

# Gaseous nitric oxide for the local treatment of bacterial keratitis in mice

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Received October 10, 2016; Accepted November 11, 2016

DOI: 10.3892/br.2016.821

**Abstract.** The successful treatment of severe bacterial keratitis continues to be a challenge in animals and humans. In the present study the aim was to assess gaseous therapy using gaseous nitric oxide (gNO) in a murine model of *Pseudomonas aeruginosa* keratitis. The cornea of anesthetized mice was mechanically scratched and covered with a bacterial suspension of *P. aeruginosa*. One day later, the infected eyes were exposed to 200 ppm NO for 30 min. Three to seven days later the mice were sacrificed and the bulbi were obtained and processed for light microscopy. The read out parameter was the maximal corneal thickness and the severity of the hypopyon. The therapy with NO did not result in either a reduction of inflammation concerning the maximal corneal thickness or the severity of the hypopyon. The bacterial load was not investigated due to technical limitations. Thus, exposure to gNO did not reduce the local inflammation in *P. aeruginosa* induced murine keratitis at the investigated time-points. This does not exclude effects of NO on the bacterial load, and in experimental and human keratitis.

## Introduction

Bacterial keratitis is a major cause of blindness worldwide with a high incidence (1). This is due to low hygiene standards and water quality with a greater risk of infection in poor countries. In addition, the widespread use of contact lenses and associated complications in wealthier countries contributes to severe keratitis (2,3). *Staphylococcus aureus* and *Pseudomonas aeruginosa* are two predominant gram-positive and gram-negative bacterial strains responsible for causing this disease (1,4,5). The

resistance of bacteria to antibiotics is reducing the efficacy of bacterial keratitis treatment. Therefore, research into the development of novel therapeutic approaches is essential. In this regard photodynamic inactivation has been in experimental and clinical use throughout the last decade (6-8). Research on cystic fibrosis established an antimicrobial effect of gaseous nitric oxide (gNO) in infections with *P. aeruginosa* using a rat model (9). Furthermore, a study in white rabbits demonstrated that a treatment using 200 ppm gNO significantly reduced the bacterial load in wounds of the dorsal skin surface (10). Thus, in view of the possibility of a surface treatment for keratitis, this therapeutic strategy demonstrated a feasible approach.

The aim of the current study was to evaluate gNO application as a potential treatment of experimental keratitis *in vivo* for the first time. However, this series of experiments did not serve as proof of the anti-inflammatory effect of gNO.

## Materials and methods

**Animals and anaesthesia.** Young (age, 9-10.5 weeks) female C57BL/6 mice [n=19; standard housing conditions (3-5 animals per cage) with 12-h light/dark cycle, minimum 70% humidity and chow/water were provided *ad libitum*; Janvier Labs, Saint-Berthevin Cedex, France] were used in the present study. All animal care and experimental procedures were approved by the local governmental animal care committee (permit no. 58/2013) and were conducted in accordance with European legislation on the protection of animals (Directive 2010/63/EU) and National Institutes of Health (NIH) guidelines on the care and use of laboratory animals (NIH publication no. 85-23 Rev. 1985). Animals were anesthetized via i.p. injection of ketamine (75 mg kg<sup>-1</sup> body weight; Pharmacia GmbH, Erlangen, Germany) and xylazine (15 mg kg<sup>-1</sup> body weight; Rompun, Bayer, Leverkusen, Germany).

**Infection and treatment.** Three scratches (length, 2 mm) were made in the centre of the cornea using a 27-gauge syringe, subsequently 5 µl of the inoculum was pipetted onto the cornea [according to (4)]. Carprofen (Rimadyl®; Zoetis Schweiz GmbH, Zürich, Switzerland) was injected during anaesthesia. For the inoculum, the multi drug resistant *P. aeruginosa*

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**Key words:** keratitis, gaseous nitric oxide, treatment

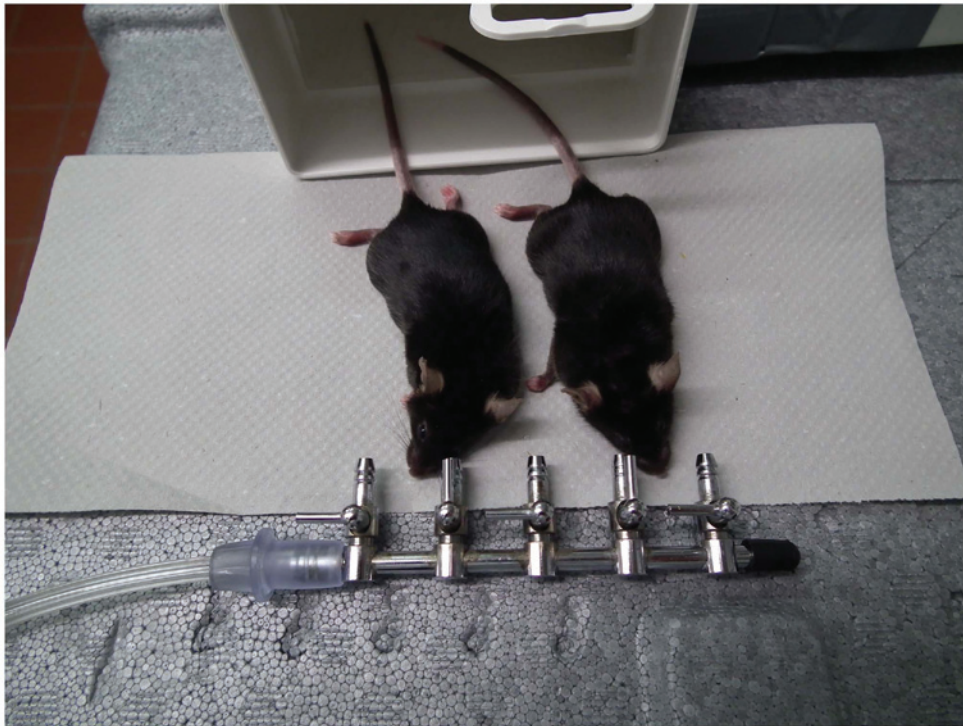


Figure 1. Application of gaseous nitric oxide was performed as a gas flow directed at the infected eye of the mice.

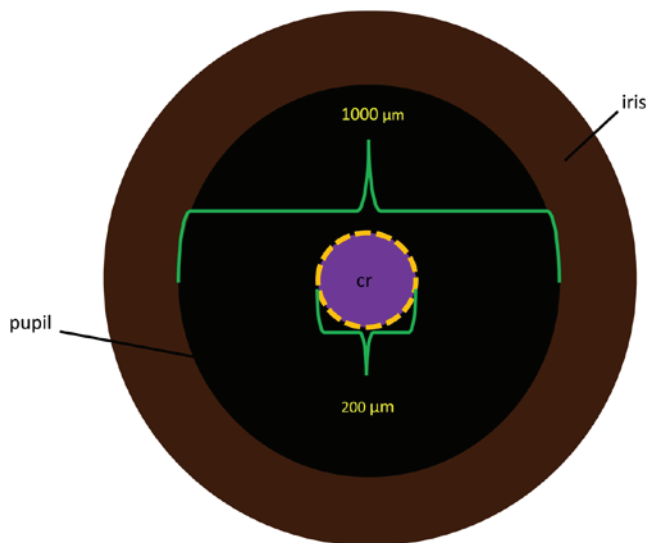


Figure 2. Schematic for obtaining the histological sections. The diameter of the pupil was  $\sim 1,000 \mu\text{m}$ . The sections were obtained from the CR of the cornea, which had a diameter of  $\sim 200 \mu\text{m}$ . CR, centre region.

strain 54 (clinical isolate, collected in 2009 at the Institute of Medical Microbiology and Hygiene, Saarland University Medical Center, Homburg, Germany. The resistance profile of PA54 was determined using the automated VITEK 2 system (BioMérieux GmbH, Nürtingen, Germany) was cultured on blood agar plates (Trypticase Soy Agar II, 5% Sheep Blood; BD GmbH, Heidelberg, Germany) for 24 h, and suspended in 10 ml 2% Luria Bertani (LB) broth (LB Broth; Difco, Saint Egrève, France) for an overnight culture ( $37^\circ\text{C}$ ). The overnight culture ( $100 \mu\text{l}$ ) was diluted in 10 ml 2% LB for another 3 h of culturing ( $37^\circ\text{C}$ ) and finally the suspension was adjusted using

a photometer to an optical density of 10 at a wavelength of 600 nm and then used for inoculation. Only one eye from each animal was infected, with certain non-infected eyes analyzed to serve as controls. Eyes were either not infected or infected, and the infected eyes were either treated or not treated (uninfected/untreated,  $n=6$  eyes; infected/treated,  $n=11$  eyes; infected/untreated,  $n=8$  eyes).

Twenty-four hours post infection the animals were treated under deep anesthesia as described above. For NO treatment, the infected eyes were exposed to gas (Praxair Deutschland GmbH, Düsseldorf, Deutschland) consisting of nitrogen and NO (200 ppm) for 30 min under anesthesia. A gNO stream with a velocity of 5 m/sec was directed to each eye using a nozzle. This nozzle was placed at a distance of 1 cm from the eye (Fig. 1). Three days after the infection (in two cases, 7 days after the infection to assess whether treatment required  $>3$  days to develop an effect) the animals were sacrificed by cervical dislocation.

**Histology and evaluation.** Their eyes were enucleated, histologically processed, and stained with hematoxylin and eosin. From each eye, five subsequent sections (thickness,  $5 \mu\text{m}$ , with a distance of  $50 \mu\text{m}$  from each other) from the center of the cornea (middle of the pupil) were obtained for evaluation (Fig. 2). The maximum corneal thickness was measured (CellSens Standard 1.8.1; Olympus Deutschland GmbH, Hamburg, Germany) and the mean value was taken. The severity of hypopyon was evaluated with a self-defined score, from 0 to 3 (0, no hypopyon; 1, mild hypopyon; 2, moderate hypopyon; 3, severe/massive hypopyon; Fig. 3).

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The Mann-Whitney U test or the  $\chi^2$  test were used

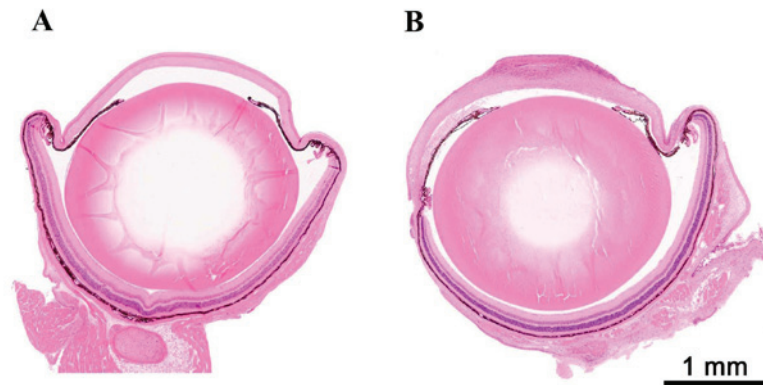


Figure 3. Examples for the severity of hypopyon, stained with hematoxylin and eosin. Score (A) 0 and (B) 1.

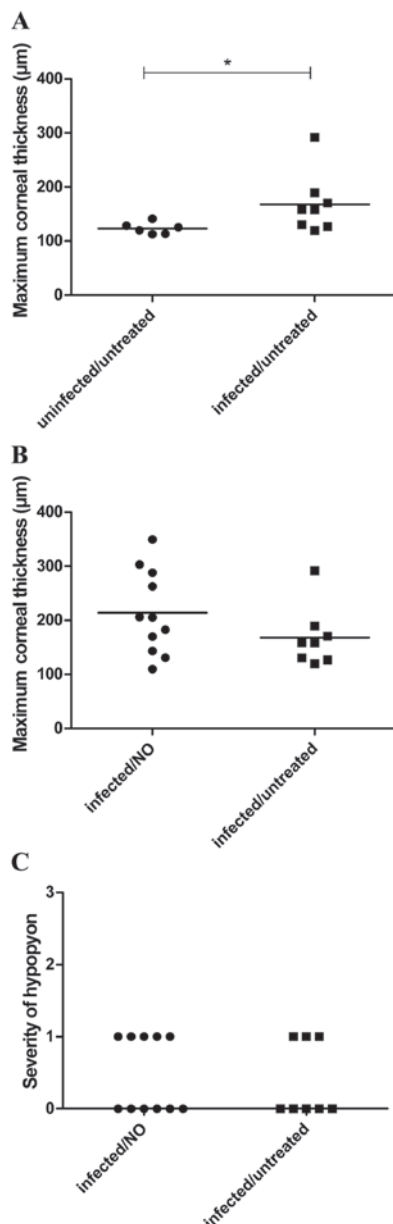


Figure 4. (A and B) Comparison between maximum corneal thickness in the different groups (infected/treated eyes, n=11; infected/untreated eyes, n=8; uninfected/untreated eyes, n=6). The bars represent mean values. \*P<0.05. (C) Comparison of the severity of hypopyon in NO-treated and untreated infected mice. Each point or square presents the mean value of one mouse. NO, nitric oxide.

for inferential statistics. Mean values and standard errors of the mean (SEM) were calculated and two-sided P<0.05 was considered to indicate a statistically significant difference.

## Results

*Maximum corneal thickness as an indication of corneal inflammation.* Concerning the maximum corneal thickness, the experiments resulted in mean values of  $124 \pm 4 \mu\text{m}$  (mean  $\pm$  SEM) for uninfected/untreated,  $168 \pm 20 \mu\text{m}$  (mean  $\pm$  SEM) for infected/untreated and  $214 \pm 23 \mu\text{m}$  (mean  $\pm$  SEM) for infected/treated animals (Fig. 4A and B). The infection led to a significant increase in the maximum corneal thickness. A significant influence of the gNO treatment was not identified for maximum corneal thickness.

*Occurrence and severity of hypopyon.* Similarly, a significant influence of the gNO treatment was not identified with regard to the severity of hypopyon (Fig. 4C).

## Discussion

Treatment with gNO is a novel and innovative approach as no resistance has yet been identified for this bactericidal mechanism. This initial in vivo study did not indicate an anti-inflammatory effect using gNO. In a previous study regarding bacterial pneumonia that was induced by *P. aeruginosa*, no influence of NO treatment was observed on the inflammation parameters, which was consistent with the present results (9). By contrast, the authors identified a marked reduction in the bacterial load of pseudomonades. The present study hypothesized that i) bacteria were killed and ii) corneal inflammation was reduced following gNO treatment. It might be a similar situation in our keratitis model as in the pneumonia model described in the study by Miller *et al* (9) where inflammation was not reduced in contrast to the bacterial load. The bacterial load was not determined in the present study, as bacterial load can only be determined in the whole globes, but not separately within the cornea alone, due to the small structure of the murine cornea. However, the hypothesis remains that gNO application kills bacteria within the cornea, but alternative experimental approaches are required. Another aspect of the study was the technical administration of gNO treatment. This treatment strategy was established specifically

for the current study. The gas application could be improved in future to ensure that a constant gNO concentration is applied to the surface of the cornea, for example, using an eye chamber, although this may be difficult due to the size of the mouse eye.

In conclusion, the aim of the current study was to evaluate gNO application as a treatment for experimental keratitis; this is the first study, to the best of our knowledge, to attempt to treat keratitis using this method. No anti-inflammatory effects were observed; however, this method should be investigated further to evaluate the bactericidal effects *in vivo*.

### Acknowledgements

The authors would like to thank Ms. Ann Soether for language editing and Ms. Tina Wiesen-Philipps for help with the manuscript.

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