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Short Communication

Reinforcement of the bactericidal effect of ciprofloxacin on *Pseudomonas aeruginosa* biofilm by hyperbaric oxygen treatment

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ABSTRACT

Chronic *Pseudomonas aeruginosa* lung infection is the most severe complication in cystic fibrosis patients. It is characterised by antibiotic-tolerant biofilms in the endobronchial mucus with zones of oxygen (O_2) depletion mainly due to polymorphonuclear leucocyte activity. Whilst the exact mechanisms affecting antibiotic effectiveness on biofilms remain unclear, accumulating evidence suggests that the efficacy of several bactericidal antibiotics such as ciprofloxacin is enhanced by stimulation of the aerobic respiration of pathogens, and that lack of O_2 increases their tolerance. Reoxygenation of O_2-depleted biofilms may thus improve susceptibility to ciprofloxacin possibly by restoring aerobic respiration. We tested such a strategy using reoxygenation of O_2-depleted *P. aeruginosa* strain PAO1 agarose-embedded biofilms by hyperbaric oxygen treatment (HBOT) (100% O_2, 2.8 bar), enhancing the diffusive supply for aerobic respiration during ciprofloxacin treatment. This proof-of-principle study demonstrates that biofilm reoxygenation by HBOT can significantly enhance the bactericidal activity of ciprofloxacin on *P. aeruginosa*. Combining ciprofloxacin treatment with HBOT thus clearly has potential to improve the treatment of *P. aeruginosa* biofilm infections.
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1. Introduction

Pseudomonas aeruginosa persists and grows in biofilms in the endobronchial mucus in cystic fibrosis (CF) patients [1], and the endobronchial secretions of CF patients with chronic *P. aeruginosa* lung infection exhibit a stratified microenvironment that imposes physiological constraints on the pathogens [2–4]. Polymorphonuclear leukocytes (PMNs) accumulating around *P. aeruginosa* biofilm aggregates can induce intense oxygen (O_2) consumption during their respiratory burst and the formation of nitric oxide, thereby impeding aerobic respiration of *P. aeruginosa* [5,6]. However, *P. aeruginosa* can adapt to such PMN-induced microenvironmental changes via anaerobic respiration by denitrification in the anoxic zones of endobronchial secretions [3,4]. A PMN-imposed restriction of O_2 availability is further verified by a positive correlation between PMN density and slow growth of *P. aeruginosa* in infected CF lungs [7], where hypoxia, anaerobic respiration and fermentation cause slower growth by *P. aeruginosa* than under normoxic conditions [8,9].

The tolerance of *P. aeruginosa* against the active host response and intense antibiotic treatment in the CF lungs is believed to be enhanced by the formation of biofilm [10] and limited O_2 availability due to bacterial consumption [11], but the significance of O_2 availability in the environment surrounding the biofilm, including the role of PMNs, remains to be studied in detail.

Several bactericidal antibiotics such as β-lactams, aminoglycosides and fluoroquinolones target bacterial aerobic respiration [12–14]. Their efficacy may thus be highly sensitive to the availability of O_2 and is has been suggested that the metabolic status of pathogenic bacteria can affect their susceptibility towards bactericidal antibiotics [11–13]. It is well known that hyperbaric oxygen treatment (HBOT) of planktonic *P. aeruginosa* can enhance the efficacy of antibiotic treatment [15,16]. In this study, we established an agar-embedded *P. aeruginosa* biofilm model in order to immobilise *P. aeruginosa* in zones of anoxia resembling the observations

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of microbial activity in the anoxic parts of sputum from CF patients [3,4].

This model was used to investigate whether anoxic agar-embedded *P. aeruginosa* biofilms resilient to ciprofloxacin could be sensitised by HBOT alleviating O_2 limitation and increasing the diffusive O_2 supply and thus aerobic respiration in the biofilm.

2. Materials and methods

2.1. Bacterial strain, media and antibiotics

Wild-type *P. aeruginosa* strain PAO1 was obtained from the *Pseudomonas* Genetic Stock Centre (http://www.pseudomonas.med.ecu.edu). The minimum inhibitory concentration (MIC) of the strain was 0.125 mg/L as determined by Etest (bioMérieux, Ballerup, Denmark). The strain was grown in Luria–Bertani (LB) broth [5 g/L yeast extract (Oxoid, Roskilde, Denmark), 10 g/L tryptone (Oxoid) and 10 g/L NaCl (Merck, Rahway, NJ), pH 7.5], incubated overnight at 37 °C and shaken at 170 rpm. The bactericidal antibiotic ciprofloxacin (Bayer GmbH, Leverkusen, Germany) was used. For determination of bacterial CFU counts, solid lactose agar plates ('Blue plates' based on a modified Conradi–Drigalski medium containing 10 g/L detergent, 1 g/L $Na_2S_2O_3{\cdot}H_2O$, 0.1 g/L bromothymol blue, 9 g/L lactose and 0.4 g/L glucose, pH 8.0; Statens Serum Institut, Copenhagen, Denmark) were used to select for Gram-negative bacteria. All plates were incubated overnight at 37 °C.

2.2. Susceptibility testing of mature biofilm

Survival curves were assayed to investigate the effect of HBOT on *P. aeruginosa* biofilms treated with ciprofloxacin. Microtitre plates (Nucleon Delta Surface; Thermo Fisher Scientific, Roskilde, Denmark) were loaded with 200 μL of overnight culture of PAO1 with an optical density at 600 nm (OD_{600}) adjusted to 0.4 before 100-fold dilution in LB medium supplemented with 2% 2-hydroxyethyl-agarose (Sigma–Aldrich, Brøndby, Denmark) to achieve a cell loading of ca. 10^6 cells/mL. To ensure metabolic activity during the study, the medium was supplemented with $NaNO_3$ (1 mM) (Sigma–Aldrich) to enable anaerobic respiration. The supernatant was replaced daily with 50 μL of LB medium supplemented with 1 mM $NaNO_3$. In addition, part of the sample medium was supplemented with 0.002% methylene blue (Sigma) used as a proxy for the presence of O_2. Microtitre plates were covered with Parafilm and lid and were incubated under normoxic conditions at 37 °C for 3 days to establish mature biofilms. Treatment with ciprofloxacin (Bayer GmbH) was initiated by replacing the supernatant with 50 μL of a ciprofloxacin solution in LB medium (supplemented with 1 mM NO_3^- and in part of the samples with 0.002% methylene blue) in fourfold dilutions from 0 to 16 mg/L. The plates were then further incubated for 2 h or 4 h under normoxic conditions or were exposed to HBOT. At the termination of experiments, the supernatant was discarded and agarose-embedded PAO1 biofilms were placed in 2.8 mL of phosphate-buffered saline (PBS) (Substrate Department, Panum Institute, Copenhagen, Denmark) and were re-suspended for 15–20 s in a homogeniser (SilentCrusher M; Heidolph, Schwabach, Germany). The actual bacterial content was estimated by CFU counting after serial dilution in 0.9% NaCl and plating on blue plates and incubation overnight at 37 °C.

2.3. Hyperbaric oxygen treatment

Agarose-embedded bacteria were exposed to HBOT (100% O_2) at a pressure of 280 kPa (2.8 bar) at room temperature in a hyperbaric oxygen chamber (OXYCOM 250 ARC; Hypcom Oy, Tampere, Finland). The HBOT sequence consisted of pressurisation over 5 min to a pressure of 280 kPa. The pressure was applied for 2 h or 4 h, followed by 5 min of decompression.

2.4. Microsensor measurements of O_2

To verify a link between methylene blue staining and O_2 concentration, 0.5 mL of overnight culture of PAO1 in LB medium was adjusted to an OD_{600} of 0.4, was diluted 100-fold dilution in LB medium supplemented with 2% 2-hydroxyethyl-agarose, 1 mM NO_3^- and 0.002% methylene blue to achieve ca. 10^6 cells/mL and was added to 2 mL glass vials (Schuett-Biotec, Göttingen, Germany). Samples were incubated at 37 °C during normoxic conditions and were positioned in a heated metal rack. Immediately after inoculation and after 24 h of incubation, vertical O_2 concentration profiles were recorded in the samples with an amperometric O_2 microsensor (OX25; Unisense A/S, Århus, Denmark) mounted in a motorised PC-controlled profiling setup (MM33 and MC-232; Unisense A/S). The microsensors (tip diameter 25 μm) were connected to a picoammeter (PA2000; Unisense A/S) and were positioned manually onto the upper surface of the samples. Profile measurements were taken by movement of the sensor in vertical steps of 100 μm through the samples. Positioning and data acquisition were controlled by dedicated software (SensorTrace PRO 2.0; Unisense A/S). The software was set to wait 3 s in each measurement depth before actual measurement and subsequent movement of the sensors to the next measuring depth. The interval between each cycle of profile measurements was 5 s.

The O_2 microsensor was linearly calibrated by measuring the sensor signal in an alkaline sodium ascorbate solution (zero O_2) and in air-saturated free PBS at experimental temperature and salinity as previously described [3].

2.5. Statistical methods

Statistical significance was evaluated by ordinary two-way analysis of variance (ANOVA) followed by Sidak's multiple comparison test. A *P*-value of <0.05 was considered statistically significant. Tests were performed with GraphPad Prism 6.1 (GraphPad Software Inc., La Jolla, CA) and Microsoft Excel (Microsoft Corp., Redmond, WA).

3. Results

3.1. Effect of hyperbaric oxygen treatment on the survival of Pseudomonas aeruginosa biofilms during ciprofloxacin treatment

Significantly less bacteria survived 4 h of treatment with ciprofloxacin when HBOT was applied ($P = 0.0008$), whereas no effect of HBOT on survival was observed after 2 h of ciprofloxacin treatment, indicating a time-dependent increased killing by supplemental HBOT (Fig. 1). Compared with normoxic conditions, HBOT did not affect the density of *P. aeruginosa* in untreated biofilms, and prolongation of HBOT from 2 h to 4 h did not increase the density of *P. aeruginosa* in untreated biofilm (Fig. 1).

3.2. Reoxygenation of Pseudomonas aeruginosa biofilms by hyperbaric oxygen treatment during ciprofloxacin treatment

The effect of HBOT on the penetration of O_2 through the *P. aeruginosa* biofilms was examined by visual inspection of the distribution of methylene blue used as a proxy for O_2 presence. Before HBOT, methylene blue staining could be localised to the supernatant only, whilst the agarose-embedded *P. aeruginosa* biofilm remained unstained, indicative of anoxia in the biofilm. After 2 h of HBOT, methylene blue staining was seen in the major part of the agarose-embedded *P. aeruginosa* biofilm, and after 4 h of HBOT

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