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MULTIPLE SYSTEM ORGAN RESPONSE INDUCED BY HYPEROXIA IN A CLINICALLY RELEVANT ANIMAL MODEL OF SEPSIS

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ABSTRACT—Oxygen therapy is currently used as a supportive treatment in septic patients to improve tissue oxygenation. However, oxygen can exert deleterious effects on the inflammatory response triggered by infection. We postulated that the use of high oxygen concentrations may be partially responsible for the worsening of sepsis-induced multiple system organ dysfunction in an experimental clinically relevant model of sepsis. We used Sprague-Dawley rats. Sepsis was induced by cecal ligation and puncture. Sham-septic controls (n = 16) and septic animals (n = 32) were randomly assigned to four groups and placed in a sealed Plexiglas cage continuously flushed for 24 h with medical air (group 1), 40% oxygen (group 2), 60% oxygen (group 3), or 100% oxygen (group 4). We examined the effects of these oxygen concentrations on the spread of infection in blood, urine, peritoneal fluid, bronchoalveolar lavage, and meninges; serum levels of inflammatory biomarkers and reactive oxygen species production; and hematological parameters in all experimental groups. In cecal ligation and puncture animals, the use of higher oxygen concentrations was associated with a greater number of infected biological samples ($P < 0.0001$), higher serum levels of interleukin-6 ($P < 0.0001$), interleukin-10 ($P = 0.033$), and tumor necrosis factor-α ($P = 0.034$), a marked decrease in platelet counts ($P < 0.001$), and a marked elevation of reactive oxygen species serum levels ($P = 0.0006$) after 24 h of oxygen exposure. Oxygen therapy greatly influences the progression and clinical manifestation of multiple system organ dysfunction in experimental sepsis. If these results are extrapolated to humans, they suggest that oxygen therapy should be carefully managed in septic patients to minimize its deleterious effects.

KEYWORDS—Sepsis, oxygen therapy, inflammation, multiple system organ dysfunction, infection, reactive oxygen species

INTRODUCTION

Sepsis is a complex clinical syndrome that results from a systemic inflammatory response to live bacteria and/or bacterial products and develops when the initial appropriate host response to an infection becomes amplified and is then dysregulated (1). Sepsis is frequently followed by multiple system organ dysfunction (MSOD) and remains the most common cause of death in intensive care units worldwide (2). There are no effective specific therapies, and many patients survive the initial infection only to die of subsequent MSOD. Previous studies have established that the prognosis is directly related to the number of organ systems failing (1, 3–5).

The cellular and molecular mechanisms underlying MSOD are unclear. The lungs are one of the first organs primed by local and/or systemic cytokine storm during sepsis, and because they are damaged both morphologically and functionally, hypoxemia is relatively common. As such, supplemental oxygen is often used as a supportive treatment to improve tissue oxygenation in septic patients. Several experimental studies have reported beneficial effects of hyperoxia in sepsis models, with modulation of inflammation and improvement in survival (6), attenuation of lung inflammatory responses (7), improvement of organ function, and decrease of apoptosis (8). However, there are important concerns with the use of hyperoxia because of the increased formation of reactive oxygen species (ROS) (9, 10). Oxidative stress exerts deleterious effects in sepsis, contributing to inflammation and tissue injury (11). Several studies have demonstrated the clinical significance of oxidative stress mechanisms in sepsis, showing an association between lower antioxidant potential and unfavorable outcome (12), greater oxidative stress in patients with the systemic inflammatory response syndrome (13), a relationship between plasma redox status and severity in critically ill patients (14), and lipid peroxidation and sepsis-induced MSOD (15).

Because the role of oxygen therapy during sepsis remains controversial, we postulated that the use of high oxygen

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concentrations may be partially responsible for the worsening or progression of sepsis-induced MSOD. We examined this hypothesis by investigating the effects of different oxygen concentrations on the spread of infection, inflammatory biomarkers, and hematological parameters in an experimental, validated, clinically relevant model of sepsis induced by cecal ligation and puncture (CLP).

METHODS

This study was approved by the ethical committee for animal experimentation of the Hospital Universitario Dr. Negrín (CEEBA 003/2012). The use of animals and the experimental protocols were performed in compliance with standard operating and quality procedures following guidelines by the European Commission (2010/63/EU). We followed the ARRIVE guidelines (16) for reporting standards for preclinical animal studies.

Experimental animals

Experiments were performed in male, pathogen-free, Sprague-Dawley rats, 13 weeks old, weighing 257 ± 21 g. Routine microbiological monitoring was performed in accordance with the recommendations of the Federation of European Laboratory Animal Associations and revealed no evidence of bacterial or parasitic infections.

Blood samples

Animals were anesthetized with an s.c. cocktail of fentanyl (Fentanest; Kern Pharma, Barcelona, Spain) and medetomidine (Domtor; Esteve, Barcelona, Spain), both at 0.3 mg/kg. Baseline blood samples (0.7 mL) were taken from the jugular vein before performing the CLP technique and placed into an EDTA K_3 tube (Becton Dickinson, Madrid, Spain) for hematological examination.

CLP model

Sepsis was induced by CLP, a widely used model for experimental sepsis, which closely mimics human sepsis (17). A detailed description of the experimental model used in this study is provided elsewhere (18). After closing the abdomen, each animal received 5 mL normal saline per 100 g/kg body weight, s.c., for fluid resuscitation. Sham-CLP rats served as controls and underwent the same surgical procedures as CLP rats: the cecum was exposed (but not ligated nor punctured) and returned to the abdominal cavity, and the abdominal wall was then sutured.

Study groups and oxygen exposure

Cecal ligation and puncture were performed in a total of 51 rats randomly allocated to four oxygen concentration groups (21%, 40%, 60%, 100%). Multiple rats were put into the O_2 chambers at the same time. However, only the first eight rats surviving the 24-h septic period were considered for analysis (n = 32). Sham-CLP was performed in 16 rats. Thus, eight groups of rats were studied: CLP (n = 8 per group) or sham-operated (n = 4 per group), breathing 21%, 40%, 60%, or 100% O_2.

After the surgical procedures, septic and control rats were placed into a sealed Plexiglas cage continuously flushed with medical air (21% oxygen) or with oxygen appropriately mixed to reach stable concentrations of 40%, 60%, and 100% of oxygen. Oxygen concentration and temperature inside the cage were continuously monitored (24 h) by an oximeter (Maxtec, Salt Lake City, Utah) and a thermometer, respectively. Eight groups of animals were studied: CLP spontaneously breathing 21%, 40%, 60%, or 100%O_2 and sham-operated animals breathing 21%, 40%, 60%, or 100% O_2. Animals received food and water *ad libitum*. At the end of the experiment (24 h after CLP or sham-CLP), surviving animals were sacrificed for biological examination.

Biological sample collection

Biological samples from blood, urine, peritoneal fluid (PF), bronchoalveolar lavage (BAL), and meninges were collected in all experimental groups at the end of the 24-h experimental period under general anesthesia. Blood (2 mL) was withdrawn from the jugular vein: 1 mL was placed into an EDTA K_3 tube for hematological study, and 1 mL was placed into a hemoculture tube (BD Bactec Peds Plus/F Culture Vials; Becton Dickinson). In addition, 4 to 5 mL of blood were collected by puncturing the heart through the diaphragm, placed into a separating tube (Becton Dickinson), and, after centrifugation at 3,000*g* for 10 min, serum was frozen and stored at minus 80°C for further analysis.

Cecal ligation and puncture and sham animals underwent laparotomy, and the bladder was punctured to collect 50 μL of urine that was placed into a sterile container for microbiological analysis. Through the laparotomy, 10 mL of sterile saline solution was introduced into the abdominal cavity. The zone was carefully "massaged" to allow better fluid distribution; 5 mL of peritoneal fluid was recovered, and 50 μL was placed into a sterile tube for microbiological analysis.

After performing sternotomy, the lungs were exposed to perform a BAL through a cannula (1.2 mm inner diameter) inserted into the trachea. Lavage fluid (5 mL) was slowly injected into the lungs using a sterile syringe and then recovered by gentle suction to avoid tissue damage. The procedure was repeated four times. Then, 100 μL of collected BAL was placed into a sterile tube for microbiological analysis. Finally, the skull was carefully dissected, and small samples of meninges were removed using sterile conditions and placed into a sterile tube for microbiological analysis.

Analytical determinations

Interleukin-6 (IL-6) was measured in serum using a commercially available enzyme-linked immunosorbent assay kit (Abcam, Cambridge, UK) following the manufacturer's instructions. Tumor necrosis factor-α (TNF-α) and IL-10 were measured in serum using the CBA Flex Set cytometric bead array (BD Biosciences, Madrid, Spain). Variation coefficients were always below 5%.

Blood leukocytes and platelets were assessed using a Cell-Dyn Sapphire (Abbott, Madrid, Spain) cell analyzer. Changes in hematological parameters were defined as the difference between levels at baseline minus levels at 24 h.

We assessed the oxidative status at the end of the 24-h experimental period by measuring total ROS levels in stored samples of serum of all experimental groups (control and CLP rats) using the Oxiselect *in vitro* assay kit (Cell Biolabs, San Diego, Calif) following the manufacturer's instructions. This assay quantifies four free radicals that are produced in excess (nitric oxide, hydrogen peroxide, peroxyl radical, peroxynitrite) during sepsis and hyperoxia. Free radicals in the samples react with a specific probe that is converted into the highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). Therefore, fluorescence intensity is proportional to ROS levels in the samples. Fluorescence was read at 480 nm excitation/530 nm emission using an FLx800 microplate reader (BioTek), and the concentration of total ROS was calculated using the DCF standard curve.

Microbiological and analytical determinations were performed by investigators blinded to experimental procedures.

Statistical analysis

For the statistical power analysis for sample size calculations in each oxygen category of CLP animals, we estimated to detect an absolute 50% increase in the total number of infected samples in animals treated with 100% oxygen compared with 21% or 40% oxygen, with an $\alpha = 0.05$ and a power greater than 0.80. The number of animals alive needed to be tested at the end of the 24-h treatment period in each CLP group is 8. Because the reported 24-h mortality rate of CLP rats is in the range of 25% to 40%, we added three to six animals in each oxygen group for ensuring an adequately powered confirmatory study.

Data are expressed as mean ± SD. Comparisons between experimental groups were performed with analysis of variance following *post hoc* tests for pairwise comparisons and linear trends using statistical software Prism 5 (GraphPad, La Jolla, Calif). Proportions were compared with the linear-by-linear association test using SPSS 15.0 exact test module. A two-tailed value of $P < 0.05$ was considered statistically significant.

RESULTS

Cecal ligation and puncture induced typical signs of disease, including lethargy, chromodacryorrhea, ruffled fur, generalized weakness, and reduced gross motor activity. Cecal ligation and puncture rats had an average weight loss of 7.6% ± 3.1% at 24 h, with no significant differences related to oxygen concentration. At 24 h, no differences in mortality rates for CLP rats allocated to 21%, 40%, 60%, and 100% O_2 were found: 27.3%, 26.6%, 27.3%, and 28.5%, respectively ($P = 0.99$). All 16 sham-operated rats survived the 24-h experimental period. Because we chose to study only eight animals in each subgroup to avoid any bias in assigning the animals according to power analysis and randomization processes, making sure that the groups were balanced and comparable, we collected blood and tissue samples from three excess animals exposed to 40% oxygen and two from the group on 100% oxygen for further studies.

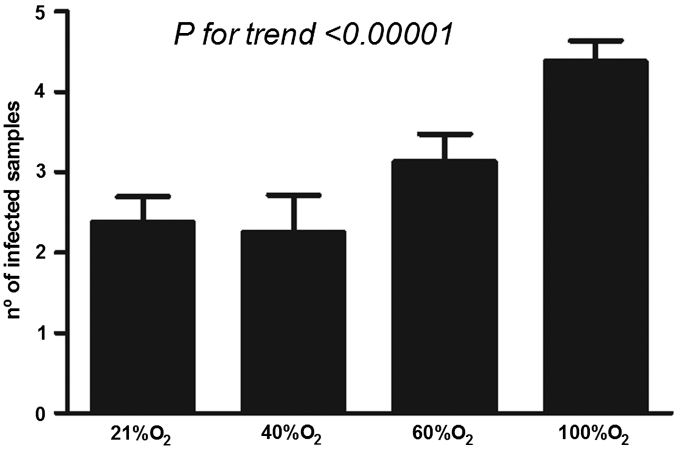

FIG. 1. **Effects of inspired oxygen on the number of infected samples after experimental sepsis**. The number of infected samples was different among different septic groups (n = 8 per group).

Effects of oxygen on infection spreading

The number of infected samples differed among the CLP groups. There was a significant dose response relating the number of infected samples (combining blood, BAL, PF, urine, and meninges) and oxygen concentration ($P < 0.00001$) (Fig. 1). All sham animals had negative bacterial cultures.

All peritoneal fluids from CLP animals were infected. Bronchoalveolar lavage fluids were also infected but did not exhibit any relationship with oxygen concentration. In general, blood, urine, and meninges showed greater infection rates after exposure to 100% O_2, although the number of positive urocultures was the only one demonstrating a significant trend with increasing oxygen concentration ($P < 0.021$) (Fig. 2). CLP animals on 100% oxygen had the highest ranges of colony-forming units in BAL and urine (data not shown). Also, CLP animals on 100% oxygen had the highest bacterial colony counts in meninges by semiquantitative analysis (data not shown).

Escherichia coli was the only type of bacteria isolated in 84.5% of CLP rats. *Enterococcus faecalis* was isolated in 3% of CLP rats, and both species were isolated in 12.5% of CLP animals.

IL-6, TNF-α, and IL-10 serum levels

Serum levels of IL-6 were different among septic groups and showed a significant increasing linear trend with oxygen concentration ($P < 0.0001$) (Fig. 3A). Oxygen concentrations modified TNF-α serum levels in CLP groups ($P = 0.034$) (Fig. 3B). CLP rats exposed to 40% or 60% O_2 had lower mean TNF-α serum levels compared with CLP–21% O_2 and CLP–100% O_2 group. There were no significant changes in TNF-α levels in sham animals (see Table S1 in Supplemental Digital Content 1, at http://links.lww.com/SHK/A213). TNF-α levels in sham–40% O_2 and sham–60% O_2 were below the detection limit of our assay.

Serum levels of IL-10 varied among CLP groups ($P = 0.033$) (Fig. 3C). CLP rats exposed to 100% O_2 had significantly higher serum levels of IL-10 than those exposed to 21%, 40%, or 60%O_2, with no significant differences among them. There were no significant differences in IL-10 levels among sham groups.

Blood leukocyte and platelet counts

At 24 h of oxygen exposure, CLP and sham-CLP rats had leukopenia and thrombocytopenia (Fig. 4). Among CLP groups, there was no effect of oxygen concentration on the change in leukocyte counts from baseline (Fig. 4A). Cecal ligation and puncture rats had greater decreases in leukocyte counts compared with their respective sham groups ($P < 0.01$ for 21% and 40% O_2; $P < 0.001$ for 40% and 100% O_2). Of note, the decrease in leukocyte counts was different among sham groups and showed a significant linear trend ($P = 0.009$), with a lower decrease at greater oxygen concentrations, suggesting a dose-dependent effect of oxygen on leukocyte count, that even leads to leukocytosis in 100% O_2 sham rats (see Figure S1 in SDC).

We found a significant dose-dependent effect of oxygen on the change in platelet numbers in the CLP groups, with a significant linear trend between changes in platelet counts and oxygen concentration ($P < 0.001$) (Fig. 4B). No significant differences were found between CLP and each respective sham group (see Figure S1 in in Supplemental Digital Content 1, at http://links.lww.com/SHK/A213).

Reactive oxygen species

In CLP animals, total ROS levels increased significantly with every increase in oxygen concentration ($P = 0.0006$) (Fig. 5). Reactive oxygen species levels were significantly higher in CLP compared with those in sham animals. No significant changes of ROS serum levels were found among sham groups (see Figure S2 in Supplemental Digital Content 1, at http://links.lww.com/SHK/A213).

DISCUSSION

The major finding of our study is that the concentration of inspired oxygen has profound effects on the spread of infection to several organ systems during experimental sepsis. To the best of our knowledge, this is the first study exploring the effects of inspired oxygen on dissemination of bacterial infection in five different systems (blood, urine, BAL, PF, and meninges).

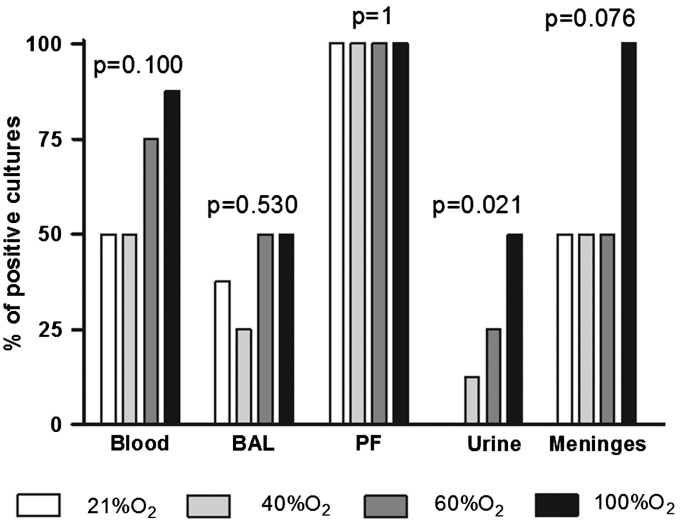

FIG. 2. **Effects of oxygen on the percentage of positive cultures in different samples analyzed**. The number of positive urocultures showed a significant association with inspired oxygen concentration, and the same tendency was observed in meningeal samples (n = 8 per group).

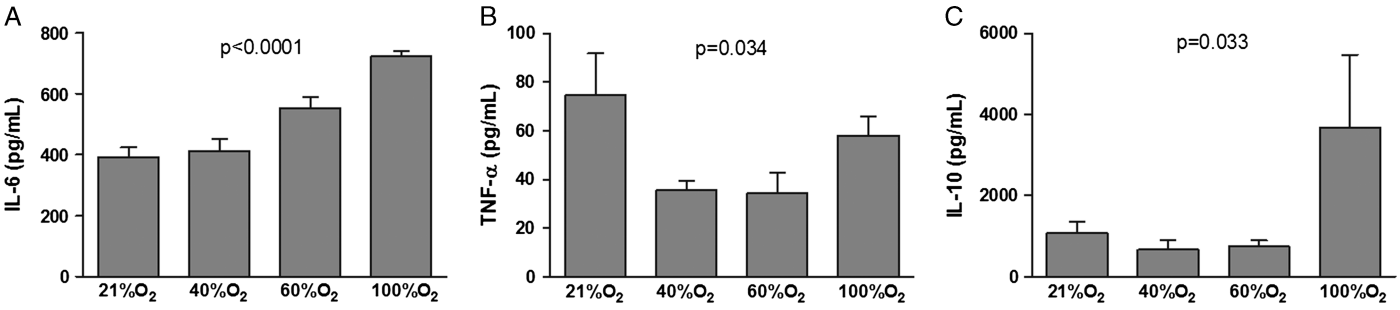

FIG. 3. **Effects of inspired oxygen concentration on IL-6 (A), TNF-α (B), and IL-10 (C) serum levels in septic rats (n = 8 per group).**

Our data could have translational implications for patient care during the early phases of the septic process. Although a recent update of the guidelines of the Surviving Sepsis Campaign (19) set the foundations for the acute management of sepsis, the panel of experts did not mention any recommendation related to targeting the bundles for oxygen therapy in septic patients.

Sepsis is currently the leading cause of MSOD and death among noncardiac critically ill patients (1, 2). Because the effects of hyperoxia during sepsis management are unclear (10), we used CLP as a clinically relevant and well-characterized animal model to explore the role of oxygen therapy during the first 24 h of sepsis. Cecal ligation and puncture induced a reproducible and consistent septic condition in accordance with previous studies (17). Live bacteria were identified in several biological samples, and CLP animals had increased serum levels of inflammatory biomarkers, leukopenia, and thrombocytopenia. In addition, exposure to high oxygen concentrations led to a greater spread of the infection, suggesting that oxygen influences cellular and molecular pathways that regulate host response to bacterial infection, eventually resulting in a more intense systemic dissemination of the pathogen and/or bacterial products. There are conflicting results regarding the use of oxygen in the management of sepsis. Using a similar model of CLP as ours, Waisman et al. (7) evaluated the effects of hyperoxia on pulmonary inflammation by comparing continuous inhalation with 100% O_2 for 20 h or 70% O_2 for 48 h, with intermittent 100% O_2 applied for 6 h daily over 48 h. They reported that intermittent hyperoxia markedly attenuated lung inflammation, with no differences in mortality among groups, although no animals died at 20 h after CLP and mortality in the intermittent O_2 group was 57.8% (7). Buras et al. (6) reported that hyperbaric treatment resulted in improvement in survival compared with control CLP mice breathing air, although this survival effect was not sustained beyond 24 h. In contrast, the study by Thiel et al. (20) showed that excess oxygen worsens organ inflammation during endotoxemia. They induced lung inflammation in two groups of mice by intratracheal injection of both lipopolysaccharide and staphylococcal enterotoxin: one group did not receive supplemental oxygen and the other group was treated with 100% for 48 to 60 h. The majority (73%) of oxygenated animals died, whereas 87% of the nonoxygenated control mice survived the 60-h observational period. The exacerbation of acute lung injury in their model suggests that excessive oxygenation inhibits the physiological tissue-protecting mechanism that limits lipopolysaccharide-induced MSOD. It is not possible to ascertain why our results were different to these (6, 7, 20) and other studies (21, 22), although differences in study design, timing and dosage of administered oxygen, species (rat versus mice), method of inducing sepsis, and the type of treatment (normobaric versus hyperbaric) are reasonable candidates.

Our study did not demonstrate a relationship between mortality and inspired oxygen concentration. We did not perform a longer follow-up because mortality was not our primary end point. As previously reported, lethality in the CLP model begins at 18 to 24 h after the induction of sepsis (17, 18). Although the problem of oxygen-induced lung damage is well known, the biochemical processes leading to tissue damage have not been fully elucidated. Reactive oxygen species are chemically reactive molecules derived from normal metabolism of oxygen and

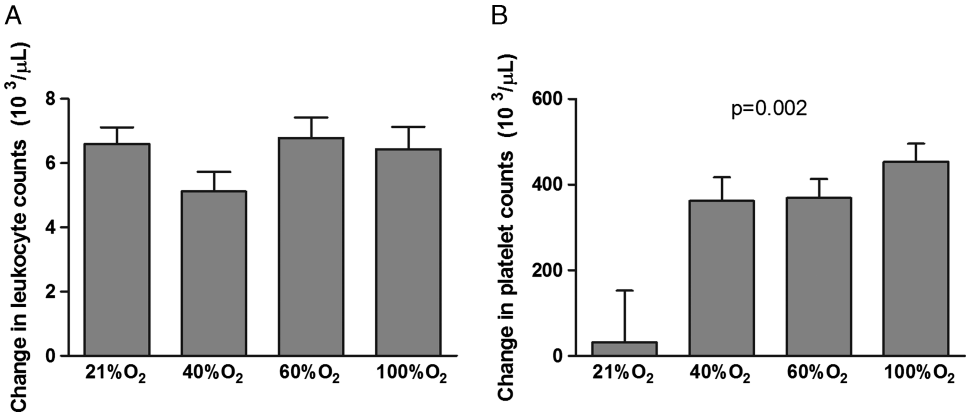

FIG. 4. **Effects of 24 h of exposure to oxygen in septic animals on changes in: (A) blood leukocyte and (B) platelet counts compared with baseline**. Data are presented as mean ± SD. See text for more details.

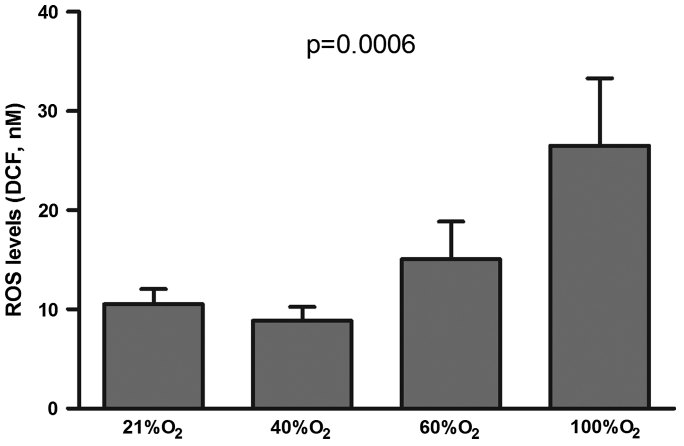

FIG. 5. **Reactive oxygen species serum levels in septic animals after 24 h of exposure to different oxygen concentrations**. DCF, 2′,7′-dichlorodihydrofluorescein.

have important roles in cell signaling. Increased ROS levels may result in significant damage to cell structures. Because we have found increased serum levels of ROS in all CLP animals, we can speculate that high oxygen concentrations, by promoting oxidative stress, could be linked in some manner to the observed proinflammatory status and multiorgan bacterial colonization.

We found that all peritoneal fluids were positive for enteric bacteria, as expected in the CLP model, and that a high concentration of inspired oxygen increases bacterial translocation to blood. This may suggest that bacterial load in blood and/or mechanisms of bacterial clearance are significantly influenced by oxygen. Half of the brains from the septic animals on 21%, 40%, and 60% O_2 grew bacteria, whereas all samples from the 100% O_2 group were infected. It is known that sepsis affects blood-brain barrier integrity (23), and although our results did not reach statistical significance ($P = 0.07$), these findings suggest that oxygen may exacerbate sepsis-induced injury by mechanisms related to oxidative stress involved in blood-brain barrier dysfunction (24), thus spreading the infection and contributing to brain dysfunction. Microbiological cultures of BAL did not show a clear relationship with oxygen concentration, although enteric bacteria often reach the lungs in this model. Previous CLP studies in rodents have reported positive blood cultures containing mixed enteric bacteria that are detectable as early as 6 h after CLP (25), but we are not aware of any reports regarding affected organs. It is possible that samples taken at different time points would show different results because bacteria may reach some organs faster than others, and cellular responses as well as clearance of pathogens do not occur at the same rate in all tissues. The aim of the present study was to examine the effect of 24 h of oxygen exposure in sepsis rather than to further characterize the extensively investigated CLP model.

Interleukin-6 is a major cytokine involved in sepsis and an independent predictor of outcome after CLP (26). In our study, we found that the greater the oxygen concentration, the higher the IL-6 serum levels. Given the crucial proinflammatory role of IL-6 and the numerous studies showing an association between high levels of IL-6 and increased mortality in septic patients (27), we speculate that hyperoxia-induced inflammation may exacerbate inflammatory and oxidative stress pathways in sepsis, contributing to the spread of infection and MSOD that ultimately leads to death. Paradoxically, we found that treatment with 40% or 60% O_2 decreases CLP-induced elevation of serum TNF-α, although TNF-α levels after 100% O_2 were not significantly different than on 21% O_2. This may suggest that moderate hyperoxia was able to reduce TNF-α expression in sepsis, possibly attenuating inflammatory effects mediated by this cytokine. Overall, these data suggest that oxygen concentration and length of exposure are crucial determinants when evaluating biological effects of oxygen in sepsis. Concentrations of 40% O_2 or 60% O_2 did not have effects on the anti-inflammatory cytokine IL-10, whereas 100% O_2 induced a marked increase in serum IL-10 levels. By contrast, previous results from Buras et al. (6) showed that hyperbaric oxygen did not modify serum IL-10 levels of septic mice but increased IL-10 in macrophages. High IL-10 levels observed after 100% O_2 exposure may support the concept of deleterious consequences of unbalanced cytokine production, in line with previous studies reporting significant associations between increased IL-10 and mortality in septic patients (28–31). Nevertheless, we cannot determine whether the elevated IL-10 levels in the septic 100% O_2 group are a direct consequence of oxygen exposure or a physiological response. Data from sham groups suggest that, in noncritical conditions, increased oxygen levels may not exert significant effects on cytokine release and/or physiological responses can counteract them.

Oxygen therapy did not have any effect on CLP-induced leukopenia, whereas it did on sham animals. Oxygen attenuated the decrease in leukocyte counts of sham-operated rats in a dose-dependent manner, and the 100% O_2 group developed leukocytosis. This finding suggests a plausible modulator role for oxygen with respect to white blood cells despite the fact that we did not observe an oxygen-induced elevation of cytokines in sham rats. Given the key role of leukocytes on immunoinflammatory mechanisms, future studies are necessary to confirm and extend this novel finding. We also found a direct relationship between oxygen concentration and platelet counts. Septic rats developed thrombocytopenia, more pronounced with higher concentrations of oxygen. Thrombocytopenia is a hematological manifestation of MSOD frequently caused by disseminated intravascular coagulation and formation of small blood clots inside the vessels throughout the body, thus reducing blood flow and indicating severe disease (32).

Our study has some limitations. First, although our aim was to analyze biological responses after 24 h of oxygen exposure, it is possible that a different temporal profile may provide additional information because it is known that cytokines peak at earlier time points (33). Second, we did not measure blood gases. However, although Waisman et al. (7) reported normal PaO_2 values on 70% and 100% O_2 at 6 h after CLP, Thiel et al. (20) found severe impairment of gas exchange in mice breathing 100% O_2 when returned to ambient air. A major strength of our study is that we measured ROS in the serum of all experimental groups and found that hyperoxia aggravated the sepsis-induced oxidative stress.

In summary, our findings suggest that oxygen therapy greatly influences clinically relevant parameters in sepsis. Treatment

with concentrations of 40% and 60% inspired O_2 led to moderate changes, whereas 100% O_2 was associated with a greater spread of infection, a marked increase of cytokines, and thrombocytopenia, with no effect on 24-h mortality. We can only speculate that these changes could be linked to oxidative stress induced by hyperoxia. Thus, our results suggest that the use of supplemental oxygen in the clinical setting should be carefully selected to achieve a proper balance between beneficial and detrimental effects. In this context, our study may add a piece to the complex puzzle of oxygen-mediated mechanisms of MSOD in sepsis.

REFERENCES

1. Blanco J, Muriel-Bombin A, Sagredo V, Taboada F, Gandía F, Tamayo L, Collado J, García-Labattut A, Carriedo D, Valledor M, et al: Grupo de Estudios y Análisis en Cuidados Intensivos. Incidence, organ dysfunction and mortality in severe sepsis: a Spanish multicentre study. *Crit Care* 12(6):R158, 2008.
2. Cohen J: The immunopathogenesis of sepsis. *Nature* 420(6917):885–891, 2002.
3. Carrico CJ, Meakins JL, Marshall JC, Fry D, Maier RV: Multiple-organ-failure syndrome. *Arch Surg* 121(2):196–208, 1986.
4. Villar J, Manzano JJ, Blazquez MA, Quintana J, Lubillo S: Multiple system organ failure in acute respiratory failure. *J Crit Care* 6(2):75–80, 1991.
5. Riedermann NC, Guo RF, Ward PA: The enigma of sepsis. *J Clin Invest* 112(4):460–467, 2003.
6. Buras JA, Holt D, Orlow D, Belikoff B, Pavlides S, Reenstra WR: Hyperbaric oxygen protects from sepsis mortality via an interleukin-10–dependent mechanism. *Crit Care Med* 34(10):2624–2629, 2006.
7. Waisman D, Brod V, Rahat MA, Amit-Cohen BC, Lahat N, Rimar D, Menn-Josephy H, David M, Lavon O, Cavari Y, et al: Dose-related effects of hyperoxia on the lung inflammatory response in septic rats. *Shock* 37(1):95–102, 2012.
8. Barth E, Bassi G, Maybauer DM, Simon F, Gröger M, Oter S, Speit G, Nguyen CD, Hasel C, Möller P, et al: Effects of ventilation with 100% oxygen during early hyperdynamic porcine fecal peritonitis. *Crit Care Med* 36(2):495–503, 2008.
9. Altemeier WA, Sinclair SE: Hyperoxia in the intensive care unit: why more is not always better. *Curr Opin Crit Care* 13(1):73–78, 2007.
10. Asfar P, Calzia E, Huber-Lang M, Ignatius A, Radermacher P: Hyperoxia during septic shock—Dr. Jekyll or Mr. Hyde? *Shock* 37(1):122–123, 2012.
11. Nathan C, Cunningham-Bussel A: Beyond oxidative stress: an immunologist's guide to reactive oxygen species. *Nat Rev Immunol* 13(5):349–361, 2013.
12. Cowley HC, Bacon PJ, Goode HF, Webster NR, Jones JG, Menon DK: Plasma antioxidant potential in severe sepsis: a comparison of survivors and non-survivors. *Crit Care Med* 24(7):1179–1183, 1996.
13. Alonso de Vega JM, Díaz J, Serrano E, Carbonell LF: Oxidative stress in critically ill patients with systemic inflammatory response syndrome. *Crit Care Med* 30(8):1782–1786, 2002.
14. Alonso de Vega JM, Díaz J, Serrano E, Carbonell LF: Plasma redox status relates to severity in critically ill patients. *Crit Care Med* 28(6):1812–1814, 2000.
15. Ware LB, Fessel JP, May AK, Roberts LJ 2nd: Plasma biomarkers of oxidant stress and development of organ failure in severe sepsis. *Shock* 36(1):12–17, 2011.
16. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG: Improving bioscience research reporting: The ARRIVE guidelines for reporting animal research. *PLoS Biology* 8(6):e1000412, 2010.
17. Rittirsch D, Huber-Lang MS, Flierl MA, Ward PA: Immunodesign of experimental sepsis by cecal ligation and puncture. *Nat Protoc* 4(1):31–36, 2009.
18. Villar J, Ribeiro SP, Mullen JBM, Kuliszewski M, Post M, Slutsky AS: Induction of the heat shock response reduces mortality rate and organ damage in a sepsis-induced acute lung injury model. *Crit Care Med* 22(6):914–921, 1994.
19. Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky JE, Sprung CL, Douglas IS, Jaeschke R, et al: Surviving Sepsis Campaign Guidelines Committee including the Pediatric Subgroup: Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2012. *Crit Care Med* 41(2):580–637, 2013.
20. Thiel M, Chouker A, Ohta A, Jackson E, Caldwell C, Smith P, Lukashev D, Bittmann I, Sitkovsky MV: Oxygenation inhibits the physiological tissue-protecting mechanism and thereby exacerbates acute inflammatory lung injury. *PLoS Biol* 3(6):e174, 2005.
21. Hou L, Xie K, Qin M, Peng D, Ma S, Shang L, Li N, Li S, Ji G, Lu Y, et al: Effects of reactive oxygen species scavenger on the protective action of 100% oxygen treatment against sterile inflammation in mice. *Shock* 33(6):646–654, 2010.
22. Xie K, Fu W, Xing W, Li A, Chen H, Han H, Yu Y, Wang G: Combination therapy with molecular hydrogen and hyperoxia in a murine model of polymicrobial sepsis. *Shock* 38(6):656–663, 2012.
23. Gofton TE, Young GB: Sepsis-associated encephalopathy. *Nat Rev Neurol* 8(10):557–566, 2012.
24. Avtan SM, Kaya M, Orhan N, Arslan A, Arican N, Toklu AS, Gürses C, Elmas I, Kucuk M, Ahishali B: The effects of hyperbaric oxygen therapy on blood-brain barrier permeability in septic rats. *Brain Res* 1412:63–72, 2011.
25. Flierl MA, Rittirsch D, Gao H, Hoesel LM, Nadeau BA, Day DE, Zetoune FS, Sarma JV, Huber-Lang MS, Ferrara JL, et al: Adverse functions of IL-17A in experimental sepsis. *FASEB J* 22(7):2198–2205, 2008.
26. Gao M, Zhang L, Liu Y, Yang M, Wang N, Wang K, Ou D, Liu M, Chen G, Liu K, et al: Use of blood urea nitrogen, creatinine, interleukin-6, granulocyte-macrophage colony stimulating factor in combination to predict the severity and outcome of abdominal sepsis in rats. *Inflamm Res* 61(8):889–897, 2012.
27. Petillä V, Hynninen M, Takkunen O, Kuusela P, Valtonen M: Predictive value of procalcitonin and interleukin 6 in critically ill patients with suspected sepsis. *Intensive Care Med* 28(9):1220–1225, 2002.
28. van Dissel JT, van Langevelde P, Westendorp RG, Kwappenberg K, Frölich M: Anti-inflammatory cytokine profile and mortality in febrile patients. *Lancet* 351(9107):950–953, 1998.
29. Gogos CA, Drosou E, Bassaris HP, Skoutelis A: Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis* 181(1):176–180, 2000.
30. Monneret G, Finck ME, Venet F, Debard AL, Bohé J, Bienvenu J, Lepape A: The anti-inflammatory response dominates after septic shock: association of low monocyte HLA-DR expression and high interleukin-10 concentration. *Immunol Lett* 95(2):193–198, 2004.
31. Kellum JA, Kong L, Fink MP, Weissfeld LA, Yealy DM, Pinsky MR, Fine J, Krichevsky A, Delude RL, Angus DC; GenIMS Investigators: Understanding the inflammatory cytokine response in pneumonia and sepsis: results of the Genetic and Inflammatory Markers of Sepsis (GenIMS) Study. *Arch Intern Med* 167(15):1655–1663, 2007.
32. Semeraro N, Ammollo CT, Semeraro F, Colucci M: Sepsis, thrombosis and organ dysfunction. *Thromb Res* 129(3):290–295, 2012.
33. Ayala A, Chaudry IH: Immune dysfunction in murine polymicrobial sepsis: mediators, macrophages, lymphocytes and apoptosis. *Shock* 6(Suppl 1): S27–S38, 1996.