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Hyperbaric oxygen treatment impacts oxidative stress markers in patients with necrotizing soft-tissue infection

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ABSTRACT

Necrotizing soft-tissue infection (NSTI) is a rare, severe, and fast-progressing bacterial infection associated with a high risk of developing sepsis or septic shock. Increasing evidence indicates that oxidative stress is crucial in the development and progression of sepsis, but its role in NSTI specifically has not been investigated. Some patients with NSTI receive hyperbaric oxygen (HBO_2) treatment as the restoration of oxidative stress balance is considered an important mechanism of action, which HBO_2 facilitates. However, a gap in knowledge exists regarding the effect of HBO_2 treatment on oxidative stress in patients with NSTI. In the present observational study, we aimed to investigate HBO_2 treatment effects on known markers of oxidative stress in patients with NSTI. We measured plasma myeloperoxidase (MPO), superoxide dismutase (SOD), heme oxygenase-1 (HO-1) and nitrite+nitrate in 80 patients with NSTI immediately before and after their first HBO_2 treatment, and on the following day. We found that HBO_2 treatment was associated with a significant increase in MPO and SOD by a median of 3.4 and 8.8 ng/mL, respectively. Moreover, we observed an HBO_2 treatment-associated increase in HO-1 in patients presenting with septic shock (n=39) by a median of 301.3 pg/mL. All markers were significantly higher in patients presenting with septic shock compared to patients without shock, and all markers correlated with disease severity. High baseline SOD was associated with 90-day mortality. In conclusion, HBO_2 treatment was associated with an increase in MPO and SOD in patients with NSTI, and oxidative stress was more pronounced in patients with septic shock.

Significance of this study

What is already known about this subject?

- There is a lack of knowledge of necrotizing soft-tissue infection (NSTI) pathophysiology. Therefore, treatment of NSTI often relies on available evidence from patients without NSTI with sepsis.
- Oxidative stress is key in the development and progression of sepsis. However, markers of oxidative stress have never been examined in septic patients with necrotizing soft-tissue infection.
- Hyperbaric oxygen (HBO_2) treatment—an adjunct treatment modality for patients with NSTI—is in general considered to induce oxidative stress, but its oxidative effects in NSTI have not been described.

What are the new findings?

- This study reports on the oxidative stress markers myeloperoxidase (MPO), superoxide dismutase (SOD), heme oxygenase-1 (HO-1) and nitrite+nitrate in patients with NSTI before and after receiving HBO_2 treatment.
- We show that HBO_2 treatment is associated with an increase in plasma MPO and SOD.
- High baseline SOD is associated with increased 90-day mortality in NSTI.

How might these results change the focus of research or clinical practice?

- These findings increase our understanding of both NSTI pathophysiology and the mechanism of action of HBO_2 treatment.

INTRODUCTION

Necrotizing soft-tissue infection (NSTI) is a rare, severe and rapidly progressing bacterial infection.[1] The incidence of NSTI is approximately two per 100,000 inhabitants/year and appears to be increasing.[2 3] Most patients with NSTI have sepsis at the time of diagnosis, and approximately 30%–50% deteriorate to septic shock.[2 4] The severity of the disease is marked by an amputation rate of 22% and a 90-day mortality of 18%[4]; survivors often have to cope with prolonged rehabilitation stays[5] and functional limitations.[6]

There is a lack of knowledge of NSTI pathophysiology. Therefore, treatment of NSTI often relies on available evidence from patients without NSTI with sepsis. Oxidative stress has been suggested as a major factor in the development and progression of sepsis and septic shock.[7–9] However, it remains unknown if oxidative stress also plays an essential role

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in NSTI and to what extent interventions during NSTI management induce oxidative stress.

HBO_2 treatment is used as an adjunctive intervention to standard care in NSTI, due in part to its antimicrobial mechanisms.[10 11] HBO_2 treatment regulates oxidative stress by the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS),[12] which are believed to destroy the invading bacteria.[13] Interestingly, markers of oxidative stress are increased in sepsis non-survivors.[14] It has been suggested that ROS plays a crucial part in the innate immune response and that some of their by-products have protective effects in the acute inflammatory response.[15 16]

Myeloperoxidase (MPO), an oxidative stress marker and key component of the innate immune response and neutrophil-mediated bacterial killing,[17 18] is associated with increased mortality in patients with sepsis.[19] It is of note that MPO seems to be important in the HBO_2-mediated regulation of oxidative stress, which is required for inhibition of β2-integrin in the endothelial adherence by neutrophils.[20 21] Moreover, HBO_2-mediated alterations of the two antioxidants, heme oxygenase-1 (HO-1) and superoxide dismutase (SOD), have been demonstrated in experimental models of sepsis associated acute lung injury, in sepsis, and as part of a protective mechanism against oxidative DNA damage.[22–27] Multiple HBO_2 treatments have been shown to elevate RNS by enhancing production of nitrite+nitrate in selected cohorts,[28] but the systemic effect on RNS remains unresolved.[29 30] Of notable interest is a SOD-mediated regulatory function of NO expression[31] and an inhibitory function of HO-1 on the NO synthase expression,[32] indicating closely interconnected regulatory mechanisms between RNS and ROS.

Oxidative stress in patients with NSTI has not been investigated extensively.[33] It is therefore unclear if high levels of markers of oxidative stress are associated with increased mortality in NSTI, and if HBO_2 treatment could impact these key factors of the innate immune response. Clinical treatment protocols of HBO_2 involve short bursts of HBO_2, and the resultant innate antioxidant defences have been demonstrated to be sufficient for making biochemical ROS stress a reversible process after HBO_2 treatment.[24 34–38] This antioxidant defence does not lead to human innate immunocompromise when HBO_2 treatment is used in bacterial infection control and sepsis.[21 39] Therefore, intermittent hyperoxia mediated by HBO_2 treatment and the resulting elevation in ROS production is considered reversible and harmless to the patient while having the effect of stimulating endogenous antioxidant systems and providing antimicrobial activity. However, the human data on the pathophysiological effects of HBO_2 treatment in patients with NSTI are scarce, and a better understanding of the effects in this group of patients is needed.

We aimed to investigate HBO_2-induced modifications of the oxidative stress markers MPO, SOD, HO-1 and nitrite+nitrate in patients with NSTI, and to associate baseline levels with severity of disease and outcome. We hypothesized that HBO_2 treatment may affect markers of oxidative stress as well as antioxidant scavengers, and that high baseline levels of MPO and SOD are associated with increased mortality in NSTI.

This article was prepared following the Strengthening the Reporting of Observational Studies in Epidemiology guidelines.[40]

STUDY DESIGN AND SUBJECTS

This was an observational cohort study of adult patients with NSTI admitted to Copenhagen University Hospital (Rigshospitalet) between February 2013 and March 2017. The cohort consisted of the Danish subgroup of patients that participated in the international multicenter INFECT study (ClinicalTrials.gov, NCT01790698) and included adult patients (≥18 years) with a diagnosis of NSTI, based on surgical findings of necrosis engaging any layer of the soft-tissue compartment; those patients where surgical findings did not reveal signs of NSTI were excluded.[4] The present study included 80 randomly selected subjects from this cohort using a computer-generated allocation table from which some data have been presented elsewhere.[41]

Patient management and data collection

All patients were treated according to our standardized treatment protocol, as previously described in detail elsewhere.[4] The treatment protocol aimed to perform a minimum of three sessions of HBO_2 treatment of 90 min duration at 284 kPa (18 m seawater equivalent), with a compression/decompression rate of 5–15 min. It was preferable that two sessions were performed within the first 24 hours from admission. No exact criteria for the termination of HBO_2 treatment was stipulated, but in general, HBO_2 therapy was provided for at least three sessions or could be continued until infection control. All HBO_2 sessions were performed in an intensive care unit (ICU)-capable multichamber (Drass Galeazzi S.p.A., Type HPO4000, HPE50.2.A).

Data on patient characteristics, biochemistry, microbiological findings, disease severity scores (Simplified Acute Physiology Score II (SAPS II) and Sequential Organ Failure Assessment (SOFA) score), amputation, renal-replacement therapy (RRT) and vital status at day 90 were obtained from an electronic database.[42] Dedicated personnel sampled blood at scheduled time points through an arterial line. Blood samples were collected in EDTA tubes, placed on ice and centrifuged at 3500 rpm (2400 G) for 10 min. Thereafter, plasma was pipetted into cryotubes and stored at −80°C until analysis.

The present study analyzed plasma samples collected immediately before the first session of HBO_2 treatment (baseline), immediately post the first session of HBO_2 treatment and on the following day (between 08:00 and 12:00).

ELISA

Plasma MPO, SOD and HO-1 were measured using ELISAs (MPO, 501410, Cayman Chemical, Michigan, USA; SOD, ab119520, Abcam, Amsterdam, Netherlands. *HO-1*: ab207621, Abcam, Amsterdam, Netherlands) according to manufacturers' instructions. All samples from one patient were measured in duplicates on the same plate. The analysts were blinded from patient and outcome. Samples were prepared in 96-well microtiter plates and absorbances were recorded in a plate reader at 450 nm (Multiskan FC, Thermo Scientific, Copenhagen, DK).

Chemiluminescent assay nitrite and nitrate concentrations were measured from the thawed plasma samples. The frozen samples were thawed and placed in 96-well microtiter plates and analyzed using a modified Griess nitrite/nitrate colorimetric assay (780001, Cayman Chemicals, Michigan, USA) according to manufacturer's instructions and as previously described .[43] In short, nitrite concentration was estimated by incubation with the Griess reagent, converting nitrite to a purple azo-compound detected by absorbance at 540 nm in a plate reader (Multiskan FC, Thermo Scientific, Copenhagen, DK). Nitrate was first converted to nitrite by incubation with nitrate reductase and subsequent addition of the Griess reagent to detect total nitrate plus nitrite. The background absorbance was estimated as the absorbance before staining with the Griess reagent and was subtracted from the absorbance of samples stained with the Griess reagent. The nitrate concentration was calculated as the difference between the total nitrite and nitrate concentration.

Outcome measures

The primary outcome was change in MPO before and after HBO_2 treatment. Secondary analyses included changes in SOD, HO-1 and nitrite+nitrate before and after HBO_2 treatment, associations of baseline levels of MPO, SOD, HO-1, nitrite/nitrate with microbiological findings, SOFA score, SAPS II, blood lactate, presence of septic shock at admission, RRT, amputation and 90-day mortality.

Sample size

To our knowledge, plasma levels of MPO have never been examined in patients with NSTI. We based our sample size calculation on a plasma MPO of 60 ng/mL, as found previously in 124 septic intensive care patients that were believed to be comparable to our cohort.[19] An HBO_2 treatment mediated modification of MPO activity of approximately 40% has been observed in patients with chronic wounds.[20] Using G*Power V.3.1.9.2 (Kiel, Germany) for sample size calculation in a paired design (power 0.90 at a 5% significance level), we found that a minimum of 41 patients should be included. In order to use all wells in the ELISA plate, thereby streamlining the laboratory analyses and to comply with the above-stated uncertainties, we chose to include 80 patients. This number of subjects was defined in a parallel experiment.[41]

Statistical analysis

Categorical data were presented as absolute numbers with percentage (%). Continuous data were presented as medians with IQR. As data were not normally distributed, we used Wilcoxon rank-sum test for non-paired continuous data and Wilcoxon signed-rank test for paired data (before/after HBO_2 treatment). Correlations were examined using Spearman's rank correlation test. Logistic regression analysis using the Youden Index optimal cut-off point[44] was performed to assess the association between baseline MPO, SOD, HO-1, nitrite+nitrate levels and 90-day mortality, and the results were addressed with OR and 95% CI. Receiver operating characteristic (ROC) curves were analyzed, and diagnostic accuracy in predicting 90-day mortality were evaluated using area under the curve (AUC), sensitivity, specificity, positive-predictive value and negative-predictive value (NPV). Patient lost to follow-up were excluded from regression analysis.

P values were reported as exact unless <0.001. P values below 0.05 were considered statistically significant. Statistical analyses were performed using RStudio V.1.0.153 (RStudio). Figures were prepared in GraphPad Prism V.8.0.2. (GraphPad, La Jolla, California, USA).

RESULTS

A total of 260 patients with NSTI were included at the Rigshospitalet, Copenhagen University Hospital, Denmark, between February 2013 and March 2017. Of these, 224 (86%) received HBO_2 treatment as part of their standardized NSTI treatment protocol, and from this group, we randomly selected 80 patients for inclusion in the present study. Patient characteristics are presented in table 1. Baseline blood samples (before the first HBO_2 treatment) were collected after a median of 4 hours and 42 min (3 hours 20 min–8 hours 15 min) from admission. One patient was lost to follow-up at day 90, resulting in an overall follow-up rate of 98.8%. Additional description of the included cohort has been published previously.[41]

Baseline MPO, SOD, HO-1 and nitrite +nitrate was 160.5 ng/mL (101.4—230.4), 118.1 ng/mL (74.5—270.2), 7575 pg/mL (5,113—13,416) and 3.6 µM (1.6—6.9) respectively. Correlations between baseline MPO, SOD, HO-1 and nitrite +nitrate are presented in online supplemental materials (online supplemental table 2).

Effects of HBOT on ROS and RNS

Paired analysis of MPO found a significant increase from baseline to post-HBO_2 treatment by a median of 3.4 ng/mL, and a subsequently decreased of −11.0 ng/mL on the following day (table 2). No significant difference was observed from baseline HBO_2 treatment to the following day (data not shown, p=0.30).

In addition, SOD increased significantly from baseline to post-HBO_2 treatment with a subsequent decrease on the following day (table 2). SOD was significantly lower on the following day, compared with baseline by a median of −14.5 ng/mL (p=0.02).

No immediate effect was observed on HO-1 in the overall cohort, but in the subgroup of patients presenting with septic shock (n=39), HO-1 was found to increase significantly from baseline to post-HBO_2 treatment (table 2). No difference was observed from baseline to the following day (data not shown, p=0.27). No differences were observed in nitrite+nitrate levels (table 2).

Association between ROS and RNS levels and NSTI severity and outcome

Patients with septic shock at admission (n=39) had significantly higher baseline MPO, SOD, HO-1 and nitrite+nitrate compared with patients without shock (MPO: 197.3 vs 140.1 ng/mL, SOD: 207.2 vs 89.3 ng/mL, HO-1: 10 402 vs 6692 pg/mL, nitrite+nitrate: 4.6 vs 2.7 µM; figure 1). This was in line with the correlation analysis that demonstrated significant correlations between MPO, SOD, HO-1, nitrite+nitrate and SOFA score (table 3). Additionally, SOD, HO-1 and nitrite+nitrate was found

Table 1 Patients' characteristics

Characteristics	NSTI, n=80
Age (years)	61 (52—68)
Sex, male	52 (65)
BMI (kg/m^2)	26 (24—31)
Comorbidities	
Cardiovascular disease	35 (44)
Chronic kidney disease	5 (6)
COPD	6 (8)
Diabetes	21 (26)
Immune deficiency	2 (3)
Liver cirrhosis	1 (1)
Malignancy	7 (9)
Peripheral vascular disease	11 (14)
Rheumatoid disease	4 (5)
Polyicrobial versus monomicrobial	
Monomicrobial infections	30 (37)
Polymicrobial infections	42 (53)
Negative findings	8 (10)
Biochemistry	
Leukocyte count (10^9/L)	15 (10–22)
C reactive protein (mg/L)	221 (148–324)
Creatinine (µmol/L)	109 (78–181)
Lactate (mmol/L)	2.3 (1.3–3.3)
Other	
SOFA score*	8 (6–10)
SAPS II†	44 (35–51)
LRINEC‡	8 (7–10)
Septic shock on admission§	39 (49)
Amputation within 7 days	13 (16)
RRT within 7 days	12 (15)
Mortality, day 90	12 (15)

Continuous data are presented as medians (IQR) and categorical data as absolute numbers (percentage, %).
*SOFA score, day 1; data were missing for three (4%) patients.
†Data were missing for three (4%) patients.
‡Data were missing for eight (10%) patients.
§Septic shock defined as lactate>2 mmol/L and use of vasopressor or inotrope.
BMI, Body Mass Index; COPD, chronic obstructive pulmonary disease; LRINEC, Laboratory Risk Indicator for Necrotizing Fasciitis Score; NSTI, necrotizing soft-tissue infection; RRT, renal-replacement therapy; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment.

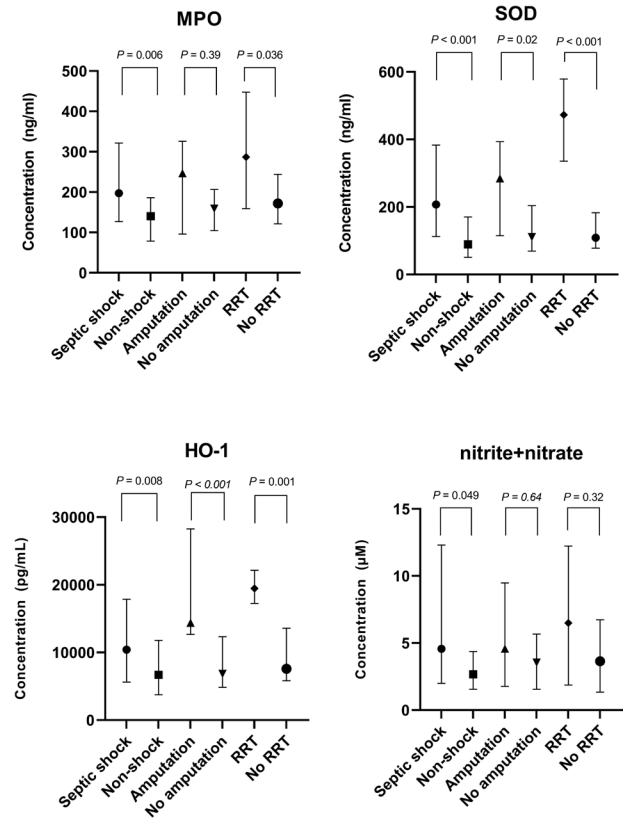

Figure 1 Baseline MPO, SOD, HO-1 and nitrite +nitrate levels according to; septic shock vs non-shock, amputation vs no amputation and RRT vs no RRT. Medians with interquartile ranges are illustrated. HO-1, heme oxygenase-1; MPO, myeloperoxidase; RRT, renal-replacement therapy; SOD, superoxide dismutase.

to correlate with SAPS II, whereas MPO, SOD and HO-1 correlated with baseline blood lactate levels (table 3).

Patients undergoingng an amputation had higher levels of SOD (284.5 vs 110.6 ng/mL) and HO-1 (14 376 vs 6829 µM) at admission compared with non-amputee patients (figure 1). Patients who received RRT within the first 7 days from admission had higher baseline MPO (286.9 vs 172.0 ng/mL), SOD (472.8 vs 108.7 ng/mL) and HO-1 (19 471 vs 7575 pg/mL) compared with those not who did not receive RRT (figure 1).

Table 2 Effects of HbO_2 treatment on ROS and RNS

	Median difference					
	Immediately before to after HBO_2			After HBO_2 to follow-up sample		
Plasma ROS/RNS	All	Septic shock	Non-shock sepsis	All	Septic shock	Non-shock sepsis
MPO (ng/mL)	3.4* ↑	4.6	3.4* ↑	−11.0* ↓	−14.2* ↓	1.6
SOD (ng/mL)	8.8* ↑	−1.9	14.4	−19.1** ↓	−24.4* ↓	−10.1* ↓
HO-1 (pg/mL)	17.9	301.3* ↑	−172.3	−11.6	−513.5	65.6
Nitrite+nitrate (µM)	0.05	0.16	0.01	−0.19	0.01	−0.20

Data are compared immediately before and after the first HbO_2 treatment session (left), as well as after the first HbO_2 treatment session and following day (right). Differences are presented as median differences across the overall cohort (n=80), patients admitted in septic shock (n=39) and patients without septic shock (n=41). Superscripts indicate level of statistical significance performed by Wilcoxon signed-rank test. A positive value indicates significant upregulation (↑); a negative value indicates significant downregulation (↓).
**P<0.05, **P<0.01.
HBO_2, hyperbaric oxygen; HO-1, heme oxygenase-1; MPO, myeloperoxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase.

Table 3 Spearman rank correlation between severity of disease and baseline ROS and RNS levels

	SAPS II		SOFA		Lactate	
	Rho	P value	Rho	P value	Rho	P value
MPO	0.16	0.15	0.40	<0.001	0.33	0.003
SOD	0.55	<0.001	0.70	<0.001	0.59	<0.001
HO-1	0.35	0.003	0.68	<0.001	0.39	<0.001
Nitrite+nitrate	0.27	0.02	0.25	0.03	0.15	0.19

HO-1, heme oxygenase-1; MPO, myeloperoxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; SAPS II, Simplified Acute Physiology Score II; SOD, superoxide dismutase; SOFA, Sequential Organ Failure Assessment.

In univariate and age-sex adjusted logistic analysis, high baseline SOD was found to be an independent predictor of 90-day mortality (table 4). However, in age–sex-SOFA score adjusted analysis no markers were found to be associated to 90-day mortality. Diagnostic accuracy of the studied markers in predicting mortality is shown in table 5. ROC curves of 90-day mortality according to baseline MPO, SOD, HO-1 and nitrite+nitrate are displayed in online supplemental figure 1.

Microbiological findings and levels of ROS and RNS

Forty-two (n=42) patients had polymicrobial infection, 30 monomicrobial infection and eight patients had no microbial findings in culture from either blood or soft tissue. No differences were found at baseline between polymicrobial and monomicrobial infections (MPO: 159.6 vs 174.9 ng/mL, p=0.87; SOD: 112.8 vs 157.5 ng/mL, p=0.48; HO-1: 7733 vs 9585 pg/mL, p=0.35; nitrite +nitrate: 2.95 vs 3.82 µM, p=0.36). Patients with *group A Streptococcus* NSTI (n=19) had higher levels of baseline MPO compared with non-group A *Streptococcus* NSTI (223.4 vs 160.5 ng/mL, p=0.01). No differences were found in SOD (p=0.21), HO-1(p=0.32) and nitrite +nitrate levels (p=0.06) in patients infected with *group A Streptococcus compared with* to other microbes (data not shown).

DISCUSSION

This observational study of 80 patients with NSTI is the first to demonstrate that HBO_2 treatment is associated with an overall increase in MPO and SOD, and an increase in HO-1 in a subgroup of patients with NSTI with septic shock. Moreover, we observed markers of oxidative stress to be associated with disease severity, and a high baseline SOD to be associated with 90-day mortality.

MPO and SOD are essential in oxygen-dependent bacterial killing by human neutrophils.[45 46] Studies indicate that oxidative stress is crucial for the release of MPO from neutrophils, monocytes and macrophages,[47 48] and that superoxide may serve as a substrate that MPO use to elicit killing of the ingested bacteria in the neutrophil phagosomes.[49 50] SOD possesses fundamental antioxidant effects[51] and its activity has been found to be decreased in patients with sepsis admitted to intensive care.[52] In the present study, we observed that HBO_2 treatment was associated with an increase in levels of MPO and SOD, suggesting that HBO_2 treatment may promote immunomodulatory effects by inducing oxidative stress and increase antioxidant capacity in patients with NSTI, as demonstrated by the initial upregulation of MPO and SOD.

HO-1 plays a fundamental role in control of bacterial infections.[53] Experimental sepsis models have indicated that deficiency of HO-1 increases mortality risk.[54 55]

Table 4 Univariate and multivariate logistic regression analyses of 90-day mortality based on low versus high baseline ROS and RNS levels according to the optimal cut-off values

	Unadjusted			Adjusted analysis: age and sex			Adjusted analysis: age, sex and SOFA Score		
	OR	95% CI	*P*	OR	95% CI	P value	OR	95% CI	P value
MPO									
Low <320.4	1 Ref.			1 Ref.			1 Ref.		
High ≥320.4	1.87	0.37 to 7.62	0.41	1.51	0.28 to 6.55	0.59	0.38	0.04 to 2.26	0.31
SOD									
Low <114.9		1 Ref.			1 Ref.			1 Ref.	
High ≥114.9	12.1	2.16 to 226.92	0.02	9.91	1.67 to 190.41	0.04	4.24	0.52 to 92.97	0.23
HO-1									
Low <7891		1 Ref.			1 Ref.			1 Ref.	
High ≥7891	4.88	1.11 to 33.95	0.06	3.66	0.75 to 26.74	0.14	1.14	0.13 to 11.22	0.90
Nitrite+nitrate									
Low <18.62		1 Ref.			1 Ref.			1 Ref.	
High ≥18.62	1.48	0.20 to 7.02	0.65	1.73	0.23 to 8.99	0.54	0.94	0.11 to 5.41	0.95

HO-1, heme oxygenase-1; MPO, myeloperoxidase; Ref., reference; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SOFA, Sequential Organ Failure Assessment.

Table 5 Accuracy of high baseline biomarker (defined by being above the optimal cut-off point) level in predicting 90-day mortality

	MPO	SOD	HO-1	Nitrite+nitrate
Sensitivity	0.25 (0.05–0.57)	0.92 (0.62–1.00)	0.80 (0.44–0.97)	0.17 (0.02–0.48)
Specificity	0.85 (0.74–0.92)	0.52 (0.40–0.65)	0.55 (0.42–0.68)	0.88 (0.78–0.95)
PPV	0.23 (0.05–0.54)	0.26 (0.14–0.42)	0.22 (0.10–0.39)	0.20 (0.03–0.56)
NPV	0.85 (0.74–0.92)	0.97 (0.85–1.00)	0.94 (0.81–0.99)	0.86 (0.75–0.93)
AUC-ROC	0.48 (0.31–0.66)	0.69 (0.51–0.87)	0.69 (0.49–0.88)	0.61 (0.43–0.79)

Data are presented as fractions (95% CI).
AUC-ROC, area under the receiver operating characteristic curve; HO-1, heme oxygenase-1; MPO, myeloperoxidase; NPV, negative predictive value; PPV, positive predictive value; SOD, superoxide dismutase.

Interestingly, HBO_2 treatment has been shown to increase HO-1 in sepsis models.[22 23] We were unable to detect any overall fluctuations in plasma HO-1 in relation to the first HBO_2 treatment session. However, we did observe significant differences in HO-1 responses between patients in septic shock and those not in septic shock, suggesting that the effect of HBO_2 treatment may differ across patients with various degrees of host response to infection. In this context, clinical studies indicate that the treatment effect of HBO_2 may be more pronounced in the most severely affected patients with NSTI.[56 57] We also observed that patients in septic shock had the highest level of MPO and SOD at baseline, as well as the greatest decrease after HBO_2 to the following day but without any significant increase immediately before or after HBO_2, which is in contrast to the patients without shock admitted with a lower level of oxidative stress, but who were able to increase in MPO and SOD from before to after HBO_2.

It should be noted that in patients with septic shock, HBO_2 treatment caused super induction of HO-1; this enzyme is known to stimulate endogenous anti-inflammatory cytokines and antioxidant defense mechanisms,[58] to be associated with improved survival in both preclinical polymicrobial models and human sepsis,[59] and to be favorably stimulated by HBO_2 treatment causing higher sepsis survival.[23] These results may indicate a two-sided effect of HBO_2 with the ability to increase oxidative stress and thereby bacterial death in patients without shock, whereas in patients with shock in whom the level of oxidative stress is already highly expressed at baseline, HO-1 induction suppresses the deleterious effects of free-heme produced during the course of the infection, rather than from an direct antibacterial effect.[60] Thus, it can be show, that the HBO_2 effect is mainly anti-inflammatory in patients with shock but enhances the oxidative stress-mediated immune response and therefore bacterial death in patients without shock.

A possible pathophysiological pathway involving nitric oxide in NSTI has been suggested previously,[61] and multiple HBO_2 treatments have shown an increase in nitrite and nitrate in selected cohorts.[28] Therefore, we evaluated changes in nitrite+nitrate in patients with NSTI; however, we were unable to observe any changes in nitrite+nitrate levels after one session of HBO_2 treatment. Previous reports have demonstrated high baseline nitrite and nitrate levels in patients with NSTI.[61] A theoretical explanation for the absence of a nitrite+nitrate response in our cohort could be that nitric oxide synthase systems were already highly expressed at baseline.

Antibiotics such as those administered to patients with NSTI use different mechanisms of action to cause bacterial death, including bacterial cell wall disruption by β-lactam antibiotics, disruption of protein synthesis by aminoglycosides, or blocking of the bacterial DNA/RNA synthesis by fluoroquinolone. These antibiotics induce metabolic changes of the bacteria's Krebs cycle, leading to an accumulation of toxic hydroxyl oxygen radicals.[62] These ROS can subsequently react with bacterial DNA, lipids or proteins, resulting in lethal bacterial effects, in addition to the stated target-specific killing effects. The formation of ROS is dependent on the presence of oxygen. Therefore, bactericidal antibiotics have reduced activity in infectious foci with poor oxygen supply, such as abscesses, biofilm infections or during high consumption of oxygen due to polymorphonuclear leukocytes influx.[63] The infected tissue in NSTI is characterized by the presence of local tissue edema and hypoxia at the infection site,[1] which may be corrected by HBO_2 treatment.[64]

Recently, attention has been drawn specifically to the effects of HBO_2 treatment-mediated tissue reoxygenation on bacterial infections with biofilm formation.[64] In the biofilm milieu, polymorphonuclear leukocytes that are activated by bacteria consume available oxygen during the process of converting oxygen to ROS and during the formation of RNS by inducible nitric oxide synthases.[64 65] This local deprivation of tissue oxygen causes a dormant state of bacterial anaerobic respiration, which in turn will cause the antibiotics targeting metabolic active bacteria to become less efficient because of reduced uptake and downregulation of the antibiotic targets.[64–66] Moreover, amplification of endogenous ROS will increase the bacteria's sensitivity to oxidative attack.[65] The effects of HBO_2 treatment with reoxygenation of biofilm-related infections have been demonstrated in a *Pseudomonas aeruginosa* biofilm model and experimental *Staphylococcus aureus* endocarditis.[67–69] HBO_2 treatment may have similar reoxygenation capabilities in human tissues with insufficient oxygen partial pressures.[70 71] Furthermore, biofilm formation has been documented in human group A streptococcal patients with NSTI.[72]

We found high baseline levels of SOD to be associated with increased mortality in univariate and age-sex adjusted analyses, with an acceptable diagnostic accuracy of approximately 0.7 AUC and sensitivity of 92%. High levels of MPO are associated with increased risk of dying in patients with sepsis[19]; however, in the present age-sex-SOFA score-adjusted analysis, we observed a trend to an improved survival in those presenting with high levels of MPO. Although this result did not reach statistical significance, it might indicate a possible pathophysiological difference between patients with sepsis and NSTI. Moreover, it highlights the importance of future research in NSTI as current pathophysiological reasoning and treatment recommendations often relies on available evidence from non-NSTI sepsis.

We assessed severity of disease by SOFA score, SAPS II score and blood lactate level, all of which have previously been associated with mortality in patients with NSTI.[4 73 74]

We observed that all markers were correlated with SOFA score, and SOD, HO-1 and nitrite+nitrate were correlated with SAPS II, whereas MPO, SOD and HO-1 correlated with blood lactate. To our knowledge, these are the first results that demonstrate correlations between markers of oxidative stress and severity of disease and patient outcome in NSTI. In accordance with the presented results, previous studies have demonstrated significantly higher levels of MPO in patients with septic shock compared with patients without septic shock,[19] as well as higher levels of nitrite+nitrate in patients with septic shock,[75 76] whereas the activity of SOD has been reported to negatively correlate with SOFA score in patients with septic shock.[52]

We found that patients with group A *Streptococcus* NSTI had higher baseline MPO. This finding may reflect that a larger fraction of patients with group A *Streptococcus* NSTI were in septic shock,[4] with consequently higher levels of MPO. We observed significant correlation between baseline SOD and nitrite+nitrate, indicating a potential regulatory mechanism between SOD and the nitric oxide pathway as previously reported.[31]

Our present study has a number of strengths: clinical data and blood were prospectively sampled by dedicated research personnel following a predefined protocol[42]; the laboratory analysts were blinded for patient outcome; and our follow-up was above 98%. Blood sample selection before and after HBO_2 treatment was performed within a well-defined time span around treatment, and therefore confounding events related to other treatment modalities are unlikely. Still, several limitations need to be taken into consideration. First, the present study did not have a matched non-HBO_2 control group, and therefore our observations may only be reported as associations. Even though the fluctuations observed at the time of the HBO_2 intervention makes the presence of other confounders unlikely, they may simply reflect the overall changes in ROS during disease progression and their associations to NSTI severity (SAPS II, lactate, RRT, amputation and mortality). Second, SOD's wide CIs in the multivariate analyses make the interpretation of the results difficult. Third, we have adjusted for numerous confounders (age, sex and SOFA score) that are among the most likely factors to influence disease severity and mortality. However, a series of other potential confounders, (eg, comorbidity score and blood lactate) were not included in the multivariate models because only 12 patients did not survive until day 90, which limited the number of confounders to consider. Finally, there may have been some level of selection bias favoring the less severe cases, as patients with the highest level of hemodynamic instability may have been too unstable to be moved from the ICU, and therefore have not been offered HBO_2 treatment. Thus, caution is required when extrapolating the present results to other non-selected cohorts.

In conclusion, we found HBO_2 treatment to be associated with an immediate increase in MPO and SOD after the first HBO_2 treatment session and with an increased HO-1 in patients with NSTI with septic shock. Patients with high baseline SOD were found to have increased 90-day mortality. MPO, SOD, HO-1 and nitrite+nitrate correlated with disease severity.

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Contributors Study planning: MH, JV, PØJ, CM, and OH; laboratory analysis: PØJ, CM and Lars Christophersen; data analysis: MH and OH; results interpretation: MH, JV PØJ, CM, and OH; drafting the manuscript: MH and OH; revision and approval of final version of the manuscript: MH, JV, PØJ, CM, and OH.

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Patient consent for publication Not required.

Ethics approval The present study abided by the principles of the Declaration of Helsinki and was approved by the Regional Ethical Committee of the Capital Region (H-19016085) and the Danish Data Protection Agency (VD-2019–179). All patients or their legal surrogates gave oral and written informed consent.

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