at least 3 h. Studies of NO release from NO-loaded, zinc-exchanged zeolite powder have demonstrated that nearly all NO is released within 1 h of contact with moisture (Wheatley *et al.*, 2008). Mixing the zeolite powder into a hydrophobic ointment base slows the transport of water to the zeolite molecules, reducing the rate at which NO is released. The ointment base also makes the powder easy to apply to skin or wounds.

The organisms used for microbicidal testing were selected to demonstrate effectiveness against a broad spectrum of common wound pathogens, including Gram-negative bacteria (*E. coli* and *A. baumannii*), Gram-positive bacteria (*S. epidermidis* and MRSA) and a fungus (*C. albicans*). Although the *in vitro* tests conducted in the present study do not represent the complexity of an infected wound, the results of MMC and log-reduction tests indicate that clinically relevant reductions in micro-organism concentrations can be achieved with NO–zeolite ointment. The control zeolite ointment did not impair the growth of any of the micro-organisms tested. These results are consistent with those of Fox *et al.* (2010), who demonstrated the antimicrobial effect of NO-loaded, zinc-exchanged zeolite mixed with a polytetrafluoroethylene polymer. This compound released most of its NO within 30 min of exposure to water and caused 1–2 log reductions in bacteria concentrations after 45 min. In the present study, 5–8 log reductions in bacteria concentrations were observed after 6–8 h of exposure to NO–zeolite ointment.

In vitro cytotoxicity testing demonstrated that more than 90 % of fibroblasts treated with control zeolite ointment were viable, while approximately one-third of cells treated with NO–zeolite ointment were viable after 24 h. These data indicate that the materials in the zeolite ointment are not toxic to fibroblasts, but that the amount of NO released by the ointment impairs fibroblast viability *in vitro*. However, significant fibroblast toxicity *in vitro* is unsurprising in light of the reported toxicity of other antimicrobial treatments. For example, *in vitro* cytotoxicity testing of some commercially available silver wound dressings resulted in less than 25 % fibroblast viability (Burd *et al.*, 2007). Fibroblast exposure to 1 % povidone–iodine resulted in approximately 5 % viability after 24 h (Balin & Pratt, 2002), yet a systematic review of the use of iodine in wound-treatment clinical trials concluded that iodine does not impair wound healing (Vermeulen *et al.*, 2010). A recent review of iodine and chlorhexidine in acute wound management highlights the apparent discrepancy among the results of preclinical *in vitro* cytotoxicity testing and those of clinical trials of these antiseptic agents (Eardley *et al.*, 2012). Therefore, the results from NO–zeolite ointment *in vitro* cytotoxicity testing are presented in order to demonstrate the similarity of NO–zeolite ointment to other broad-spectrum antimicrobial treatments. Moreover, the present study demonstrated the ability of NO-loaded zeolite ointment to enhance healing in full-thickness cutaneous wounds in obese Zucker rats. Wounds that were treated with NO–zeolite ointment did not show external signs of inflammation such as erythema or swelling in comparison with wounds that were treated with control dressings. Treatment with NO–zeolite ointment resulted in earlier wound closure when compared with wounds treated with control ointment, suggesting that, in contrast to the *in vitro* studies, NO–zeolite ointment does not have a cytotoxic effect on healing wounds and could potentially be used as a treatment for infected wounds.

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