

RESEARCH ARTICLE | *Translational Physiology*

## Early hyperbaric oxygen therapy improves survival in a model of severe sepsis

Jonathan L. Halbach,<sup>1</sup> James M. Prieto,<sup>1</sup> Andrew W. Wang,<sup>1</sup> Dennis Hawisher,<sup>2</sup> David M. Cauvi,<sup>2</sup> Tony Reyes,<sup>3</sup> Jonathan Okerblom,<sup>4</sup> Israel Ramirez-Sanchez,<sup>5</sup> Francisco Villarreal,<sup>5</sup> Hemal H. Patel,<sup>4</sup> Stephen W. Bickler,<sup>6</sup> George A. Perdrizet,<sup>7</sup> and Antonio De Maio<sup>2,8</sup>

<sup>1</sup>Department of Surgery, Naval Medical Center San Diego, San Diego, California; <sup>2</sup>Division of Trauma, Critical Care, Burns, and Acute Care Surgery, Department of Surgery, School of Medicine, University of California San Diego, La Jolla, California; <sup>3</sup>University of California San Diego Initiative for Maximizing Student Development Program, University of California San Diego, La Jolla, California; <sup>4</sup>Department of Anesthesiology, School of Medicine, University of California San Diego, and Veterans Affairs San Diego Healthcare System, La Jolla, California; <sup>5</sup>Department of Medicine, School of Medicine, University of California San Diego, La Jolla, California; <sup>6</sup>Division of Pediatric Surgery, Rady Children's Hospital, San Diego, California; <sup>7</sup>Department of Emergency Medicine, School of Medicine, University of California San Diego, La Jolla, California; and <sup>8</sup>Department of Neurosciences, School of Medicine, University of California San Diego, La Jolla, California

Submitted 19 March 2019; accepted in final form 8 May 2019

**Halbach JL, Prieto JM, Wang AW, Hawisher D, Cauvi DM, Reyes T, Okerblom J, Ramirez-Sanchez I, Villarreal F, Patel HH, Bickler SW, Perdrizet GA, De Maio A.** Early hyperbaric oxygen therapy improves survival in a model of severe sepsis. *Am J Physiol Regul Integr Comp Physiol* 317: R160–R168, 2019. First published May 15, 2019; doi:10.1152/ajpregu.00083.2019.—Sepsis is a major clinical challenge, with therapy limited to supportive interventions. Therefore, the search for novel remedial approaches is of great importance. We addressed whether hyperbaric oxygen therapy (HBOT) could improve the outcome of sepsis using an acute experimental mouse model. Sepsis was induced in male CD-1 mice by cecal ligation and puncture (CLP) tailored to result in 80–90% mortality within 72 h of the insult. After CLP, mice were randomized into two groups receiving HBOT or not at different times after the initial insult or subjected to multiple HBOT treatments. HBOT conditions were 98% oxygen pressurized to 2.4 atmospheres for 1 h. HBOT within 1 h after CLP resulted in 52% survival in comparison with mice that did not receive the treatment (13% survival). Multiple HBOT at 1 and 6 h or 1, 6, and 21 h displayed an increase in survival of >50%, but they were not significantly different from a single treatment after 1 h of CLP. Treatments at 6 or 21 h after CLP, excluding the 1 h of treatment, did not show any protective effect. Early HBO treatment did not modify bacterial counts after CLP, but it was associated with decreased expression of TNF- $\alpha$ , IL-6, and IL-10 expression in the liver within 3 h after CLP. The decrease of cytokine expression was reproduced in cultured macrophages after exposure to HBOT. Early HBOT could be of benefit in the treatment of sepsis, and the protective mechanism may be related to a reduction in the systemic inflammatory response.

cytokines; hyperbaric oxygen therapy; sepsis; systemic inflammatory response

### INTRODUCTION

Sepsis is a major health problem at the level of morbidity, mortality, and economic burden to the healthcare system, impacting more than 1.5 million people/yr in the United States. The mortality rate is between 30% and 50% and is triggered particularly by the development of secondary conditions such as septic shock and multiple organ failure (3, 20). In addition, the healthcare costs associated with the treatment of sepsis exceed more than \$20 billion per year, which is likely to continue to rise (50). Sepsis is currently defined as a life-threatening organ dysfunction condition caused by a dysregulated host response to infection and injury (18), in which homeostasis is not restored (21). Therapeutic interventions directed at neutralizing single factors have failed (3), probably due to the multifactorial characteristics of this condition, which is modulated by several factors, including the initial insult, sex, age, environment, and genetics (19). In addition, the therapeutic window for disease resolution is unclear. Supported therapy, such as the administration of antibiotics and fluids, remains the only clinical option. The Surviving Sepsis Campaign calls for early administration of supported therapy to ameliorate the disease (<http://www.survivingsepsis.org>), suggesting that early events may be responsible for triggering the condition. Indeed, prior studies using an experimental model of sepsis showed that the therapeutic window is restricted to early events after the insult (13). Consequently, the search for novel systemic interventions that could ameliorate sepsis at earlier stages is crucial to curbing this devastating condition.

Sepsis is characterized by an increase in the inflammatory response and dramatic changes in systemic metabolism with an early hypermetabolic phase that progresses to a preterminal low-flow shock condition (30, 45). Inadequate oxygen delivery to various tissue systems is likely to contribute to organ failure during septic shock (46). Consequently, improving tissue oxygenation may ameliorate organ dysfunction and the return to homeostasis. In this regard, hyperbaric oxygen therapy

Address for reprint requests and other correspondence: A. De Maio, Univ. of California San Diego School of Medicine, 9500 Gilman Dr., No. 0739, La Jolla, CA 92093-0739 (e-mail: ademai@ucsd.edu).

(HBOT) that consists of exposure to 100% oxygen under increased atmospheric pressure is a potential intervention to prevent septic shock. HBOT was first used as a radio-sensitizing agent during radiation therapy for cancer (16), but it has gained great interest as an agent for the treatment of decompression illness (28). HBOT has also been used as a remedy for many other conditions (48), including severe pancreatitis (15), diabetic foot ulcers, and radiation-related tissue injuries (17). HBOT has also been shown to improve survival in preclinical animal models of infection (48, 49), endotoxemia (35), zymosan toxicity (37), and sepsis (9). In the present study, we tested the efficacy of HBOT in an acute experimental murine model of sepsis induced by cecal ligation and puncture (CLP). We found that early HBOT within 1 h of CLP improved survival after the insult. However, HBOT at later time points even within 6 h of CLP failed to improve the outcome. These observations are consistent with our prior observations that the therapeutic window to reverse the outcome from CLP is constrained to the first hours after the procedure (13), reflecting the surgical concept of the “golden hour.”

## METHODS

**Animals.** Male CD-1 mice were obtained from Charles River Laboratories (San Diego, CA) and maintained in pathogen-free conditions at the University of California San Diego (UCSD) Animal Facility (La Jolla, CA). Experiments were conducted on 8-wk-old animals and approved by the UCSD Institutional Animal Care and Use Committee.

**Cecal ligation and puncture.** Male CD-1 mice were fasted for 16 h before the procedure. Animals were anesthetized with isoflurane, and a 2-cm laparotomy was made exposing the cecum. The cecum was ligated 1.5 cm from the end with a silk suture, and a 16-G needle was used to make a single puncture at the tip. The cecum was placed back into the peritoneum. The peritoneal wall was closed, a temperature probe was placed under the skin, and the skin was closed over the

probe. Mice were continuously monitored for changes in core body temperature and mortality for 72 h after surgery. We have previously shown that core body temperature after CLP is a reliable surrogate marker for survival. Animals that drop below 28°C in the postoperative period will expire shortly thereafter, and thus a drop below this temperature is used as a marker for mortality (13).

**Hyperbaric oxygen therapy.** Mice selected for HBOT were placed in a hyperbaric chamber (Hyperbaric Technologies, Amsterdam, NY) that was pressurized with 98% oxygen to 2.4 atmospheres. The mice were kept at this pressure for 60 min. Three different treatment schedules were chosen for the experimental groups: 1, 6, and 21 h post-CLP, 1 h post-CLP (early treatment only), and 6 and 21 h post-CLP (delayed treatment only).

**Cytokine expression.** A group of mice was selected for cytokine expression analysis. Control mice were subjected to CLP, and the liver was collected at 3 and 8 h after surgery. Three hours was chosen based on previous experience with cytokine expression in this model (13, 29). Liver samples were collected from experimental animals at 3 h after a single HBOT exposure at 1 h after CLP or at 8 h after two HBOT sessions at 1 and 6 h post-CLP. Liver tissue was homogenized using an Ultra-turrax T25 (IKA, Wilmington, NC) in TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was purified using the manufacturer's protocol, and DNA contamination was removed (DNA-free kit; Ambion, Austin, TX). RNA was then reverse transcribed to cDNA using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The cDNA was then measured by quantitative real-time PCR (qPCR) using QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) with QuantiTect-validated primer sets [IL-6: QT00098875; IL-10: QT00106169; TNF $\alpha$ : QT00104006 (all from Qiagen)]. The 7500 Fast Real-Time PCR System (Applied Biosystems) was utilized for all qPCR reactions. To ensure amplification specificity, melting curve analysis was utilized for each primer, and corresponding standard curves were added in each reaction. To normalize data to cDNA inputs, the housekeeping gene GAPDH (QT01658692; Qiagen) was used. Results are expressed as fold increase over control animals or as copy number of target gene per copy number of GAPDH.

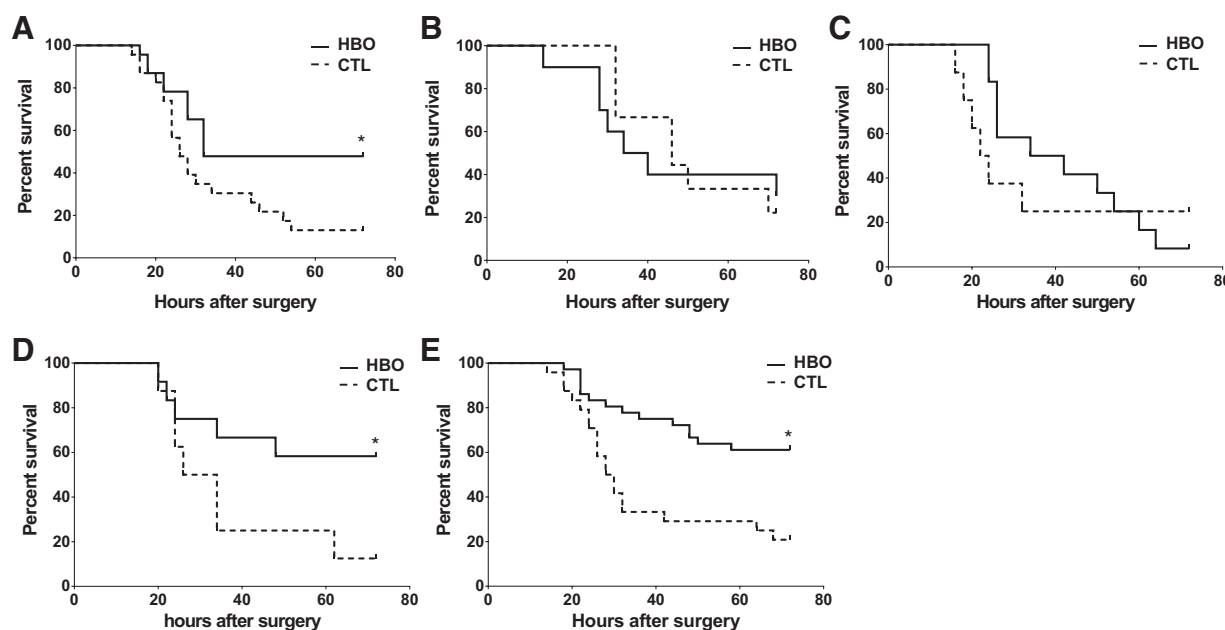


Fig. 1. Early hyperbaric oxygen (HBO) therapy (HBOT) improved survival from sepsis. Male CD-1 mice (8 wk old) were subjected to cecal ligation and puncture (CLP) and treated by HBOT or not (2.4 atm. for 1 h) at 1 ( $n = 23$ /group,  $*P = 0.0159$ ; A), 6 ( $n = 10$ /group; B), 21 ( $n = 10$  for CLP and  $n = 12$  for CLP + HBOT; C), 1 + 6 ( $n = 10$  for CLP and  $n = 12$  for CLP + HBOT,  $*P = 0.0396$ ; D), or 1 + 6 + 21 h post-CLP ( $n = 24$  for CLP and  $n = 36$  for CLP + HBOT,  $*P = 0.0008$ ; E). Survival was continuously monitored for 72 h. Statistical significance was analyzed by the log rank test.

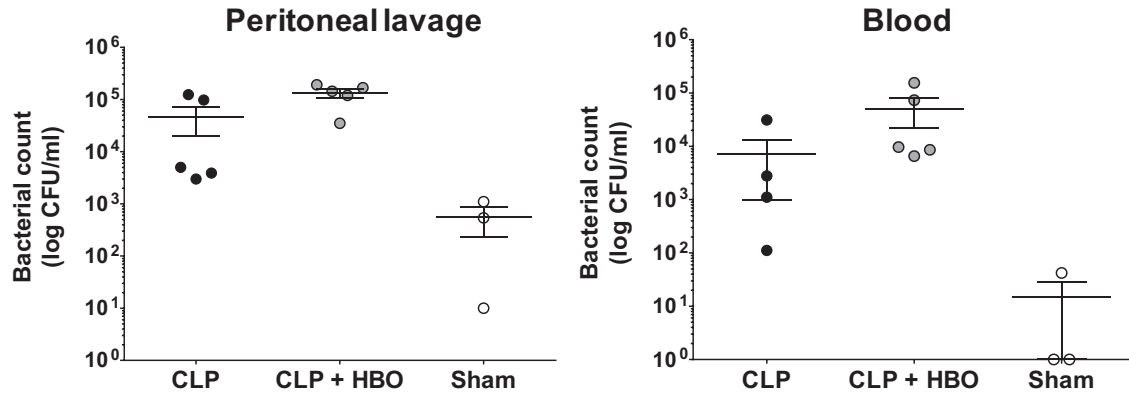


Fig. 2. Hyperbaric oxygen (HBO) therapy did not reduce the bacterial load within the peritoneum and blood after cecal ligation and puncture (CLP). Male CD-1 mice (8 wk old) were subjected to CLP ( $n = 5$ ), CLP + HBO therapy (2.4 atm. for 1 h at 1 h post-CLP;  $n = 5$ ), or sham operation ( $n = 3$ ). Mice were subjected to peritoneal lavage and blood collection at 6 h post-CLP or sham operation. Peritoneal lavage and blood samples were serially diluted in PBS spread on trypticase soy agar plates containing 5% sheep blood. All plates were incubated for 24 h at 37°C. The no. of bacterial colonies was counted and expressed as colony-forming units (CFU)/ml blood or peritoneal lavage fluid. Values are means  $\pm$  SE. Statistical analysis for the comparison between groups was performed by 1-way ANOVA, followed by Tukey's multiple-comparison test.

**High-resolution mitochondrial respirometry.** Mice were subjected to CLP and exposed to HBOT 1 h after, as described above, and euthanized at 3 h after the initial insult. Sham-operated mice were used as control. Liver samples were obtained immediately after euthanasia and placed in preservation solution (10 mM  $\text{Ca}^{2+}$ -EGTA buffer, 20 mM imidazole, 50 mM  $\text{K}^{+}$ -4-morpholineethanesulfonic acid, 0.5 mM dithiothreitol, 6.56 mM  $\text{MgCl}_2$ , 5.77 mM ATP, and 15 mM phosphocreatine, pH 7.1) at 4°C until measurements were made within 2 h of euthanasia. Tissue samples ( $\sim 1$  mg) were weighed using a microbalance and transferred into a calibrated respirometer (Oxygraph 2 k; Oroboros Instruments, Innsbruck, Austria) containing 2 ml of media in each chamber. Respirometry was performed in duplicate at 37°C in stirred media (MiRO5) containing 0.5 mM EGTA, 3 mM  $\text{MgCl}_2$ , 60 mM K-lactobionate, 20 mM taurine, 10 mM  $\text{KH}_2\text{PO}_4$ , 20 mM HEPES, 110 mM sucrose, and 1 g/l bovine serum albumin essentially fatty acid free, adjusted to pH 7.1. Oxygen in the media was kept between 300 and 500 pmol/ml. A simplified substrate-uncoupler-inhibitor-titration protocol was used to assess maximum ADP-stimulated oxidative phosphorylation (OXPHOS) (44), including 10 mM glutamate and 2 mM malate to support electron entry through complex I (GM; "leak" state), 5 mM ADP to stimulate OXPHOS, 10 mM succinate to maximize convergent electron flux at the Q-junction, and 10  $\mu\text{M}$  cytochrome *c* to test for outer mitochondrial membrane integrity as a quality control ( $>15\%$  cytochrome *c* response was excluded).

**Statistical analysis.** Graphpad Prism (GraphPad Prism Software, San Diego, CA) was utilized for data analysis. The significance of survival curve results was determined through a log rank test, and a  $P$  value of  $<0.05$  was used to determine statistically significant survival difference. Statistical analysis for the comparison between treatment groups was performed by one-way ANOVA followed by Tukey's multiple-comparison test or two-way ANOVA followed by the Bonferroni's multiple-comparison test. A  $P$  value of  $<0.05$  was considered statistically significant.

## RESULTS

**Early HBOT improved survival from sepsis.** We investigated the effect of HBOT on mortality after sepsis induced by CLP. Mice were exposed to HBOT (2.4 atm for 1 h) at 1, 6, or 21 h after CLP. Changes in core body temperature and mortality were continuously monitored for 72 h. There was a significant improvement in survival under HBOT after 1 h of CLP (52% survival) in comparison with mice after CLP without the

treatment (13% survival; Fig. 1A). In contrast, there was no improvement in survival if HBOT was performed after 6 or 21 h post-CLP (Fig. 1, B and C). Repeated HBOT was performed to determine whether survival could be improved, including 1 and 6 h after CLP or a triple treatment (1, 6, and 21 h). These multiple treatments also resulted in a  $>50\%$  survival increase, which was not significantly different from the effect observed after 1 h post-CLP single treatment (Fig. 1, D and E, respectively). These results illustrate the efficacy of HBOT in improving the outcome from sepsis, and they also delineate the importance of early therapeutic interventions in this experimental model of sepsis.

**HBOT did not reduce the bacterial load within the peritoneum and blood after CLP.** It was likely that improved survival after HBOT and CLP could be due to reducing the bacterial

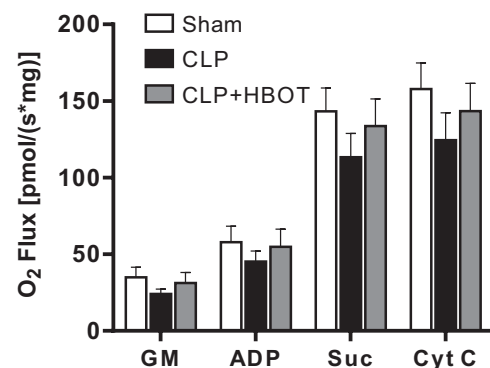


Fig. 3. Hyperbaric oxygen therapy (HBOT) did not impact mitochondrial function after cecal ligation and puncture (CLP). Liver tissues from male CD-1 mice subjected to sham operation ( $n = 8$ ), CLP + HBOT (2.4 atm. for 1 h at 1 h post-CLP,  $n = 7$ ), or CLP ( $n = 7$ ) were collected 3 h post-CLP and immediately used to measure oxygen flux under saturating conditions of the following substrates: glutamate and malate (GM) to measure complex I respiration, ADP to measure state III respiration, complex II substrate succinate (Suc) to measure complex I, and complex II respiration combined and cytochrome *c* (Cyt *c*) as a quality control to ensure mitochondrial outer membrane integrity. Oxygen flux is expressed as  $\text{pmol}\cdot\text{s}^{-1}\cdot\text{mg}^{-1}$  protein. Values are means  $\pm$  SE. Statistical analysis for the comparison between groups was performed by 2-way ANOVA followed by the Tukey's multiple-comparison test. No statistical significance was found.

load associated with the insult. Mice were exposed to HBOT or not after 1 h CLP, and bacterial counts were determined by colony-forming units (CFU) in samples obtained from the peritoneum as well as from blood at 6 h after CLP. No differences in CFU were observed between the two groups of mice (Fig. 2). Furthermore, bacterial cultures directly exposed to HBOT for 1 h did not show a significant decrease in the organism viability.

*HBOT did not affect mitochondrial function after CLP.* There is extensive literature indicating mitochondrial dysfunction during late stages of sepsis that leads to the development of multiple organ failure (34). Part of this mitochondrial dysfunction has been associated with a decrease in oxygen delivery to cells and tissues. Because it is expected that HBOT will increase the input of oxygen into organs, we investigated whether mitochondrial function was affected by HBOT during sepsis induced by CLP. Mice were exposed to HBOT or not after 1 h of CLP, and liver samples were collected 3 h post-CLP. The 3-h time point was selected based on the window of protection from CLP observed after HBOT. Mitochondrial function was then immediately assessed by high-resolution respirometry. Oxygen flux was determined after the addition of glutamate (10 mM) and malate (2 mM) to trigger electron transfer through mitochondria complex I. The process was followed by the addition of ADP (5 mM) to stimulate oxidative phosphorylation and continued with the addition of

succinate (10  $\mu$ M) to maximize convergent electron flux at the Q-junction. Exposure to cytochrome *c* (10  $\mu$ M) was used to test for outer mitochondrial membrane integrity as a quality control. We did not observe any significant differences in oxygen flux at any experimental conditions between mice that were exposed to HBOT or not after CLP and sham-operated animals (Fig. 3). Consistent with these observations, we did not detect any differences in citrate synthase activity, a component of the citric acid cycle, in mice under HBOT after CLP. These observations suggest that HBOT does not result in the formation of products that can adversely impact mitochondrial function.

*Cytokine gene expression was altered in mice that underwent HBOT and CLP.* The improvement in survival after CLP in mice exposed to HBOT could be due to a decrease in the inflammatory response. Prior studies have shown that measuring cytokine gene expression in target organs (e.g., liver) by quantitative (q)RT-PCR is a great indicator of the early inflammatory response correlating very well with the outcome from the insult (13, 29). Male CD-1 mice were exposed to HBOT or not 1 h after CLP, liver samples were harvested after 3 or 6 h of CLP, TNF- $\alpha$ , and IL-6, and IL-10 expressions were measured by qRT-PCR, corresponding to the peak of cytokine expression levels in this model (29). A significant decrease in the expression of TNF- $\alpha$ , IL-6, and IL-10 was observed in liver samples in the HBOT group in comparison with mice that were

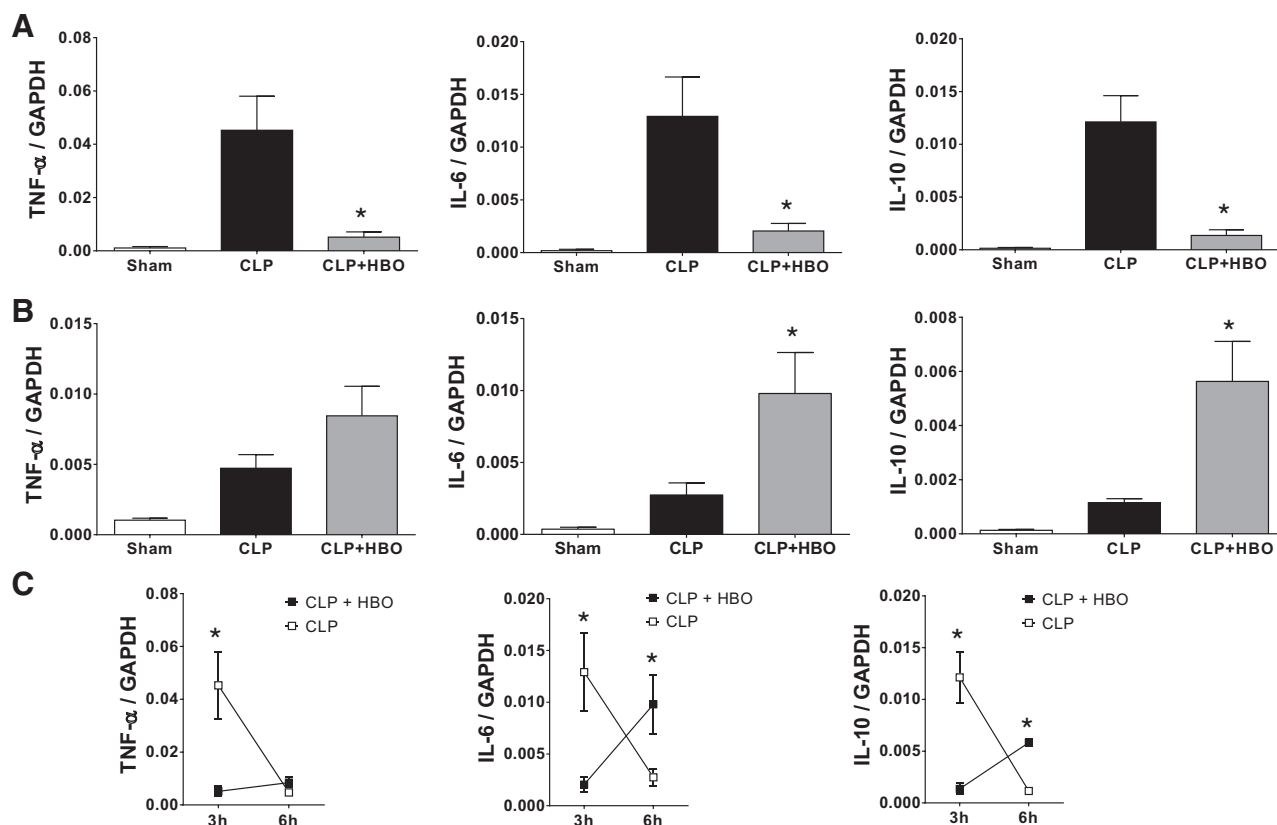


Fig. 4. Inflammatory cytokine expression was altered in mice that underwent hyperbaric oxygen therapy (HBOT) and to cecal ligation and puncture (CLP). Male CD-1 mice (8 wk old) were subjected to CLP ( $n = 5$ ), CLP + HBOT (2.4 atm, for 1 h at 1 h post-CLP;  $n = 5$ ), or sham operation ( $n = 4$ ), and liver tissues were collected at 3 or 6 h post-CLP. *A* and *B*: cytokine mRNA levels (TNF $\alpha$ , IL-6, and IL-10) were measured by quantitative real-time PCR (qPCR) in the liver samples obtained at 3 (*A*) or 6 h (*B*) post-CLP. *C*: kinetic expression of TNF $\alpha$ , IL-6, and IL-10 is depicted. Values are means  $\pm$  SE, and statistical analysis for the comparison between groups was performed by 1-way ANOVA followed by Tukey's multiple-comparison test (*A* and *B*) or 2-way ANOVA followed by the Bonferroni's multiple-comparison test (*C*). \* $P < 0.05$ , comparing CLP and CLP + HBO groups.

not exposed to HBOT at 3 h after CLP (Fig. 4A). TNF- $\alpha$  levels decreased 6 h after CLP and did not change in mice treated with HBOT (Fig. 4, B and C). In contrast, both IL-6 and IL-10 increased in the HBOT group after 6 h of CLP, whereas these cytokines decreased after 6 h of CLP in the absence of HBOT (Fig. 4, B and C).

The reduction in the inflammatory response could be due to a direct effect of HBOT on the pathway involved in the induction of the inflammatory response. First, we investigated whether HBOT altered the population of peritoneal cells in mice. Mice were exposed to HBOT for 1 h, and peritoneal cells were harvested by lavage after 1 h of the treatment and analyzed by flow cytometry. The normal peritoneal cell population is composed mainly of macrophages (CD19-CD11b+F4/80+), B1 cells (CD19+CD11b+), B2 cells (CD19+CD11b-), and other cells, including T and mast cells (CD11b-CD19-). We did not observe any differences in cell populations in mice exposed to HBOT or not (Fig. 5). Then, we evaluated whether or not HBOT affected the expression of cell surface receptors involved in the recognition of agents that trigger the inflammatory response. Peritoneal macrophages were isolated by lavage from naïve mice that were exposed to HBOT or not (1 h), and the expression of Toll-like receptor 4 (Tlr4) and CD14 was measured by flow cytometry. We did not observe any change in the expression of these receptors in macrophages isolated from mice exposed to HBOT or not (Fig. 6).

*Exposure of macrophages to HBOT resulted in a reduction of LPS-induced cytokine levels.* To further evaluate the mechanism that HBOT influences the inflammatory response, we investigated the effect of this treatment in culture cells. Macrophages (J744 cell-line) were exposed to HBOT or not for 1 h in the presence of LPS (100 ng/ml), returned to normal culture conditions for an additional 2 h, and lysed for the determination of cytokine levels (TNF- $\alpha$ , IL-6, and IL-10) by qRT-PCR. A significant reduction in TNF- $\alpha$ , IL-6, and IL-10

levels was observed after HBOT in comparison with cells that were not exposed to HBOT (Fig. 7, A–C).

## DISCUSSION

Sepsis remains a major health problem, the incidences of which have not declined during the last few years despite improvements in the clinical care of patients. Thus, supportive therapy, such as the use of antibiotics and fluids, remains the only available intervention in the critical care setting. Therefore, the development of novel therapeutic interventions to ameliorate sepsis is of critical importance. In the present study, we tested whether or not HBOT could be protective in an acute model of sepsis induced by CLP. We found that a single early treatment after the induction of sepsis resulted in a dramatic improvement of survival that could not be obtained if the treatment is postponed until 6 h after the insult. Multiple treatments did not improve the outcome significantly. Buras et al. (9) previously evaluated multiple dosages of HBOT at various oxygen pressures, finding that 2.5 atmospheres every 12 h resulted in significant improvement in survival after CLP, whereas other treatments did not improve survival, and high oxygen pressures were toxic. Thus, our results are consistent with these prior findings. However, it should be noted that Buras et al. (9) exposed mice to HBOT multiple times, whereas we obtained a similar result with a single treatment early after CLP. In this regard, a recent report showed the lack of protection if HBOT was performed 24 h after CLP in rats (5), which is again consistent with our observations indicating that only an early HBOT alleviated the outcome from sepsis. The early protective effect of HBOT echoes a prior study defining the therapeutic window for sepsis resolution in this model of CLP within <6 h of the insult (13). In addition, a robust presence of neutrophils within the peritoneum before CLP reduced the bacterial load substantially and improved mouse

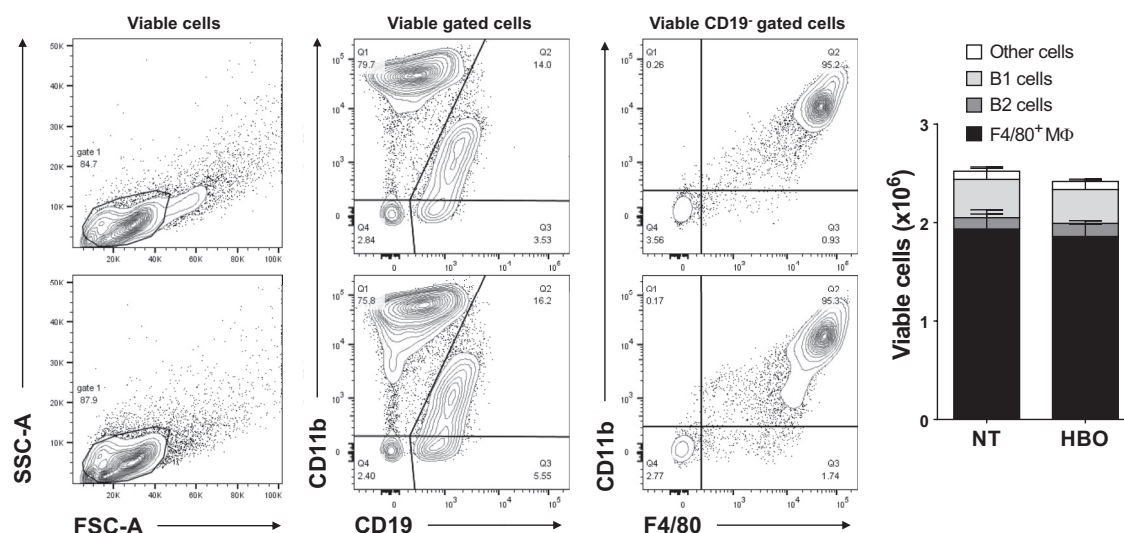


Fig. 5. Hyperbaric oxygen therapy (HBOT) treatment did not modify the peritoneal cellular composition. Male CD-1 mice ( $n = 3/\text{group}$ ) were subjected to HBOT treatment (2.4 atm. for 1 h) or left untreated, and peritoneal cells were obtained by lavage of the peritoneum at 1 h post-HBOT treatment. Cells were centrifuged for 10 min at 300 g, resuspended in PBS without  $\text{Ca}^{2+}/\text{Mg}^{2+}$  supplemented with 0.5% BSA, and counted. Cells were stained as described in METHODS, and flow cytometry was performed using a FACSCanto II flow cytometer with FACSDiva software. The data were analyzed using FlowJo software version 10.1. Representative FSC/SSC and CD11b/CD19 contour plots and Ly6G/F4/80 contour plots of CD19-CD11b+ gated viable cells are shown. Bar graph showing the proportion of the main cell populations within the peritoneum following HBOT treatment is also presented. Values are means  $\pm$  SE. NT, not treated.

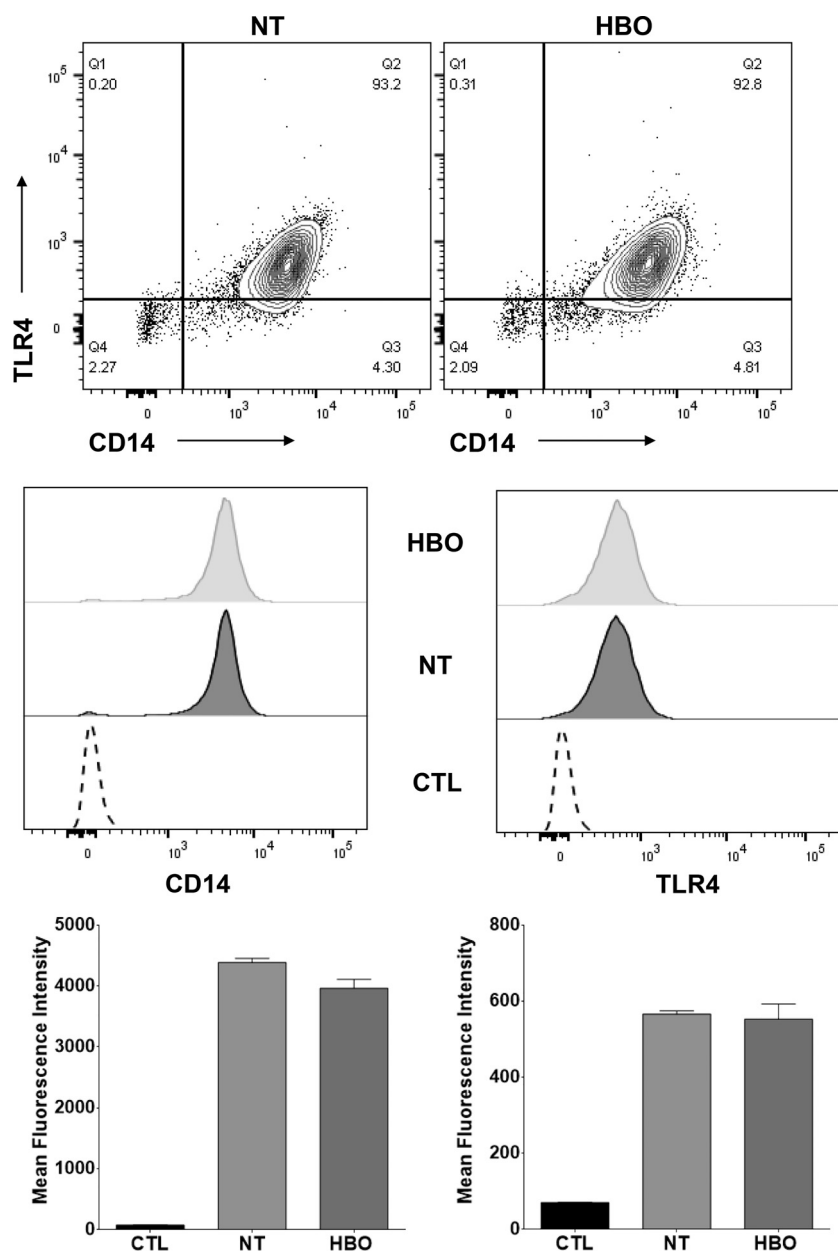


Fig. 6. Hyperbaric oxygen therapy (HBOT) treatment did not change the expression of CD14 and Toll-like receptor 4 (Tlr4) on peritoneal macrophages. Male CD-1 mice ( $n = 3/\text{group}$ ) were subjected to HBOT treatment (2.4 atm. for 1 h) or left untreated, and peritoneal cells were obtained by lavage of the peritoneum at 1 h post-HBOT treatment. Cells were centrifuged for 10 min at 300 g, resuspended in PBS without  $\text{Ca}^{2+}/\text{Mg}^{2+}$  supplemented with 0.5% BSA, and counted. Cells were stained as described in METHODS, and flow cytometry was performed using a FACSCanto II flow cytometer with FACSDiva software. Data were analyzed using FlowJo software version 10.1. Representative CD14/Tlr4 contour plots in addition to CD14 and Tlr4 histogram plots are shown. Control staining (CTL) was obtained by using the appropriate isotype control for each antibody. Bar graphs showing the mean fluorescence intensity of CD14 and Tlr4 expressions on peritoneal macrophages obtained from mice treated or not treated (NT) with HBOT are also presented. Values are means  $\pm$  SE.

survival, demonstrating that early source control is critical for sepsis resolution (12). Therefore, we have hypothesized that early events at the immunological and metabolic level are responsible for determining the final outcome of sepsis. Consequently, we suggest that sepsis resolution could be achieved by early robust interventions directed at eliminating pathogens, amending tissue damage and ameliorating the inflammatory response to restore homeostasis. Indeed, the “Sepsis Campaign” is calling for early interventions within 6 to 72 h of diagnosis to improve the clinical outcome from this devastating condition (<http://www.survivingsepsis.org>). Thus, the protective effect of HBOT that we report in this study is consistent with the idea of early interventions to mitigate sepsis. However, the therapeutic window observed in this preclinical experimental model is likely to depend on the severity of the initial insult.

Prior investigations have shown that HBOT provided a salutary effect after infections of *Escherichia coli*, *Streptococcus faecalis*, *Bacteroides fragilis* (49), and *Pseudomonas aeruginosa* (53). However, our observations demonstrate that the protective effect of HBOT was not associated with a direct bactericidal effect. We did not observe a decrease in bacterial load within the peritoneum or their translocation into the blood after CLP, which is in contrast with prior observations after CLP (9) and in a model of intestinal obstruction (1). Moreover, we did not observe the direct killing of bacteria after HBOT in culture conditions. Other studies have demonstrated that HBOT did not affect phagocytosis (31). Thus, the protective effect of HBOT is a more complex phenomenon.

Other studies have shown that HBOT improved renal function in a model of *E. coli* infection (22). In addition, HBOT has been reported to protect against LPS-induced mortality in rats

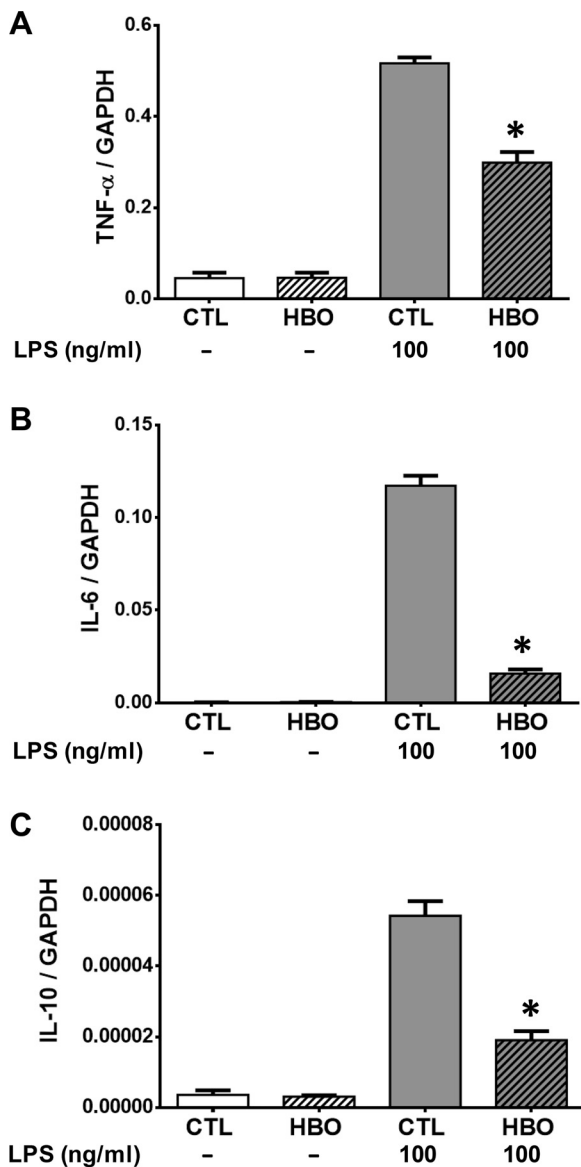


Fig. 7. Hyperbaric oxygen therapy (HBOT) treatment of macrophages resulted in a reduction of LPS-induced cytokine levels. Macrophages (J744A.1 cell line) were exposed to HBOT (2.4 atm.) for 1 h or not (CTL) in the presence of LPS or not (100 ng/ml), and cells were harvested 3 h after treatment. Total RNA was isolated and reverse-transcribed, and levels of TNF $\alpha$  (A), IL-6 (B), and IL-10 (C) were measured by quantitative real-time PCR (qPCR). Values are means  $\pm$  SE ( $n = 4$ ), and statistical analysis for the comparison between groups was performed by 1-way ANOVA followed by the Tukey's multiple-comparison test. \* $P < 0.05$ , comparing CTL and HBO groups in the presence of LPS.

(35), LPS-mediated kidney and liver injury (14), and a reduction of LPS-induced hypotension, acidosis, and NO production (43). HBOT preconditioning has also been shown to be protective in the case of ischemia-reperfusion injury (10, 54), ameliorating skin damage from UV radiation (24) and improving the resolution of diabetic foot ulcers and chronic wounds (25). Moreover, HBOT was shown to induce protection and promote repair of the endothelium (26) and reduce the damage of diabetic kidney disease (51). Despite the positive effect of HBOT in various disease conditions, the underlying protective mechanism is still unclear. HBOT has been shown to increase

subcutaneous tissue oxygenation in naïve rats (33) and necrotizing fasciitis patients (32). It also elevated oxygen levels in hypoxic tissue (2, 4). However, it is unclear how an increase in oxygen tension is protective. For example, HBOT has been reported to increase the production of reactive oxygen species (ROS) within the lung in an animal model (39, 40, 47) and in human blood (41). HBOT has also been proposed to mobilize cellular antioxidant responses (27, 39). HBOT has also been reported to display a protective role in mitochondrial function in the liver in ischemic-reperfusion injury (6). Nevertheless, we did not observe any changes in mitochondrial function after CLP in mice exposed or not to HBOT. In this regard, our analysis of mitochondrial function was performed on a whole organ (liver). Therefore, it is possible that different effects could be observed in specific cell types.

An important result from our study is the significant initial reduction in the early expression of cytokines (TNF, IL-6, and IL-10) after CLP, which may explain the protective effect that was observed. However, it is important to notice that both IL-6 and IL-10 increase after 6 h of CLP in mice under HBOT in marked contrast with a decline of these cytokines in mice that were not exposed to HBOT. Whether the increase in these cytokines may contribute to improving the outcome remains to be determined. Perhaps the preservation of the innate immune response capability of confronting subsequent insults may be critical to restoring homeostasis during sepsis. In support of this assumption, it has been shown that the protective effect of HBOT after CLP is linked to the presence of IL-10, since the deletion of this gene in a mouse line reduced the protective effect of HBOT in sepsis (9). IL-10 may play a more extensive role since, in addition to its well documented anti-inflammatory effect, it has been shown to stimulate T cell proliferation (23, 38). Other studies have also shown that HBOT interfered with cytokine production and activity, attenuating the inflammatory response (2, 7, 36). HBOT was also reported to suppress MAPK signaling and apoptotic pathways in degenerated human disc cells (42). Moreover, HBOT reduced ICAM-1 expression, impacting the adhesion of peripheral polymorphonuclear leukocytes to endothelial cells in a model of hypoxia that was proposed due to an increase of NO production (11). However, we did not observe changes in the levels of key cell surface mediators of the inflammatory response, such as CD14 and Tlr4.

In summary, we have confirmed that HBOT is a potential intervention to ameliorate sepsis if applied at the right time after the initial insult. However, there are many questions that arise from our study, including the limited reduction in mortality (50–60%) after a single treatment. It could be envisioned that protocols using different oxygen pressures, increasing the duration of the single treatment or combining HBOT with adjuvant interventions, such as antibiotics, fluids, and vaso-pressors, could improve the outcome from our model of sepsis. Thus, the protective mechanism remains to be fully elucidated, although our data and others (2, 7, 9, 36) pointed toward altering the inflammatory response, an aspect that requires further investigations. Finally, the main question is whether HBOT could be used to successfully treat human septic patients. Although evidence exists for the logistical safety of HBOT in the care of critically ill patients (8, 52), it remains cumbersome for the critical care setting (52). However, we are

optimistic that obstacles could be overcome to test whether HBOT could improve the outcome of septic patients.

### Perspectives and Significance

Sepsis remains a major clinical challenge due to the lack of successful interventions to mitigate the disease that is aggravated by a poor understanding of the therapeutic window. Sepsis, like many other diseases, is the product of multiple factors that in conjunction could result in a negative outcome. Therefore, the search for systemic interventions is critical to diminishing this multibillion-dollar health problem. Hyperbaric oxygen therapy (HBOT) that consists of exposure to 100% oxygen under increased atmospheric pressure is a potential systemic intervention to abate sepsis. In this investigation, we tested this possibility using a murine preclinical model. We indeed found a reduction in mortalities in this model that was correlated with a decreased inflammatory response. However, it is possible that this is not the only factor modulated by HBOT that could be having an impact on multiple organ systems at various cellular and metabolic events. Thus, we believe that we just scratched the tip of the iceberg. Nevertheless, the outcome of the use of HBOT in this animal model is significant. Then, the question that arises is whether this intervention may be suitable for the treatment of human sepsis. This is an important query since many successful interventions in animal models have failed in the clinical setting. Perhaps the complexity of human sepsis cannot be recapitulated in a murine model since the clinical approaches to treat human septic patients are not applied to a sick mouse. In addition, sepsis may be the product of particular responses modulated by specific human genes that are obviously not present in rodents. With these biases in mind, the success of HBOT in our murine model may still open the door for its use for the treatment of human sepsis. Indeed, HBOT has been safely and effectively utilized in the treatment of various clinical conditions in humans. However, much more work is needed to fully understand the mechanistic implication of HBOT in the context of a complex disease like sepsis, but we still hope that this intervention may one day save some lives.

### ACKNOWLEDGMENTS

We acknowledge the late W.T. “Ted” Gurmeé for his vision and commitment to hyperbaric oxygen therapy.

Current Address of G. A. Perdreizet: Dept. of Surgery, Hospital of Central Connecticut, New Britain, CT, 06052.

### GRANTS

This study was funded by the Michelle and Theodore Gurmeé Foundation and the National Institute of General Medical Sciences Grant no. R01-GM-11447.

### DISCLOSURES

Hemal H. Patel has equity as a founder in CavoGene LifeSciences Holdings, LLC.

### AUTHOR CONTRIBUTIONS

J.L.H., D.M.C., and A.D.M. conceived and designed research; J.L.H., J.M.P., A.W.W., D.H., T.R., J.O., and I.R.-S. performed experiments; J.L.H., D.M.C., F.V., G.A.P., and A.D.M. analyzed data; D.M.C., F.V., and G.A.P. interpreted results of experiments; D.M.C. and A.D.M. prepared figures; D.M.C., H.H.P., S.W.B., and G.A.P. edited and revised manuscript; H.H.P., S.W.B., G.A.P., and A.D.M. drafted manuscript; G.A.P. and A.D.M. approved final version of manuscript.

### REFERENCES

- Akin ML, Uluotku H, Erenoglu C, Ilıcak EN, Elbuken E, Erdemoglu A, Celenk T. Hyperbaric oxygen ameliorates bacterial translocation in rats with mechanical intestinal obstruction. *Dis Colon Rectum* 45: 967–972, 2002. doi:10.1007/s10350-004-6337-3.
- Al-Waili NS, Butler GJ. Effects of hyperbaric oxygen on inflammatory response to wound and trauma: possible mechanism of action. *Scientific-WorldJournal* 6: 425–441, 2006. doi:10.1100/tsw.2006.78.
- Angus DC, van der Poll T. Severe sepsis and septic shock. *N Engl J Med* 369: 840–851, 2013. doi:10.1056/NEJMr1208623.
- Babchin A, Levich E, Melamed M D Y, Sivashinsky G. Osmotic phenomena in application for hyperbaric oxygen treatment. *Colloids Surf B Biointerfaces* 83: 128–132, 2011. doi:10.1016/j.colsurfb.2010.11.019.
- Bærnethsen NF, Hansen MB, Wahl AM, Simonsen U, Hyldegaard O. Treatment with 24 h-delayed normo- and hyperbaric oxygenation in severe sepsis induced by cecal ligation and puncture in rats. *J Inflamm (Lond)* 14: 27, 2017. doi:10.1186/s12950-017-0173-4.
- Baldim LB, Nejo R Jr, Souza ME, Gomes MC, Picinato MA, Fina CF, Castro-e-Silva O. Effect of hyperbaric oxygen therapy on liver function during intermittent ischemia. *Acta Cir Bras* 28, Suppl 1: 61–65, 2013. doi:10.1590/S0102-86502013001300012.
- Benson RM, Minter LM, Osborne BA, Granowitz EV. Hyperbaric oxygen inhibits stimulus-induced proinflammatory cytokine synthesis by human blood-derived monocyte-macrophages. *Clin Exp Immunol* 134: 57–62, 2003. doi:10.1046/j.1365-2249.2003.02248.x.
- Bosco G, Garetto G, Rubini A, Paoli A, Dalvi P, Mangar D, Camporesi EM. Safety of transport and hyperbaric oxygen treatment in critically-ill patients from Padua hospitals into a centrally-located, stand-alone hyperbaric facility. *Diving Hyperb Med* 46: 155–159, 2016.
- 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: 2624–2629, 2006. doi:10.1097/01.CCM.0000239438.22758.E0.
- Buras JA, Reenstra WR. Endothelial-neutrophil interactions during ischemia and reperfusion injury: basic mechanisms of hyperbaric oxygen. *Neurol Res* 29: 127–131, 2007. doi:10.1179/016164107X174147.
- Buras JA, Stahl GL, Svoboda KK, Reenstra WR. Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS. *Am J Physiol Cell Physiol* 278: C292–C302, 2000. doi:10.1152/ajpcell.2000.278.2.C292.
- Cauvi DM, Hawisher D, Dores-Silva PR, Lizardo RE, De Maio A. Macrophage reprogramming by negatively charged membrane phospholipids controls infection. *FASEB J* 33: 2995–3009, 2019. doi:10.1096/fj.201801579R.
- Cauvi DM, Song D, Vazquez DE, Hawisher D, Bermudez JA, Williams MR, Bickler S, Coimbra R, De Maio A. Period of irreversible therapeutic intervention during sepsis correlates with phase of innate immune dysfunction. *J Biol Chem* 287: 19804–19815, 2012. doi:10.1074/jbc.M112.359562.
- Chang CK, Chang CP, Chiu WT, Lin MT. Prevention and repair of circulatory shock and cerebral ischemia/injury by various agents in experimental heatstroke. *Curr Med Chem* 13: 3145–3154, 2006. doi:10.2174/092986706778742945.
- Christophi C, Millar I, Nikfarjam M, Muralidharan V, Malcontenti-Wilson C. Hyperbaric oxygen therapy for severe acute pancreatitis. *J Gastroenterol Hepatol* 22: 2042–2046, 2007. doi:10.1111/j.1440-1746.2006.03380.x.
- Churchill-Davidson I, Sanger C, Thomlinson RH. High-pressure oxygen and radiotherapy. *Lancet* 265: 1091–1095, 1955. doi:10.1016/S0140-6736(55)90589-4.
- Clark RA. Oxidative stress and “senescent” fibroblasts in non-healing wounds as potential therapeutic targets. *J Invest Dermatol* 128: 2361–2364, 2008. doi:10.1038/jid.2008.257.
- Coopersmith CM, Deutschman CS. The new sepsis definitions: implications for the basic and translational research communities. *Shock* 47: 264–268, 2017. doi:10.1097/SHK.0000000000000763.
- De Maio A, Torres MB, Reeves RH. Genetic determinants influencing the response to injury, inflammation, and sepsis. *Shock* 23: 11–17, 2005. doi:10.1097/01.shk.0000144134.03598.c5.
- Dellinger RP, Levy MM, Rhodes A, Annane D, Gerlach H, Opal SM, Sevransky JE, Sprung CL, Douglas IS, Jaeschke R, Osborn TM, Nunnally ME, Townsend SR, Reinhart K, Kleinpell RM, Angus DC, Deutschman CS, Machado FR, Rubinfeld GD, Webb SA, Beale RJ,



- Vincent JL, Moreno R; 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: 580–637, 2013. doi:10.1097/CCM.0b013e31827e83af.
21. Deutschman CS, Tracey KJ. Sepsis: current dogma and new perspectives. *Immunity* 40: 463–475, 2014. doi:10.1016/j.immuni.2014.04.001.
  22. Edremittioğlu M, Kiliç D, Oter S, Kisa U, Korkmaz A, Coşkun O, Bedir O. The effect of hyperbaric oxygen treatment on the renal functions in septic rats: relation to oxidative damage. *Surg Today* 35: 653–661, 2005. doi:10.1007/s00595-004-3000-5.
  23. Emmerich J, Mumm JB, Chan IH, LaFace D, Truong H, McClanahan T, Gorman DM, Oft M. IL-10 directly activates and expands tumor-resident CD8(+) T cells without de novo infiltration from secondary lymphoid organs. *Cancer Res* 72: 3570–3581, 2012. doi:10.1158/0008-5472.CAN-12-0721.
  24. Fuller AM, Giardina C, Hightower LE, Perdrizet GA, Tierney CA. Hyperbaric oxygen preconditioning protects skin from UV-A damage. *Cell Stress Chaperones* 18: 97–107, 2013. doi:10.1007/s12192-012-0362-2.
  25. Gill AL, Bell CN. Hyperbaric oxygen: its uses, mechanisms of action and outcomes. *QJM* 97: 385–395, 2004. doi:10.1093/qjmed/hch074.
  26. Godman CA, Chheda KP, Hightower LE, Perdrizet G, Shin DG, Giardina C. Hyperbaric oxygen induces a cytoprotective and angiogenic response in human microvascular endothelial cells. *Cell Stress Chaperones* 15: 431–442, 2010. doi:10.1007/s12192-009-0159-0.
  27. Godman CA, Joshi R, Giardina C, Perdrizet G, Hightower LE. Hyperbaric oxygen treatment induces antioxidant gene expression. *Ann N Y Acad Sci* 1197: 178–183, 2010. doi:10.1111/j.1749-6632.2009.05393.x.
  28. Goodman MW. Decompression Sickness Treated with Compression to 2–6 Atmospheres Absolute; Report of Fourteen Cases, Discussions and Suggestions for a Minimal Pressure-Oxygen Breathing Therapeutic Profile. *Aerosp Med* 35: 1204–1212, 1964.
  29. Halbach JL, Wang AW, Hawisher D, Cauvi DM, Lizardo RE, Rosas J, Reyes T, Escobedo O, Bickler SW, Coimbra R, De Maio A. Why antibiotic treatment is not enough for sepsis resolution: an evaluation in an experimental animal model. *Infect Immun* 85: e00664-17, 2017. doi:10.1128/IAI.00664-17.
  30. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. *N Engl J Med* 348: 138–150, 2003. doi:10.1056/NEJMr0421333.
  31. Inamoto Y, Okuno F, Saito K, Tanaka Y, Watanabe K, Morimoto I, Yamashita U, Eto S. Effect of hyperbaric oxygenation on macrophage function in mice. *Biochem Biophys Res Commun* 179: 886–891, 1991. doi:10.1016/0006-291X(91)91901-N.
  32. Korhonen K. Hyperbaric oxygen therapy in acute necrotizing infections with a special reference to the effects on tissue gas tensions. *Ann Chir Gynaecol Suppl*: 7–36, 2000.
  33. Korhonen K, Kuttilla K, Niinikoski J. Subcutaneous tissue oxygen and carbon dioxide tensions during hyperbaric oxygenation: an experimental study in rats. *Eur J Surg* 165: 885–890, 1999. doi:10.1080/11024159950189401.
  34. Levy RJ. Mitochondrial dysfunction, bioenergetic impairment, and metabolic down-regulation in sepsis. *Shock* 28: 24–28, 2007. doi:10.1097/01.shk.0000235089.30550.2d.
  35. Lin HC, Wan FJ, Wu CC, Tung CS, Wu TH. Hyperbaric oxygen protects against lipopolysaccharide-stimulated oxidative stress and mortality in rats. *Eur J Pharmacol* 508: 249–254, 2005. doi:10.1016/j.ejphar.2004.12.021.
  36. Lin KC, Niu KC, Tsai KJ, Kuo JR, Wang LC, Chio CC, Chang CP. Attenuating inflammation but stimulating both angiogenesis and neurogenesis using hyperbaric oxygen in rats with traumatic brain injury. *J Trauma Acute Care Surg* 72: 650–659, 2012. doi:10.1097/TA.0b013e31823c575f.
  37. Luongo C, Imperatore F, Cuzzocrea S, Filippelli A, Scafuro MA, Mangoni G, Portolano F, Rossi F. Effects of hyperbaric oxygen exposure on a zymosan-induced shock model. *Crit Care Med* 26: 1972–1976, 1998. doi:10.1097/00003246-199812000-00022.
  38. MacNeil IA, Suda T, Moore KW, Mosmann TR, Zlotnik A. IL-10, a novel growth cofactor for mature and immature T cells. *J Immunol* 145: 4167–4173, 1990.
  39. Matsunami T, Sato Y, Hasegawa Y, Ariga S, Kashimura H, Sato T, Yukawa M. Enhancement of reactive oxygen species and induction of apoptosis in streptozotocin-induced diabetic rats under hyperbaric oxygen exposure. *Int J Clin Exp Pathol* 4: 255–266, 2011.
  40. Matsunami T, Sato Y, Sato T, Ariga S, Shimomura T, Yukawa M. Oxidative stress and gene expression of antioxidant enzymes in the streptozotocin-induced diabetic rats under hyperbaric oxygen exposure. *Int J Clin Exp Pathol* 3: 177–188, 2009.
  41. Narkowicz CK, Vial JH, McCartney PW. Hyperbaric oxygen therapy increases free radical levels in the blood of humans. *Free Radic Res Commun* 19: 71–80, 1993. doi:10.3109/10715769309056501.
  42. Niu CC, Lin SS, Yuan LJ, Chen LH, Wang IC, Tsai TT, Lai PL, Chen WJ. Hyperbaric oxygen treatment suppresses MAPK signaling and mitochondrial apoptotic pathway in degenerated human intervertebral disc cells. *J Orthop Res* 31: 204–209, 2013. doi:10.1002/jor.22209.
  43. Pedoto A, Nandi J, Yang ZJ, Wang J, Bosco G, Oler A, Hakim TS, Camporesi EM. Beneficial effect of hyperbaric oxygen pretreatment on lipopolysaccharide-induced shock in rats. *Clin Exp Pharmacol Physiol* 30: 482–488, 2003. doi:10.1046/j.1440-1681.2003.03865.x.
  44. Pesta D, Gnaiger E. High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle. *Methods Mol Biol* 810: 25–58, 2012. doi:10.1007/978-1-61779-382-0\_3.
  45. Remick DG. Pathophysiology of sepsis. *Am J Pathol* 170: 1435–1444, 2007. doi:10.2353/ajpath.2007.060872.
  46. Shoemaker WC, Appel PL, Kram HB. Role of oxygen debt in the development of organ failure sepsis, and death in high-risk surgical patients. *Chest* 102: 208–215, 1992. doi:10.1378/chest.102.1.208.
  47. Simsek K, Ay H, Topal T, Ozler M, Uysal B, Ucar E, Acikel CH, Yesilyurt O, Korkmaz A, Oter S, Yildiz S. Long-term exposure to repetitive hyperbaric oxygen results in cumulative oxidative stress in rat lung tissue. *Inhal Toxicol* 23: 166–172, 2011. doi:10.3109/08958378.2011.558528.
  48. Thom SR. Oxidative stress is fundamental to hyperbaric oxygen therapy. *J Appl Physiol* (1985) 106: 988–995, 2009. doi:10.1152/jappphysiol.91004.2008.
  49. Thom SR, Lauerma MW, Hart GB. Intermittent hyperbaric oxygen therapy for reduction of mortality in experimental polymicrobial sepsis. *J Infect Dis* 154: 504–510, 1986. doi:10.1093/infdis/154.3.504.
  50. Torio CM, Andrews RM. National Inpatient Hospital Costs: The Most Expensive Conditions by Payer, 2011: Statistical Brief #160. In: *Healthcare Cost and Utilization Project (HCUP) Statistical Briefs*. Rockville, MD: Agency for Healthcare Research and Quality, 2006–2013.
  51. Verma R, Chopra A, Giardina C, Sabbiseti V, Smyth JA, Hightower LE, Perdrizet GA. Hyperbaric oxygen therapy (HBOT) suppresses biomarkers of cell stress and kidney injury in diabetic mice. *Cell Stress Chaperones* 20: 495–505, 2015. doi:10.1007/s12192-015-0574-3.
  52. Weaver LK. Hyperbaric oxygen in the critically ill. *Crit Care Med* 39: 1784–1791, 2011. doi:10.1097/CCM.0b013e31821858d1.
  53. Weislow OS, Pakman LM. Inhibition of *Pseudomonas aeruginosa* by hyperbaric oxygen: interaction with mouse peritoneal exudate cells. *Infect Immun* 10: 546–552, 1974.
  54. Yu SY, Chiu JH, Yang SD, Yu HY, Hsieh CC, Chen PJ, Lui WY, Wu CW. Preconditioned hyperbaric oxygenation protects the liver against ischemia-reperfusion injury in rats. *J Surg Res* 128: 28–36, 2005. doi:10.1016/j.jss.2005.04.025.