Short communication

Experimental use of flow cytometry to detect bacteria viability after hyperbaric oxygen exposure: Work in progress report

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Abstract
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Introduction: Hyperbaric oxygen treatment (HBOT), based on inhaling pure oxygen under elevated ambient pressure, is used as adjuvant intervention to promote healing in infected wounds. Despite extensive clinical evidence of beneficial effects of HBOT in soft tissue infections the mechanism of action remains to be elucidated. The aim of this study was to evaluate the use of flow cytometry as a novel method to assess the viability of pathogenic bacteria after hyperbaric oxygen (HBO) exposure.
Methods: Bacterial strains associated with soft tissues infections: *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus* were exposed to oxygen at 2.8 atmospheres absolute (atm abs) (283.6 kPa) pressure for 45, 90, or 120 min, then stained with propidium iodide and thiazole orange and analysed by flow cytometry.
Results: *Escherichia coli and Staphylococcus aureus* showed no change in viability, nor morphology, the viability of *Pseudomonas aeruginosa* reduced in a dose-dependent manner and *Klebsiella pneumoniae* also showed dye uptake after HBO.
Conclusions: These initial results, indicate diverse sensitivity of bacteria to HBO, and suggest that flow cytometry can be used to monitor viability and morphological changes triggered by HBO exposure in bacteria.

Introduction

Hyperbaric oxygen treatment (HBOT) involves inhaling pure oxygen under elevated ambient pressure, and is a treatment option for wound healing, carbon monoxide intoxication, arterial gas embolism, and decompression sickness.[1–3] It is used, together with proper wound debridement and antibiotic therapy, as adjuvant treatment for various soft tissue infections, mainly anaerobic, mixed or necrotising.[2,4] Despite the clinical evidence, the underlying mechanisms are still not fully understood. Elevated generation of oxygen reactive species impeding bacteria metabolism and supporting the immune system in bacteria elimination coupled with denaturing bacterial toxins seem to be the most applicable.[5,6]

Accurate determination of live, dead, and total bacteria plays a critical role in clinical and experimental microbiology. In addition, the knowledge of bacterial cell morphology is required for the understanding of the probable mechanisms of action of biocides. Traditionally, the viability in bacteria is determined by the ability to form colonies on solid growth medium and to proliferate in liquid nutrient broths. These traditional, culture-based tests work poorly in slow-growing and non-culturable organisms and are time-consuming. Flow cytometry, a technique first applied to eukaryotic cells, has been previously adapted for quantification, viability and single cell analysis of bacteria.[7–9]

Despite extensive clinical evidence of beneficial effects of HBOT in soft tissue infections the mechanism of action remains to be elucidated. To do so, applicable molecular and analytical methods should be employed. The aim of this prelimnary study was to evaluate the use of flow cytometry as a method for the specific quantification of viable and non-viable bacteria after hyperbaric oxygen exposure.

Material and methods

BACTERIAL STRAINS

Four bacterial strains associated with soft tissues infections were chosen: *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus*. These strains can behave as opportunistic pathogens, particularly in burns, septicaemia, endocarditis, soft tissue infections, urinary tract infections, and pneumonia (see e.g.,[10]). Bacterial strains were obtained either from the Czech National Collection of Type Cultures (CNCTC) or were isolated from patients. Strains isolated from patients underwent determination in our department, which serves as the Czech National Anaerobic Bacteria Reference Laboratory in the Czech Republic.

Escherichia coli

Gram-negative, facultative anaerobe, commensal microorganism of the gut microbiota. Various serotypes of *Escherichia coli* are human pathogens.

Klebsiella pneumoniae

Gram-negative, facultative anaerobe, routinely found in the human nose, mouth, and gastrointestinal tract as a part of the normal microbiota. *Klebsiella* species are often associated with nosocomial (hospital) infections.

Pseudomonas aeruginosa

Gram-negative, aerobe, *Pseudomonas aeruginosa* is a multidrug-resistant pathogen, often causing nosocomial infections.

Staphylococcus aureus

Gram-positive, facultative anaerobe, member of the normal flora of the body, it can cause a wide variety of diseases in humans including skin infections, abscesses, respiratory infections, and food poisoning. *Staphylococcus aureus* is the primary cause of bacteraemia in humans.

HYPERBARIC OXYGEN (HBO) EXPOSURE

Each bacterial strain was inoculated four times at 1×10^8 CFU·ml^{-1} in Schaedler Broth (HiMedia, India). One sample served as a control and three other samples were exposed to 100% oxygen at 2.8 atmospheres absolute (atm abs) (283.6 kPa) in an experimental hyperbaric chamber (HAUX Testcom 400, Germany). Three exposure times (45, 90 and 120 min) were used to identify possible dose-response effects on bacteria viability. One culture of each bacterium was subjected to HBO for each of these exposure times. The compression and decompression phases were each completed over two minutes. The inner chamber temperature was monitored, and maintained at room temperature throughout the exposure. The whole sample was subsequently analysed by flow cytometry after HBO exposure.

FLOW CYTOMETRY

Flow cytometry (FC) is commonly employed for quantification of viable bacteria in laboratory cultures, environmental, clinical and food samples.[11,12] Bacterial populations can be characterized by analysing their forward scatter (FSC), as well as their side scatter (SSC). The general assumption is that FSC is correlated to cell size and SSC represents cell density or granularity. In addition, staining with a variety of probes allows for a deeper understanding of bacterial membrane integrity, membrane potential, enzymatic and metabolic activity, culture structure and dynamics.[13]

Viability of bacteria is usually determined with FC after culture staining with various fluorescent dyes. This includes DNA binding dye probes, such as propidium iodide, which is impermeable in cells with an intact membrane, and only leaks into cells with compromised membranes.[14] Detection of non-viable bacteria with propidium iodide is often coupled with thiazole orange staining, which is a permeable nucleic acid dye, which stains live and dead bacteria.[7,9]

In our study, the viability of bacterial strains was analysed by flow cytometry (FACSCalibur, BD Biosciences, USA) after each HBO exposure. To do so, 0.5 ml of each sample was mixed with a combination of propidium iodide and thiazole orange, 0.5 µL each (BD Biosciences, USA), incubated for 20 min at room temperature and processed. The FSC and SSC together with propidium iodide absorption were used to detect bacteria viability and cell membrane integrity. Data were displayed as a dot-plot diagram. Briefly, more dot-plot diagram distribution in FSC and SSC parameters indicates bacterial subpopulations with changed cell morphology (e.g. cell swelling), and when associated with corresponding heterogeneity in propidium iodide absorption indicates cells with compromised membranes (e.g., dying bacteria). The data were analysed by FACSDiva (BD Biosciences, USA) software.

Results

Bacterial strains exposed to HBO exhibited differing patterns in the measured parameters. In *Escherichia coli* and *Staphylococcus aureus* no obvious difference was observed in FSC or SSC between the control sample (normobaric air, room temperature) and samples exposed to 45, 90 and 120 min of O_2 at 2.8 atm abs. *Pseudomonas aeruginosa* reacted to increased doses of HBO with the appearance of a visible population of bacteria with changed morphology, also associated with increased propidium iodide absorption (Figure 1). HBO exposure in *Klebsiella pneumoniae* led to the appearance of a visible population of bacteria with increased propidium iodide absorption, but without

Figure 1

Flow cytometric analysis of *Pseudomonas aeruginosa* before (K) and after 45, 90 and 120 min of exposure to HBO (2.8 atm abs O_2). **A:** X axis – size of bacteria (forward scattered light, FSC), Y axis – cell granularity (side scattered light, SSC); **B:** X axis – bacteria stained with propidium iodide, Y axis – SSC; **C:** axis X – bacteria stained with thiazole orange, axis Y – SSC. Morphological and viability changes are highlighted (black squares)

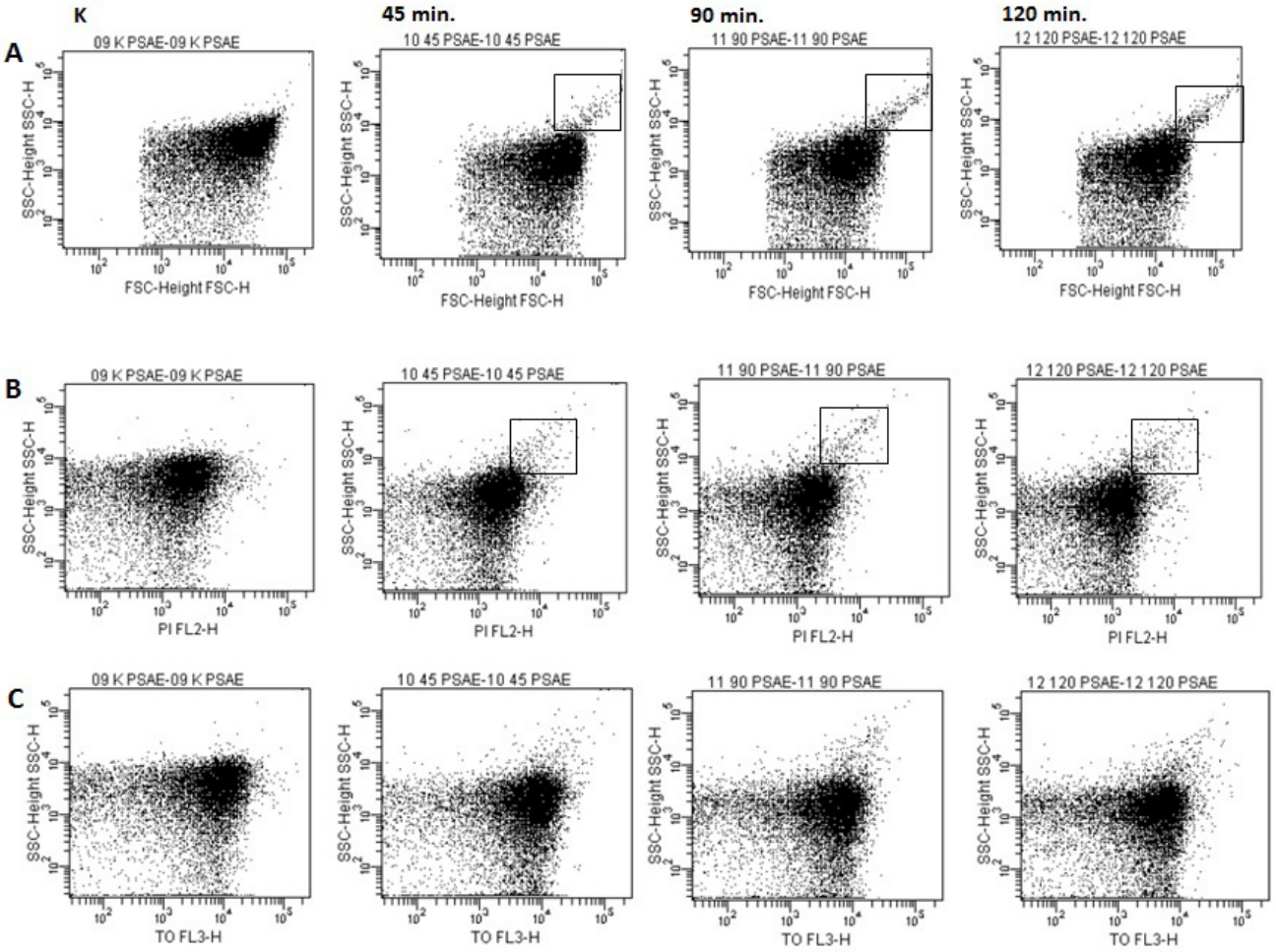

Table 1

Summary of changes in bacterial morphology and dye absorption after HBO exposure

Bacterial strain	Morphological change	Absorption of fluorescent dye
E. coli	Not visible	Not visible
S. aureus	Not visible	Not visible
P. aeruginosa	Dose-dependent	Dose-dependent
K. pneumoniae	Not visible	Increased

associated changes in bacterial morphology. The results summary is showed in Table 1.

Discussion

USE OF FLOW CYTOMETRY FOR BACTERIAL VIABILITY DETERMINATION

The conventional gold standard test for microbial contamination, a plate count method, can take up to several days to complete. Bacterial cell concentrations obtained by FC enable quantification of the entire bacterial community instead of the fraction of cultivable bacteria detected with the plate count method (typically < 1% of all bacteria). Flow cytometry measurements are reproducible with relative standard deviations below 3% and can be available within 15 min of samples arriving in the laboratory. The main advantages of FC are relevance, speed, accuracy, costs and automation potential.[15,16] Flow cytometry also facilitates understanding of physiological diversity in seemingly likewise acting populations when additional staining is applied. For practicality in microbiology, repeated division of a cell on an agar surface to produce a visible colony is usually taken as evidence of viability. Interpreting the

situation where there is an absence of colony formation is not clear-cut. Nowadays we understand that there is a discrepancy between the presence of bacteria and its ability to replicate under given conditions, so-called 'viable but non-culturable' bacteria. In this regard, it is crucial to further focus on reproductive growth, membrane potential, metabolic activities, and membrane integrity to better understand culture heterogeneity and viability. The advance of a direct single-cell examination using FC measurements accompanied by staining of bacteria with a variety of fluorescent probes facilitates this aim.[13,17]

THE SENSITIVITY OF BACTERIA TO HYPERBARIC EXPOSURE

The aim of this study was to demonstrate the use of flow cytometry in assessing viability of pathogenic bacteria after HBO exposure. In doing so, we identified apparent inter-specific differences in cytometric responses, possibly indicating diverse sensitivity of bacterial species to HBO (though see the study limitations below). These preliminary results are broadly confluent with the findings of others authors, who did not use flow cytometry to investigate effects of oxidative stress on bacteria.[18–20] Despite the relatively wide use of HBOT in soft tissue infection, data on the sensitivity of different bacterial strains to elevated partial pressures of oxygen are sparse and further work is required. We have identified FC as a plausible outcome measure in related future HBO studies.

LIMITATIONS

This study reports an attempt to utilise FC as a universal tool allowing for rapid detection of alive, impaired and dead bacteria after HBO exposure. Despite our promising results, some cautions should be taken. Firstly, we used bacterial isolates while the infected wound is usually colonised by a variety of normal and pathogenic bacterial flora. Thus, the observed reaction of bacteria to HBO might be over- or underestimated, since multi-strain colonies might behave differently. Secondly, some of the observed results might be transitory, thus evaluating the cultures at longer time points might be needed to fully understand the effect of the elevated partial pressure of oxygen on bacteria. Finally, due to the study design (one sample per bacterial species per exposure time) the results can only be considered hypothesis-generating. Further work with greater number of samples and possibly with sampling at different time intervals after HBO exposure is required to further determine the response of these bacteria to HBO.

Conclusions

To our knowledge, this is the first attempt to measure the direct effect of HBO exposure on bacteria viability with flow cytometry. The use of flow cytometry, originally designed for eukaryotic cells, has been extended to quantification, viability assessment and single cell analysis when staining with different fluorochromes. Our initial results suggest that flow cytometry can be used to monitor viability and morphological changes triggered by HBO exposure in bacteria. Nevertheless, further research is required to further investigate our results as well as to test other capabilities of this method while studying the effect of hyperbaric oxygen on bacteria.

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Conflicts of interest and funding

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