

Oxygen and Surgical Site Infection

A Study of Underlying Immunologic Mechanisms

Motaz Qadan, M.D., Ph.D.,* Christopher Battista, B.S.,† Sarah A. Gardner, B.S.,‡ Gary Anderson, Ph.D.,§ Ozan Akca, M.D.,|| Hiram C. Polk, Jr., M.D.‡

ABSTRACT

Background: Recent clinical trials investigating the role of hyperoxia in decreasing surgical site infection have reported conflicting results. Immunologic mechanisms through which supplemental oxygen could act have not been elucidated fully. The authors sought to investigate the effects of hyperoxia on previously tested and prognostically significant innate immune parameters to uncover the potential effects of hyperoxia at the cellular level.

Methods: After formal approval and informed consent, venous blood samples were collected from young healthy volunteers. Corresponding samples were incubated at 21 or 80% O₂ following a 1 ng/ml lipopolysaccharide challenge and analyzed to determine human leukocyte antigen-DR surface receptor expression, cytokine release, phagocytic capacity, and formation of reactive oxygen species. Data are presented as mean ± SD.

Results: After the 2 h of incubation at 21% O₂ (room air) and in 80% O₂ chambers, the change in human leukocyte antigen-DR mean channel fluorescence in lipopolysaccharide-stimulated monocytes was 2,177 ± 383 and 2,179 ±

338 ($P = 0.96$), respectively. Tumor necrosis factor- α concentrations were significantly lower for samples incubated at 80% O₂ when compared with 21% O₂ ($P < 0.05$). The phagocytic capacity of the innate immune system was not significantly enhanced by supplemental oxygen. However, the formation of reactive oxygen species increased by 87% ($P < 0.05$).

Conclusion: Hyperoxia exerts significant effects on multiple cellular and immunologic parameters, providing a potential mechanism for benefits from the use of supplemental oxygen. However, the ability to translate positive basic scientific findings to the operating suite or bedside require the existence of similar innate immune processes *in vivo* and the efficient transfer of oxygen to the sites where it may be used.

What We Already Know about This Topic

- ❖ Whether perioperative hyperoxia decreases surgical site infections is controversial

What This Article Tells Us That Is New

- ❖ In venous blood from healthy volunteers incubated *in vitro* with lipopolysaccharide, exposure to 80% O₂ increased reactive oxygen species generation while not or minimally affecting monocyte antigen presentation, neutrophil phagocytosis, or cytokine release compared with exposure to 20% O₂

* Post-Doctoral Surgical Research Fellow, † Medical Student, ‡ Senior Laboratory Technologist and Research Coordinator, The Price Institute of Surgical Research, University of Louisville School of Medicine, Louisville, Kentucky. § Professor of Physiology and Biophysics, Department of Physiology and Biophysics, # Ben A. Reid Professor of Surgery, Department of Surgery, University of Louisville School of Medicine. || Associate Professor of Anesthesiology, Department of Anesthesiology and Perioperative Medicine, University of Louisville School of Medicine, and the Outcomes Research Group, Louisville, Kentucky.

Received from The Price Institute of Surgical Research, and Department of Surgery, University of Louisville School of Medicine, Louisville, Kentucky. Submitted for publication September 16, 2009. Accepted for publication April 1, 2010. Supported in part by the Joint Royal College of Surgeons of Edinburgh/James and Emmeline Ferguson Research Fellowship Trust, Louisville, Kentucky. The funding source had no role in the production of this article.

Address correspondence to Dr. Qadan: The Price Institute of Surgical Research, Medical-Dental Research Building (3rd Floor), 511 South Floyd Street, Louisville, Kentucky 40202. mqadan@standford.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

IN recent years, attempts to understand innate host defense mechanisms have provided a specific focus for the reduction of surgical site infection (SSI) and associated sepsis, which extend beyond antimicrobial therapy.¹ Manipulating, or even failing to control, factors such as temperature, oxygen, and glucose, among others, have been shown to significantly influence the development of SSI and sepsis after surgery, despite the appropriate use of prophylactic antibiotics.^{2–5}

Although the importance of prevention of hypothermia is clear-cut at both the clinical and cellular levels,^{3,6} the potential benefit from oxygen supplementation is not as plain. In addition to previous data,^{7–9} a meta-analysis published earlier in the year combining trials that investigated the novel use of high inspired oxygen concentrations in an attempt to minimize SSI showed an overall significant benefit from the

treatment.¹⁰ The result was based on five trials conducted in the last decade. Within the cohort of included studies, four showed a distinct advantage in delivering high inspired oxygen concentrations.^{11–14} However, the remaining study not only failed to demonstrate the potential benefit but also suggested a higher rate of SSI in patients randomized to receive high inspired oxygen.¹⁵ That trial became the subject of intense scrutiny and criticism and is widely referenced in today's literature.

Since the publication of our meta-analysis, three further trials have emerged. A prospectively conducted randomized controlled trial in obstetric patients failed to demonstrate any benefit from providing high inspired oxygen concentrations,¹⁶ whereas another trial, which was a retrospective review of patients who had undergone spinal surgery, clearly reproduced the benefit described previously.¹⁷ Most recently, a Danish multicenter randomized controlled trial failed to reduce the risk of SSIs in patients undergoing abdominal surgery.¹⁸

With conflicting clinical results, it was unknown whether supplemented oxygen was truly being delivered to tissue. With the exception of one trial, effective modalities for the measurement of tissue oxygen levels were not used, and the efficacy of delivering high inspired oxygen concentrations was not verified. Furthermore, the underlying mechanisms by which oxygen would act were not elucidated fully. Therefore, we sought to investigate the effect of high oxygen concentrations (hyperoxia) on four well-recognized, previously tested, and prognostically significant innate immune parameters. Using *in vitro* oxygen concentrations similar to applied oxygen concentrations from previous clinical trials, we investigated the effect of hyperoxia on monocyte antigen presentation *via* surface human leukocyte antigen (HLA)-DR receptor expression, cytokine production, neutrophil phagocytosis, and the formation of reactive oxygen species (ROS), using a consistent and reproducible *in vitro* whole blood model. Although the chosen oxygen concentrations were higher than what would be expected at the wound and best mimic alveolar oxygen concentrations, we believe that our chosen concentrations may provide support for a novel mechanism by which a proximal increase in oxygen tension in the circulation "primes" innate immune cells before their arrival at the surgical site.

Materials and Methods

Whole Blood Sampling and Volunteer Screening

After approval by the University of Louisville Institutional Review Board (approval number HSPPO 08.0018, Louisville, Kentucky) and written informed consent, up to 15 ml venous blood samples were collected from a convenience sample of healthy male or female (18- to 42-yr-old volunteers). From our previous studies, it seemed that 13 volunteers were sufficient to detect large differences in HLA-DR surface expression.³ Therefore, we ensured a total pool of 15 volunteers for these experiments.

Volunteer body mass indices were all within normal limits (18–25 kg/m²). Exclusion criteria included any history of immunosuppressive disorders, diabetes mellitus, chronic medication, or pregnancy. All volunteers fasted for a minimum of 6 h before venipuncture, and blood glucose concentrations were determined using a Glucometer Elite (Bayer Corporation, Elkhart, IN). Subjects with fasting blood glucose concentrations less than 90 mg/dl or exceeding 110 mg/dl were excluded.

HLA-DR Receptor Expression

Sample Preparation. Blood was collected in EDTA Vacutainers (Becton-Dickinson and Co., Franklin Lakes, NJ). Samples were supplemented with 200 mM L-glutamine at a concentration of 10 μ l/ml of whole blood for incubation periods greater than 1 h (Sigma Chemical Co., St. Louis, MO). Aliquots of whole blood (1 ml) were subsequently transferred into 5-ml Falcon polypropylene culture tubes (VWR, Westchester, PA). A lipopolysaccharide (Sigma-Aldrich, St. Louis, MO) concentration of 1 ng/ml,³ as confirmed by our pilot experiments, provided the endotoxin challenge immediately before incubation.

Experimental Design. Corresponding lipopolysaccharide-treated samples with normal fasting glucose concentrations (<120 mg/dl) were incubated in the clinically applicable oxygen concentrations of 21% (room air) and 80%, with 5% CO₂ at 37°C, using an air-tight sealed plastic chamber. The chamber was modified by creating two lateral pin-sized holes, which permitted the insertion of tubing through the sides of the chamber. Entry and exit ports were sealed with silicone to maintain oxygenation. Entry tubing was connected to the 80% O₂ source, whereas exit tubing provided an escape route to prevent gas-pressure buildup (fig. 1). Flow

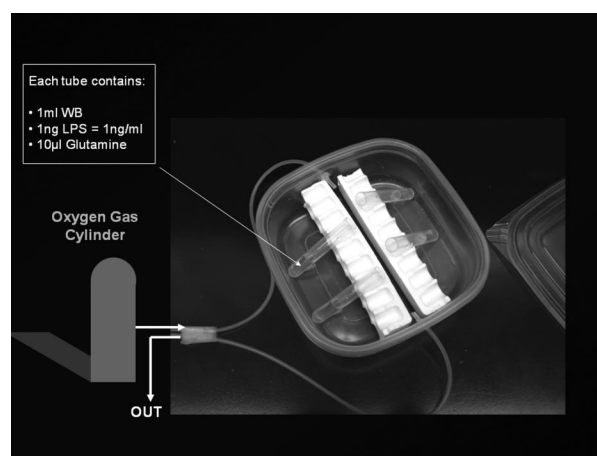


Fig. 1. This photograph demonstrates the experimental setup used. The two pin-sized holes that accommodate the tubing through the sides of the chamber are shown. The holes were sealed with silicone to maintain oxygenation once the cover is applied. Entry tubing was connected to an oxygen source, whereas exit tubing provided an escape route to prevent gas-pressure buildup. LPS = lipopolysaccharide; WB = whole blood.

rates were maintained at a constant rate between all experiments. A premixed oxygen source containing 80% O₂, 5% CO₂ gas, and 15% N₂ gas was used to provide the hyperoxic medium. Once the tubing was connected to the 80% O₂ cylinder, an adequate seal within the oxygenation chamber was confirmed *via* the exit tubing (bubbles in water). Room air incubation was used for the standard normoxic concentration (21%), with 5% CO₂ gas infused through the incubator.

To determine monocyte HLA-DR surface expression, samples were incubated for 2 h, as determined by early pilot experiments. Aliquots of cultured samples (50 μ l) from the HLA-DR assay were stained immediately before incubation (time 0) and after incubation and were then subsequently analyzed to determine baseline and final monocyte HLA-DR expression, respectively. Throughout incubation, gentle vortex was applied at 45-min intervals to ensure cellular homogeneity and even exposure to environmental variables.

Monocyte CD 14⁺/HLA-DR Staining and Extraction. Whole blood samples (50 μ l) were stained with fluorescein isothiocyanate-labeled antihuman CD14⁺ and phycoerythrin-labeled anti-HLA-DR antibodies (BD Biosciences, La Jolla, CA) to determine monocyte HLA-DR expression. Staining was carried out for 25 min in the culture environment to prevent down- or up-regulation of HLA-DR before quantitative binding. Appropriately matched isotype controls were used to determine nonspecific binding thresholds. Manufacturer instructions were precisely followed.

After staining, erythrocyte lysis was carried out for 6 min using ice-cold ammonium chloride, potassium bicarbonate, and EDTA (Sigma Chemical Co.) solution. Monocytes were pelleted by centrifugation, washed with 1 ml Dulbecco phosphate-buffered saline (PBS; Sigma Chemical Co.), and fixed in 250 μ l of 1% paraformaldehyde solution (Polyscience Inc., Warrington, PA).

Flow Cytometric Analysis. Monocyte CD14⁺ and HLA-DR surface expression were analyzed within 4 h of cell culture using a FACSCalibur flow cytometer (Becton-Dickinson and Co.). A total of 20,000 events were acquired. HLA-DR mean channel fluorescence (MCF) was analyzed in CD14⁺ monocytes using Cell Quest software (Becton-Dickinson and Co.).

Cytokine Assays

Sample Preparation and Experimental Design. Experiments were conducted to provide a time course analysis of cytokine release after endotoxin challenge. Samples were incubated for intervals of 30, 60, 120, and 240 min at 21 and 80% O₂ concentrations. Time intervals were selected to allow characterization of cytokine trends, as determined by early pilot experiments. After incubation, whole blood samples were centrifuged at 3,000 revolutions per minute for 12 min after incubation to obtain an acellular supernatant, which was stored at -81°C for subsequent analysis.

Cytokine Determinations. Plasma tumor necrosis factor (TNF)- α and interleukin-10 concentrations were quantified

using commercially available enzyme-linked immunosorbent assay kits (e-Biosciences, San Diego, CA). All enzyme-linked immunosorbent assays were carried out in 96-well plates according to the manufacturer's instructions. Samples were assayed in duplicate, with either recombinant human TNF- α or interleukin-10, to generate a standard curve. Enzyme activity was measured at a wavelength of 450 nm on a SpectraMax Plus384 spectrophotometer, and data were generated using Softmax Pro software (Molecular Devices, Sunnyvale, CA) and expressed in picograms per milliliter. The lower limits of detection for TNF- α and interleukin-10 were 4 and 2 pg/ml, respectively.

Phagocytosis Assays

Sample Preparation. Venous blood samples were collected in sodium heparin BD Vacutainers (Becton-Dickinson and Co.). Lipopolysaccharide (1 ng/ml) was supplemented, and 2 ml whole blood aliquots were preincubated for 15 min at the desired oxygen concentrations before the commencement of quantification of phagocytosis.

Preparation and Opsonization of Fluorescein Isothiocyanate-labeled *E. coli*. A stock solution of fluorescein isothiocyanate-labeled *Escherichia coli* (Molecular Probes, Eugene, OR) was prepared by diluting 5 mg lyophilized fluorescein isothiocyanate-labeled *E. coli* with Hanks Balanced Salt Solution for a final concentration of 1 mg/ml, where 1 mg of bacteria comprised 3×10^8 particles/mg. The bacteria were sonicated and stored at -81°C until required for use. When required, the labeled organisms were thawed and washed twice with PBS by centrifugation. To opsonize bacteria, a 10% pooled human serum solution was freshly prepared in PBS and was mixed with equal volumes of bacteria for a final opsonin concentration of 5%. The mixture was incubated for 25 min at 37°C, with 5% CO₂, to allow for opsonization to occur. Excess opsonins were removed by washing bacteria twice with PBS and centrifugation, followed by resuspension to the original bacterial volume.

Experimental Design. A final volume of 40 μ l/ml whole blood (40 μ g) of opsonized bacteria was quickly added to the preoxygenated aliquots. Samples were incubated at 21 and 80% O₂ concentrations. Sampling times of 2, 6, 12, 20, and 45 min were selected, which adequately characterized the hyperbolic nature of the phagocytic reaction in early pilot experiments and were sufficient to attain greater than 95% of cells contributing to phagocytosis within 45 min. At these times, 50 μ l samples were extracted with subsequent erythrocyte lysis, leukocyte pellet isolation, and fixation as described previously.

Flow Cytometric Analysis. Before acquisition using a FACSCalibur flow cytometer (Becton-Dickinson and Co.), extracellular fluorescent bacteria were quenched using 75 μ l trypan blue reagent (Molecular Probes). A total of 20,000 events were acquired for each sample. Monocytes and neutrophils were gated according to the light scattering properties, and the proportion of fluorescent cells was recorded.

ROS Assays

Sample Preparation. Venous blood samples were collected in sodium heparin BD Vacutainers (Becton-Dickinson and Co.). Aliquots of whole blood (1 ml) were transferred into 5-ml Falcon polypropylene culture tubes. Optimal phorbol myristate acetate (PMA) (Sigma-Aldrich) concentrations of 100 ng/ml were used to provide the cellular stimulus before incubation, as shown in previous studies,¹⁹ and by early pilot time-response and dose-response experiments. Working solutions of PMA were prepared by adding 5 μ l (5 μ g) of PMA to 0.5 ml of PBS. Ten microliters of the working solution were transferred into Falcon polypropylene culture tubes. Whole blood samples (940 μ l) were then transferred into the polypropylene culture tubes, which contained the added PMA to give a 100 ng/ml whole blood volume.

Experimental Design. ROS were quantified using dihydrorhodamine 123 (Invitrogen, Carlsbad, CA). A 60 μ g/ml working solution of dihydrorhodamine 123 was prepared freshly by adding 8 μ l of stock dihydrorhodamine 123 to 1,312 μ l PBS to obtain a final volume of 3 μ g/ml of whole blood. Immediately after its preparation, 50 μ l dihydrorhodamine 123 working solution was added to the combined solution of whole blood and PMA. After the addition of dihydrorhodamine 123, culture tubes were promptly placed within the oxygen chambers to minimize environmental exposure and avoid early false-positive fluorescence and contamination. Samples were incubated for 30 min, as determined in early pilot experiments, at 37°C in 21 or 80% O₂ chambers. After 30 min of incubation, 50- μ l samples were extracted with subsequent erythrocyte lysis, leukocyte pellet isolation, fixation as described previously.

Flow Cytometric Analysis. Samples were analyzed within 1 h of cell culture using a FACSCalibur flow cytometer (Becton-Dickinson and Co.). A total of 10,000 events were acquired. MCF (ROS quantity) was recorded in gated neutrophils using Cell Quest software (Becton-Dickinson and Co.).

Oxygen Partial Pressure Determination

In a separate pilot experiment, venous whole blood samples from four volunteers, who met the predetermined inclusion and exclusion criteria, were immediately incubated for 120 min at 37°C in 21 or 80% O₂ chambers. Blood sampling was carried out at 0-, 10-, 30-, 60-, and 120-min intervals to determine oxygen partial pressures. Single droplets were analyzed using an i-STAT blood gas analyzer (Abbott Laboratories, East Windsor, NJ).

Statistical Analysis

With two corresponding test groups (21 and 80% O₂ concentrations), paired *t* tests (two-tailed) were used to detect the significant differences in corresponding, same-subject, samples. Data were normally distributed and are presented as mean \pm SD. Statistical analyses were performed using Primer of Biostatistics (version 6.0, McGraw Hill, New York, NY). Significance was assigned at the 5% level.

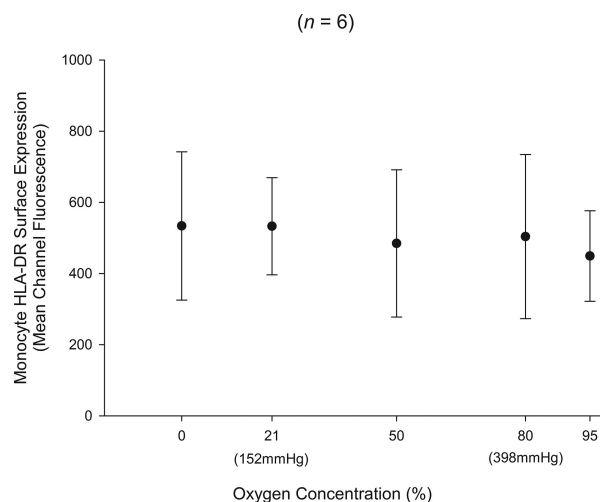


Fig. 2. The effect of 0, 21, 50, 80, and 95% oxygen concentrations on human leukocyte antigen (HLA)-DR surface expression in monocytes from early pilot experiments. A total of 0.5 ng/ml lipopolysaccharide was used. Samples were incubated for a total of 120 min. Mean channel fluorescence (arbitrary units) was used to quantify monocyte HLA-DR surface expression.

Results

After 2 h of incubation at 21% O₂ (room air), the change in HLA-DR MCF in lipopolysaccharide-stimulated monocytes was $2,177 \pm 383$. When corresponding samples (same subject) were incubated in 80% O₂ chambers, recorded MCF was $2,179 \pm 338$ ($P = 0.96$; data available on request). Figure 2 shows the effects of a wider range of oxygen concentrations on HLA-DR surface expression from early pilot experiments. There were no significant differences among 0, 21, 50, 80, and 95% O₂ concentrations in this model ($P = 0.75$).

The initial increase in TNF- α concentration was similar between the two oxygen concentrations (fig. 3A). However, at 120 min, TNF- α concentrations were statistically significantly lower for samples incubated at 80% O₂ when compared with 21% O₂ ($P < 0.05$), although probably not clinically significant. Specifically, mean changes between 60 and 120 min at 21 and 80% O₂ were +26 and -3%, respectively, with a delay in reaching peak concentration at 21% O₂ (>120 min) when compared with 80% O₂ (<120 min).

The antiinflammatory cytokine response, assessed using interleukin-10 concentrations, demonstrated a similar trend between 21 and 80% O₂ concentrations, with an early increase in interleukin-10 concentrations (fig. 3B). There were no significant differences in the early antiinflammatory response.

Figure 4 depicts the hyperbolic response characteristic of neutrophil phagocytosis. Using the selected time periods, approximately 95% neutrophils had contributed to the process of *E. coli* phagocytosis, indicating an adequate experimental response. The process is characteristically hyperbolic as early ingestion proceeds at an exponential rate followed by a slower response as neutrophils become "saturated" with

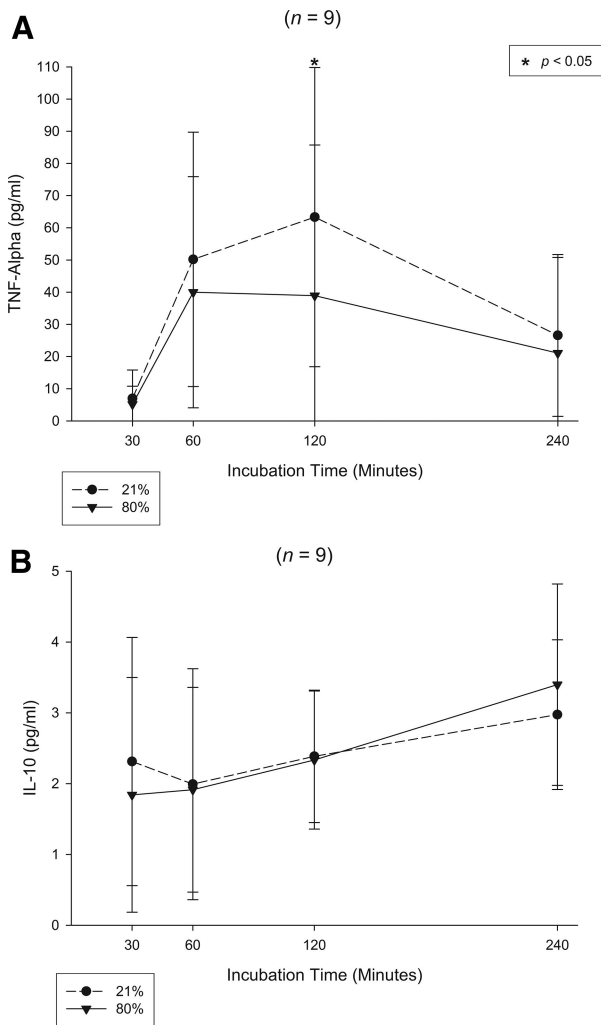


Fig. 3. (A) The effect of 21 and 80% oxygen on proinflammatory cytokine production (tumor necrosis factor [TNF]- α) by 1 ng/ml lipopolysaccharide-stimulated monocytes is shown. Samples were incubated for a total of 240 min and sampled at 30-, 60-, 120-, and 240-min intervals. Lipopolysaccharide was added at time 0. TNF- α levels between 21 and 80% oxygen at the 120-min interval are being compared for the *P* value shown. (B) The effect of 21 and 80% oxygen on anti-inflammatory cytokine production (interleukin [IL]-10) by 1 ng/ml lipopolysaccharide-stimulated monocytes is shown. Samples were incubated for a total of 240 min and sampled at 30-, 60-, 120-, and 240-min intervals. Lipopolysaccharide was added at time 0. There were no statistically significant differences in IL-10 production at any of the sampled intervals.

ingested organisms. Throughout the investigated time course, there were no differences detected in phagocytosis rates between 21 and 80% O_2 concentrations, represented by statistically similar K_m50 (time for 50% of neutrophils to contribute to phagocytosis) response rates, 3.564 and 2.997 s, respectively.

To provide a functional aspect to phagocytosis assays, ROS formation was measured and compared between 21 and 80% O_2 concentrations. Although these values do not specifically represent bacterial killing, they represent the

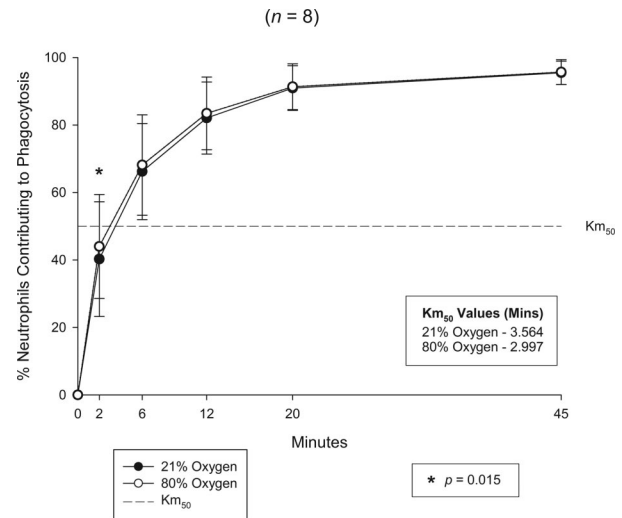


Fig. 4. The effect of 21 and 80% oxygen concentration on the rate of neutrophil phagocytosis shown by the proportion of neutrophils contributing to phagocytosis (contain fluorescent bacteria) with given time is represented. Samples were pre-incubated with lipopolysaccharide for 15 min before the addition of opsonized bacteria at time 0. The statistically significant finding, represented by the *P* value, occurred at 2 min and represents a comparison between 21 and 80% oxygen at that interval. K_m50 = time for 50% of neutrophils to contribute to phagocytosis.

amount of substrate available for the process and are thought to directly correlate with intracellular killing.²⁰ When samples were incubated at 80% O_2 when compared with 21% O_2 , there was an 87% up-regulation in ROS formation, which was quantitatively assessed using the arbitrary-valued MCF (fig. 5).

Figure 6 represents measured oxygen partial pressures with the applied oxygen concentrations used in the *in vitro* experiments. When calculating oxygen partial pressures, values from the stable segments after equilibration (*i.e.*, 30, 60, and 120 min) were used to calculate mean partial pressures.

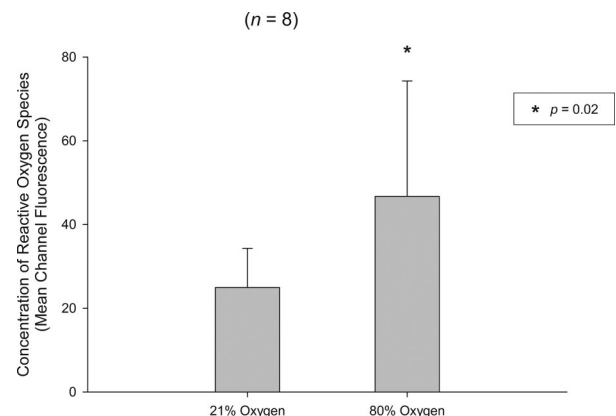


Fig. 5. The effect of 21 and 80% oxygen on reactive oxygen species formation is shown. Mean channel fluorescence was used to detect the absolute quantity, or concentration, of reactive oxygen species formed. The statistically significant difference between 21 and 80% oxygen is represented by the *P* value of 0.02.

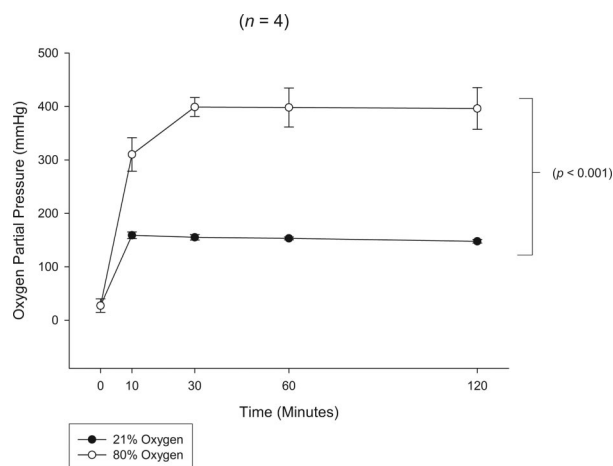


Fig. 6. Early pilot studies demonstrating oxygen equilibration times and partial pressures in whole blood after 120-min incubation at 21 and 80% oxygen concentrations are demonstrated. This figure represents measured oxygen partial pressures with the applied oxygen concentrations used in the *in vitro* experiments. When calculating oxygen partial pressures, values from the stable segments after equilibration (i.e., 30, 60, and 120 min) were used to calculate mean partial pressures.

Oxygen partial pressures at 21 and 80% O₂ concentrations were 152 and 398 mmHg, respectively.

Discussion

The benefit from the use of high inspired oxygen concentrations to minimize SSI today poses a clinical conundrum. The hypothesis that oxidative killing of pathogens is the primary mechanism of defense against surgical pathogens would suggest that its use is feasible.^{21,22} Furthermore, oxygen partial pressures and wound tissue oxygen concentrations have been shown to correlate with oxidative killing and, thus, help to predict SSI rates.²⁰ Even without great increases in wound oxygen tensions, increasing systemic and pulmonary oxygen may beneficially influence inflammatory mediators and ROS formation for intracellular killing.

Several previous studies have shown potential benefit from providing high inspired oxygen concentrations to reduce SSIs,¹⁰ without a significant increase in complications because of its use.^{23–25} However, this beneficial effect was not universal, with some studies failing to reproduce the benefit.^{15,16,18} Although several explanations were put forward to provide possible reasons for the negative trials, factors such as temperature, volume, and pain, which may indeed affect oxygen tensions, were not always adequately controlled in all trials, thereby contributing to variable tissue oxygenation. In addition, wound oxygen concentrations were not verified in several trials. Greif *et al.* did, however, document tissue oxygen levels. They noted that tissue oxygen levels were significantly less than arterial oxygen concentrations, but they demonstrated a statistically significant doubling in oxygen tension with 80% O₂ supplementation. Bearing in mind the differences in important factors such as

temperature, intravascular volume, and pain, especially when combined with an absence of wound oxygen measurements in several trials, the true comparative potential of these clinical trials is, in reality, unknown. Identifying cellular mechanisms for the use of hyperoxia could help to clarify solutions to the clinical conflict because no effects were visible at the molecular level, then it would be unlikely that any true clinical effect would exist. The converse would also be true.

By using an unchanged and reproducible *in vitro* whole blood model, our results show several significant effects outside the wound among well-recognized immunologic parameters that may be directly attributed to the high oxygen concentrations provided. Specifically, oxygen significantly promoted the formation of reactive oxygen intermediates available for intracellular killing and resulted in an earlier peak and more rapid decrease in TNF- α levels.

ROS formation represents a functional assessment of the potency by which neutrophils and monocytes are able to destroy pathogens early after contamination. In essence, it provides a quantified estimate of the capacity of innate immune system to eliminate early infection potentially before gross dissemination within the host. This small window for elimination of inevitable contamination has historically been coined the “decisive period,” and its true importance has been well described.^{26,27} Our study showed a significant doubling of the quantity of oxygen intermediates with increasing blood oxygen concentrations, in keeping with, and thereby reverifying, previous findings by Allen *et al.*²¹ Having said that, an important difference in our study is the use of significantly higher oxygen pressures, which would not have translated to an increase in ROS in the findings of Allen *et al.*, thereby suggesting that the increase at the much higher oxygen pressures in our findings represent a higher overall K_m 50 value. The functional aspect of the large increase in ROS formation is likely to be of utmost clinical significance, particularly in surgery, where several anesthetic drugs, including nitrous oxide, sevoflurane, and isoflurane, have been shown to impair the innate immune response and specifically blunt ROS formation.^{28–30} ROS formation may arguably be the most important of all immune parameters, potentially offsetting other neutral findings described at the cellular level, because the ability of cells to present antigen, produce cytokines, and contribute to phagocytosis ultimately culminate in a single process: intracellular killing *via* ROS, complemented by lysosomal enzyme killing. Conversely, the lethality of diseases such as Chediak–Higashi syndrome, characterized by remarkably effective phagocytosis but ineffective intracellular killing, represents the true clinical and functional importance of this early innate immune parameter. Although we did not study intracellular killing using dedicated killing assays, the ability to generate ROS is an alternative, potentially more relevant, parameter in the whole blood model, which is essential in the process of bacterial killing, and has been shown previously to correlate directly with intracellular killing.²⁰ Hyperoxia has also long been shown to possess microbicidal activity in several studies, dat-

ing back to the 1970s, by enhancing bacterial clearance and killing.^{31,32}

From a technical point of view, we used dihydrorhodamine 123 to detect ROS because it has been shown to detect all available intermediates more sensitively than other stains. It is considered by many to be the most sensitive technique available for accurately quantifying ROS formation and, with it, neutrophil oxidative killing potential.^{27,33}

We have previously attempted to explain the clinical significance of cytokine production and benefit derived from attenuation of release of proinflammatory proteins such as TNF- α . TNF- α is an important mediator of host defense, without which survival is severely compromised. It is thought to be an early proinflammatory cytokine to be released in a cascade of mediators and is released both locally and systemically. Its effects in the local circulation include an increase in vascular permeability, activation of lymphocytes (adaptive immune system), and reciprocal activation of macrophages, resulting in an enhancement of innate immune defense mechanisms. In the systemic circulation, TNF- α affects the hypothalamus to induce fever, the liver to produce acute-phase proteins, and the bone marrow to produce neutrophils, which are subsequently mobilized into the circulation.

Although TNF- α plays a major role in activation and enhancement of immune mechanisms, it has also been arguably linked to host tissue injury and multiorgan dysfunction from many disparate and disagreeing reports. Some evidence for this pathologic role began to emerge in 1985, when pretreatment with antiserum to TNF- α was shown to protect mice against endotoxin lethality *in vivo*.^{34,35} The concept did not, however, explain the repeated failure in reducing septic deaths when TNF- α blockade was used in human clinical trials.^{36,37} Van der Poll³⁸ later hypothesized that secretion of proinflammatory cytokines, such as TNF- α , occur in relatively short-lived and rapid bursts immediately after the insult and were followed by prolonged "refractory" compensatory increases in antiinflammatory cytokines, most notably interleukin-10. They proposed that previous human trials had intercepted septic human subjects late in the antiinflammatory phase, thereby failing to demonstrate survival benefit from antagonizing TNF- α .³⁹ Although proinflammatory cytokines exert important inflammatory effects required to eliminate infection, exaggerated or prolonged responses in the initial phases could be linked to higher morbidity and mortality rates. Similarly, the proportional degree of antiinflammation that would follow could blunt the body's inflammatory potential. In keeping with the proposed explanation, increased levels of interleukin-10 were subsequently shown to serve as a prognostic marker of impending mortality.⁴⁰ Our overall view remains that an integrated, sequential, and modulated release of both the proinflammatory and, later, antiinflammatory cytokines is more important than absolute values, and hence the need to perform time course analyses when attempting to elucidate the roles of

these highly complex proteins and their even more complex and timely interactions.

In our *in vitro* model, which uses a 1 ng/ml lipopolysaccharide challenge to emulate the introduction of an intraoperative bacterial load, we were able to reproduce the short-lasting proinflammatory "burst" release at 80% O₂, when compared with the a slower and delayed clearance at 20% O₂. These findings correlated with our previous temperature studies, where 80% O₂ resembled the pattern of release and clearance seen by beneficial fever and 21% O₂ resembled more closely the pattern seen by detrimental hypothermia.³

Although interleukin-10 results do not seem to be significantly influenced by oxygen, it is important to recognize that as a late cytokine responder, change in interleukin-10 levels would be unlikely in the relatively early stages of inflammation represented in this model. However, longer interventions would be difficult to elicit in fragile *in vitro* models, and work related to nucleic acid signaling would seem more appropriate there.

Monocyte HLA-DR antigen expression has been studied extensively in infection complicating surgery and trauma as an early prognostic indicator of outcome.⁴¹ Despite the previous findings, however, our *in vitro* results show that oxygen has no effect on surface receptor expression, which, nonetheless, is a novel scientific finding in this clinical debate.

The results of this study are encouraging for the use of hyperoxia to aid in innate immune elimination of early bacterial contaminants during surgery and after trauma. Because several cellular mechanisms seem to be significantly enhanced by hyperoxia, use in human subjects to prevent SSI and septic sequelae remains a realistic possibility. However, the limitations in this study must be borne in mind when interpreting these encouraging findings and include the use of an *in vitro* model, where clinical significance of mathematically significant findings may not be truly known outside the internal human milieu. In addition, the study focus was on arterial oxygen concentrations rather than the much lower tissue oxygen values. The inspired oxygen concentrations used in this study resemble alveolar oxygen levels, and despite the detected positive differences, they would further benefit from the inclusion of lower oxygen concentrations (10–15%) to emulate true arterial and tissue levels.

Problems in implementing and translating this research to the operating suite are thought to relate to the delivery of oxygen to the surgical site. Although the vascular supply may often be compromised at sites where oxygen is in greatest demand, an intact blood supply does not ensure appropriate delivery of supplemental oxygen either. Oxygen "sinks," which have been shown to exist both radially and longitudinally along the vascular system, are well described.⁴² In addition, hypothermia, hypovolemia, mechanical pressure, or other augmentation to the sympathetic nervous system all represent possible conditions where severe decreased tissue oxygen levels and hypoxia may occur, regardless of either the inspired oxygen level or the arterial blood oxygen concentration. Therefore, we propose the idea that priming of innate

immune cells by the very high partial pressures of oxygen in the proximal circulation, before arrival at the surgical site and before dissemination of oxygen during transit, may be an alternative mechanism.

Conclusion

Hyperoxia exerts significant effects on multiple, well-recognized, and previously tested cellular and immunologic parameters. Most importantly, the functional capacity of the innate immune response, reflected by an increase in potent killing ROS, is significantly increased by the provision of high oxygen concentrations *in vitro*. The ability to translate these positive basic scientific findings to the bedside requires the existence of similar innate immune processes *in vivo* and the efficient transfer of oxygen to the sites where it is needed most, such as the wound, or, perhaps, the more proximal circulation. Optimal and timely monitoring of oxygen to tissue is essential for effective clinical translation of the "hyperoxia hypothesis."

The authors are thankful for a substantial degree of guidance and supervision provided by the late James D. Pietsch, B.S. (Laboratory Manager, The Price Institute of Surgical Research, University of Louisville School of Medicine, Louisville, Kentucky), whose contributions included the design and production of the oxygen chamber.

References

1. Akca O, Sessler DI: Supplemental oxygen and risk of surgical site infection. *JAMA* 2004; 291:1956-7
2. Polk HC Jr, Lopez-Mayor JF: Postoperative wound infection: A prospective study of determinant factors and prevention. *Surgery* 1969; 66:97-103
3. Qadan M, Gardner SA, Vitale DS, Lominadze D, Joshua IG, Polk HC Jr: Hypothermia and surgery: Immunologic mechanisms for current practice. *Ann Surg* 2009; 250:134-40
4. Bratzler DW, Houck PM, Richards C, Steele L, Dellinger EP, Fry DE, Wright C, Ma A, Carr K, Red L: Use of antimicrobial prophylaxis for major surgery: Baseline results from the National Surgical Infection Prevention Project. *Arch Surg* 2005; 140:174-82
5. Classen DC, Evans RS, Pestotnik SL, Horn SD, Menlove RL, Burke JP: The timing of prophylactic administration of antibiotics and the risk of surgical-wound infection. *N Engl J Med* 1992; 326:281-6
6. Kurz A, Sessler DI, Lenhardt R: Perioperative normothermia to reduce the incidence of surgical-wound infection and shorten hospitalization. Study of Wound Infection and Temperature Group. *N Engl J Med* 1996; 334:1209-15
7. Dellinger EP: Increasing inspired oxygen to decrease surgical site infection: Time to shift the quality improvement research paradigm. *JAMA* 2005; 294:2091-2
8. Tornero-Campello G: Hyperoxia to reduce surgical site infection? *ANESTHESIOLOGY* 2007; 106:632-3
9. Chura JC, Boyd A, Argenta PA: Surgical site infections and supplemental perioperative oxygen in colorectal surgery patients: A systematic review. *Surg Infect (Larchmt)* 2007; 8:455-61
10. Qadan M, Akca O, Mahid SS, Hornung CA, Polk HC Jr: Perioperative supplemental oxygen therapy and surgical site infection: A meta-analysis of randomized controlled trials. *Arch Surg* 2009; 144:359-66
11. Belda FJ, Aguilera L, Garcia de la AJ, Alberti J, Vicente R, Ferrandiz L, Rodriguez R, Company R, Sessler DI, Aguilar G, Botello SG, Orti R: Supplemental perioperative oxygen and the risk of surgical wound infection: A randomized controlled trial. *JAMA* 2005; 294:2035-42
12. Greif R, Akca O, Horn EP, Kurz A, Sessler DI: Supplemental perioperative oxygen to reduce the incidence of surgical-wound infection. Outcomes Research Group. *N Engl J Med* 2000; 342:161-7
13. Mayzler O, Weksler N, Domchik S, Klein M, Mizrahi S, Gurman GM: Does supplemental perioperative oxygen administration reduce the incidence of wound infection in elective colorectal surgery? *Minerva Anestesiol* 2005; 71: 21-5
14. Myles PS, Leslie K, Chan MT, Forbes A, Paech MJ, Peyton P, Silbert BS, Pascoe E: Avoidance of nitrous oxide for patients undergoing major surgery: A randomized controlled trial. *ANESTHESIOLOGY* 2007; 107:221-31
15. Pryor KO, Fahey TJ III, Lien CA, Goldstein PA: Surgical site infection and the routine use of perioperative hyperoxia in a general surgical population: A randomized controlled trial. *JAMA* 2004; 291:79-87
16. Gardella C, Goltra LB, Laschansky E, Drolette L, Magaret A, Chadwick HS, Eschenbach D: High-concentration supplemental perioperative oxygen to reduce the incidence of postcesarean surgical site infection: A randomized controlled trial. *Obstet Gynecol* 2008; 112:545-52
17. Maragakis LL, Cosgrove SE, Martinez EA, Tucker MG, Cohen DB, Perl TM: Intraoperative fraction of inspired oxygen is a modifiable risk factor for surgical site infection after spinal surgery. *ANESTHESIOLOGY* 2009; 110:556-62
18. Meyhoff CS, Wetterslev J, Jorgensen LN, Henneberg SW, Hogdall C, Lundvall L, Svendsen PE, Mollerup H, Lunn TH, Simonsen I, Martinsen KR, Pulawska T, Bundgaard L, Bugge L, Hansen EG, Riber C, Gocht-Jensen P, Walker LR, Bendtsen A, Johansson G, Skovgaard N, Helto K, Poukinski A, Korshin A, Walli A, Bulut M, Carlsson PS, Rodt SA, Lundbeck LB, Rask H, Buch N, Perdawid SK, Reza J, Jensen KV, Carlsen CG, Jensen FS, Rasmussen LS: Effect of high perioperative oxygen fraction on surgical site infection and pulmonary complications after abdominal surgery: The PROXI randomized clinical trial. *JAMA* 2009; 302: 1543-50
19. Vowells SJ, Sekhsaria S, Malech HL, Shalit M, Fleisher TA: Flow cytometric analysis of the granulocyte respiratory burst: A comparison study of fluorescent probes. *J Immunol Methods* 1995; 178:89-97
20. Hopf HW, Hunt TK, West JM, Blomquist P, Goodson WH III, Jensen JA, Jonsson K, Paty PB, Rabkin JM, Upton RA, von Smitten K, Whitney JD: Wound tissue oxygen tension predicts the risk of wound infection in surgical patients. *Arch Surg* 1997; 132:997-1004
21. Allen DB, Maguire JJ, Mahdavian M, Wicke C, Marcocci L, Scheuenstuhl H, Chang M, Le AX, Hopf HW, Hunt TK: Wound hypoxia and acidosis limit neutrophil bacterial killing mechanisms. *Arch Surg* 1997; 132:991-6
22. Hunt TK, Linsey M, Sonne M, Jawetz E: Oxygen tension and wound infection. *Surg Forum* 1972; 23:47-9
23. Hedenstierna G: Atelectasis and its prevention during anaesthesia. *Eur J Anaesthesiol* 1998; 15:387-90
24. Rothen HU, Sporre B, Engberg G, Wegenius G, Reber A, Hedenstierna G: Prevention of atelectasis during general anaesthesia. *Lancet* 1995; 345:1387-91
25. Akca O, Podolsky A, Eisenhuber E, Panzer O, Hetz H, Lampl K, Lackner FX, Wittmann K, Grabenwoeger F, Kurz A, Schultz AM, Negishi C, Sessler DI: Comparable postoperative pulmonary atelectasis in patients given 30% or 80% oxygen during and 2 hours after colon resection. *ANESTHESIOLOGY* 1999; 91:991-8
26. Miles AA, Miles EM, Burke J: The value and duration of defence reactions of the skin to the primary lodgement of bacteria. *Br J Exp Pathol* 1957; 38:79-96
27. Wenisch C, Narzt E, Sessler DI, Parschalk B, Lenhardt R, Kurz A, Graninger W: Mild intraoperative hypothermia

- reduces production of reactive oxygen intermediates by polymorphonuclear leukocytes. *Anesth Analg* 1996; 82: 810-6
28. Bardosi L, Tekeres M: Impaired metabolic activity of phagocytic cells after anaesthesia and surgery. *Br J Anaesth* 1985; 57:520-3
 29. Moudgil GC, Pandya AR, Ludlow DJ: Influence of anaesthesia and surgery on neutrophil chemotaxis. *Can Anaesth Soc J* 1981; 28:232-8
 30. Nunn JF, O'Morain C: Nitrous oxide decreases motility of human neutrophils *in vitro*. *ANESTHESIOLOGY* 1982; 56:45-8
 31. Knighton DR, Halliday B, Hunt TK: Oxygen as an antibiotic. A comparison of the effects of inspired oxygen concentration and antibiotic administration on *in vivo* bacterial clearance. *Arch Surg* 1986; 121:191-5
 32. Mandell GL: Bactericidal activity of aerobic and anaerobic polymorphonuclear neutrophils. *Infect Immun* 1974; 9:337-41
 33. Smith JA, Weidemann MJ: Further characterization of the neutrophil oxidative burst by flow cytometry. *J Immunol Methods* 1993; 162:261-8
 34. Beutler B, Milsark IW, Cerami AC: Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. *Science* 1985; 229:869-71
 35. Tracey KJ, Fong Y, Hesse DG, Manogue KR, Lee AT, Kuo GC, Lowry SF, Cerami A: Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature* 1987; 330:662-4
 36. Abraham E, Wunderink R, Silverman H, Perl TM, Nasraway S, Levy H, Bone R, Wenzel RP, Balk R, Allred R: Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial TNF-alpha MAb Sepsis Study Group. *JAMA* 1995; 273: 934-41
 37. Abraham E, Anzueto A, Gutierrez G, Tessler S, San PG, Wunderink R, Dal NA, Nasraway S, Berman S, Cooney R, Levy H, Baughman R, Rumbak M, Light RB, Poole L, Allred R, Constant J, Pennington J, Porter S: Double-blind randomized controlled trial of monoclonal antibody to human tumour necrosis factor in treatment of septic shock. NO-RASEPT II Study Group. *Lancet* 1998; 351:929-33
 38. Van der Poll T: Immunotherapy of sepsis. *Lancet Infect Dis* 2001; 1:165-74
 39. Arndt P, Abraham E: Immunological therapy of sepsis: Experimental therapies. *Intensive Care Med* 2001; 27(suppl 1): S104-15
 40. Lekkou A, Karakantza M, Mouzaki A, Kalfarentzos F, Gogos CA: Cytokine production and monocyte HLA-DR expression as predictors of outcome for patients with community-acquired severe infections. *Clin Diagn Lab Immunol* 2004; 11:161-7
 41. Cheadle WG, Hershman MJ, Wellhausen SR, Polk HC Jr: HLA-DR antigen expression on peripheral blood monocytes correlates with surgical infection. *Am J Surg* 1991; 161:639-45
 42. Tsai AG, Johnson PC, Intaglietta M: Oxygen gradients in the microcirculation. *Physiol Rev* 2003; 83:933-63