Lack of toxic side effects in neutrophils following hyperbaric oxygen.

B. JÜTTNER[1], D. SCHEINICHEN[1], S. BARTSCH[1], J. HEINE[1], H. RUSCHULTE[1], H-A. ELSNER[2], W. FRANKO[3] and K. JAEGER[4]

[1]*Department of Anesthesiology, Hannover Medical School, D-30625 Hannover, Germany,* [2]*Department of Transfusion Medicine, Hannover Medical School, D-30625 Hannover, Germany,* [3]*Institute of Hyperbaric Medicine, D-30163 Hannover, Germany,* [4]*Department of Anesthesiology, Hospital St. Joseph-Stift, D-28209 Bremen, Germany.*

Jüttner B, Scheinichen D, Bartsch S, Heine J, Ruschulte H, Elsner H-A, Franko W, Jaeger K, Lack of toxic side effects in neutrophils following hyperbaric oxygen. Undersea Hyperb Med 2003, 30(4):305-311 - Conflicting data have been reported about the impact of repeated HBO_2 exposure on the production of superoxide radicals during the neutrophil respiratory burst (RB) and on phagocytosis. In this study we wanted to see if exposure to hyperoxia would affect human neutrophil RB and phagocytosis. Short- and long-term effects after single or repetitive HBO_2 exposure of 2.5 atmospheres absolute over a period of 90 min were studied in 40 healthy volunteers. The RB was measured by the intracellular oxidation of dihydrorhodamine after induction by *Escherichia coli (E. coli)*, or priming with recombinant tumour necrosis factor alpha (TNF-α), followed by N-formyl-methionyl-leucyl-phenylalanine (fMLP) stimulation. The phagocytic activity was determined by the intake of FITC-labelled opsonized *E. coli*. No differences could be found between RB and phagocytic activity before and after HBO_2 therapy, regardless of short- or long-term exposure. These findings indicate that exposure to hyperoxia does not impair these two important functions of the human innate host defense.

hyperbaric oxygenation, neutrophils, respiratory burst, phagocytosis, flow cytometry

INTRODUCTION

Controlled hyperbaric oxygen (HBO_2) therapy is considered clinically beneficial for the treatment of a variety of pathological conditions. Commonly accepted indications with a scientific basis include decompression sickness, arterial gas embolism, severe carbon monoxide poisoning, prevention and treatment of osteoradionecrosis, improved skin graft and flap healing, and clostridial myonecrosis [1]. The biological effects of hyperoxia derive from dissolved oxygen in plasma during and shortly following treatment, which results in a transiently increased diffusion gradient between microcirculation and surrounding tissue. It has been demonstrated that phagocytic killing is impeded in hypoxic environments [2]. If this environment can be eradicated by HBO_2, phagocytic killing is improved [3]. There are other important cellular and biochemical benefits of HBO_2 including stimulation of angiogenesis [1].

It has been amply demonstrated that leukocytes are involved in the development of inflammatory conditions. Detrimental effects of leukocytes may be due to the production of free radicals and release of destructive enzymes [4]. In spite of positive clinical data about the successful use of HBO_2, only limited investigations are available on the influence of hyperoxia on leukocyte function. Most studies have investigated the effects of HBO_2 *in vitro* or in animals. Very few clinical studies of hyperoxia have demonstrated increased free radical production in humans [5, 6]. Since so few data on the influence of HBO_2 on human neutrophil function exist, the aim of our study was to investigate its short- and long-term effects. We hypothesized that

repeated exposure to HBO_2 would increase neutrophil superoxide anion production during the respiratory burst and phagocytosis activity in healthy volunteers receiving HBO_2.

METHODS

After ethics committee approval, 28 male and 12 female healthy volunteers gave informed consent for this study. They were allocated randomly, either to group A in which volunteers (13 males, 7 females aged 18 to 56 years; mean age 36.9 ± 11.2 years; body mass 76.6 ± 14.6 kg) were HBO_2 exposed once over a period of 90 min, or to group B (15 males, 5 females aged 18 to 58 years; mean age 36.2 ± 12.6 years; body mass 74.3 ± 14.0 kg) in which volunteers were exposed for 90 min daily to HBO_2 for five consecutive days.

In each group, 100% oxygen was breathed at 2.5 ATA for three 20 min periods interspersed with 5 min of air breathing to prevent oxygen seizures. Venous blood samples were collected in lithium-heparin coated disposable blood sampling tubes (7.5 ml S-Monovette® LH, Sarstedt, Nümbrecht, Germany) before and immediately after single HBO_2 exposures (group A). Blood samples of group B were obtained prior to the first and after the fifth HBO_2 exposure.

Measurement of respiratory burst (RB)

We used the protocol of Rothe *et al*. [7]. Blood was layered on equal Ficoll quantity (Ficoll-Hypaque, density 1.077 g dl^{-1}, Biochrom, Berlin, Germany). The nucleated blood cells of the supernatant were harvested after 1 g sedimentation at 22°C for 45 min. Phosphate buffered saline (Dulbecco's PBS without Ca^{2+} and Mg^{2+}, GIBCO BRL, Eggenstein, Germany) was pipetted (1 ml) into Eppendorf cups and heated to 37°C. 30 µl of leukocyte supernatant (containing an average of 5 x 10^5 cells ml^{-1}) was incubated with 15 µl of 0.1 mM DHR (MoBiTec, Göttingen, Germany) at 37°C for 5 min. RB was induced either by 20 µl *Escherichia coli* (1 x 10^9 ml^{-1}, *E. coli* strain HB 101) or by priming with 10 ng recombinant tumour necrosis factor alpha (1 µg ml^{-1}, TNF-α, Sigma, Deisenhofen, Germany) followed by 1 x 10^{-7} mol N-formyl-methionyl-leucyl-phenylalanine (0.01 mM, fMLP, Sigma, Deisenhofen, Germany). After incubation (20 min, 37°C), the reaction was terminated by transferring the tubes to ice. To determine viability, dead cells were stained with 1 x 10^{-5} mol propidium iodide (PI, 1 mM, Serva, Heidelberg, Germany). Samples were stored on ice and analysed within 30 min using a flow cytometer (Epics® XL, Beckman-Coulter, Krefeld, Germany). Negative controls with no stimulation were carried out to detect possible artificial or spontaneous RB activity.

Measurement of phagocytosis

Whole blood phagocytosis of opsonized *E. coli* (Phagotest®, Orpegen, Heidelberg, Germany) was assessed using a commercial test kit [8]. Briefly, 100 µl of heparinized venous blood was incubated with 5 µl FITC-labelled opsonized *E. coli* (1 x 10^9 bacteria ml^{-1}) at 37°C for a period of 10 minutes. Negative controls were kept on ice. The samples were centrifuged (250 g, 4°C) for 5 minutes after addition of quenching solution (50 µl, Orpegen) to suppress the fluorescence of the bacteria adhered to the surface of the cells. After additional washing with PBS (250 g, 5 min, 4°C), lysis solution (50 µl, Optilyse®, Immunotech, Krefeld, Germany) was added and incubated for 10 min, 2 ml distilled water added, and the samples incubated for another 10 min before centrifugation (250 g, 5 min, 4°C). The remaining cell pellet was washed with 3 ml PBS (250 g, 5 min, 4°C). After discarding 2 ml of the remaining PBS and addition of

PI (5 x 10^{-5} mol, Serva, Heidelberg, Germany) to stain the DNA of the cells and bacteria, the samples were analysed within 20 min by flow cytometry.

Flow cytometry adjustment and acquisition

Both applied flow cytometry assays allowing quantification of neutrophil response at single cell level. The flow cytometer was equipped with a 488 nm argon ion laser (Epics XL®, Beckman-Coulter, Krefeld, Germany). The rhodamine emission of the RB assay and the FITC emission of the labelled *E. coli* in the phagocytosis test were measured at 515-545 nm (FL1). The photomultiplier for the red fluorescence (FL3, 650 nm) was used in both assays to measure PI emission for discrimination between vital and dead cells, or bacteria and leukocytes, respectively. Side scatter (SSC) and forward scatter (FSC) were assessed in linear mode and FL1 and FL3 in logarithmic mode, without compensation. The photomultiplier volts and gains of FSC, SSC, FL1, and FL3 were adjusted for each negative control and remained constant for the matched samples. Data files were stored in list mode and analysed in dot plots using the PC software package EXPO® 2.0 (Beckman-Coulter, Krefeld, Germany).

Analysis of RB

Erythrocytes and cell debris were excluded using a discriminator adjusted in the FSC signal. Neutrophils (PMNs) were included by setting a polygonal gate in a FSC/SSC dot plot. These gated cells were transferred to a SSC/FL3 dot plot for exclusion of dead neutrophils in which high fluorescence in FL3 results from the intracellular PI content. Finally, only vital neutrophils were included in a SSC/FL1 dot plot, and the possible effect of HBO_2 on the neutrophil RB was estimated at approximately 10,000 live neutrophils per sample. The percentage of rhodamine positive neutrophils was calculated after setting a quadrant region in the SSC/FL1 dot plot of the negative control. A data processing sample is shown in Figure 1.

Analysis of phagocytosis

A linear region on the sharp peak signal in the histogram of FL3 was set to discriminate leukocytes from bacteria. Within this linear region, bacteria and cell debris were excluded by live gating during acquisition. In each sample 20,000 events were measured. A FSC/SSC dot plot was used to gate on neutrophils. These gated cells were displayed in a SSC/FL1 dot plot. The percentage of phagocytizing neutrophils was calculated after setting a quadrant region which was adjusted on ice in the negative control. In addition, phagocytosis activity of each PMN was determined by the content of FITC-marked *E. coli* in phagocytic cells, expressed as mean channel fluorescence of FL1, calculated after setting a linear region on the peak signal of the FL1 histogram determined with the content of FITC-marked *E. coli* in the phagocytic cells, expressed as mean channel fluorescence of FL1, calculated after setting a linear region on the peak signal of the FL1 histogram.

Statistical analysis

The percentage of activated neutrophils (RB assay: rhodamine positive PMNs after induction with *E. coli* or TNF-α/fMLP, phagocytosis assay: *E. coli*-FITC positive PMNs) after HBO_2 was compared to the respective percentage (= 100 %) before HBO_2 exposure. This net effect was calculated using the formula:

$$\text{activated PMNs \%} = \frac{\% \text{ positive PMNs after hyperbaric oxygen (HBO}_2\text{) exposure}}{\% \text{ positive PMNs before hyperbaric oxygen (HBO}_2\text{) exposure}} \times 100\%$$

All numeric data showed a Gaussian distribution using the Kolmogorov-Smirnov test (SPSS/PC® V 10.0 software package, SPSS, Munich, Germany). Because not all the measurement showed skewness $|\gamma| < 0.4$ the nonparametric Wilcoxon test was used to compare the intragroup data between before and after HBO$_2$ exposure. To identify intergroup effects between group A and B the Mann-Whitney-U test was used. A probability of less than 0.05 was considered significant.
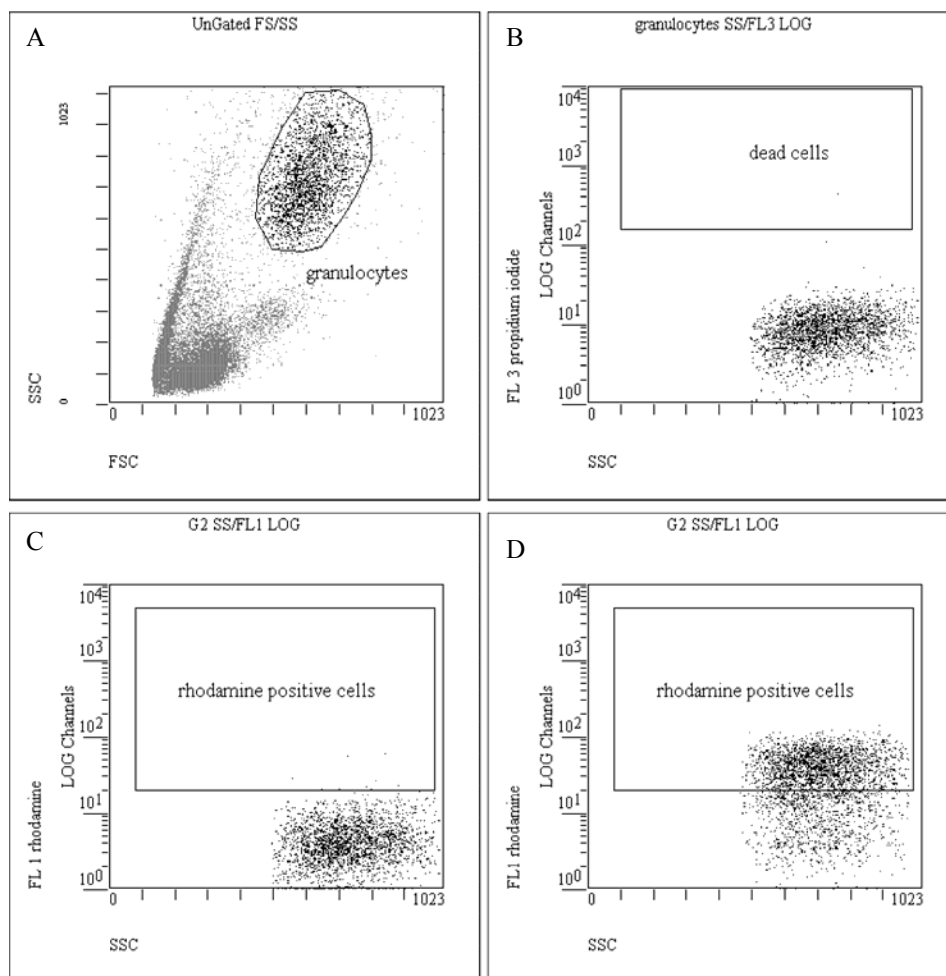

Figure 1. Adjustment of acquisition dot plots for analysis of neutrophil respiratory burst by green rhodamine fluorescence. Fig. 1A**:** Forward scatter (FSC) versus side scatter signals (SSC) were depicted as acquired on the flow cytometer. Neutrophils were localized and gated due to their high SSC signals. Only neutrophils from this region were inserted to the next dot plot. Fig. 1B: SSC versus red propidium iodide fluorescence (photomultiplier FL 3) for gating of dead cells due to their high red fluorescence. Cells from this gate were excluded for the following dot plots. Figs. 1C and 1D: SSC versus green rhodamine fluorescence (photomultiplier FL 1) for gating of rhodamine positive cells using the gate combination "granulocytes" of dot plot A but not "dead cells" of dot plot B. Fig. 1C is an example for a negative control without stimulation, whereas Fig. 1D depicts RB positive PMNs after *E. coli* stimulation.

RESULTS

Artificial or spontaneous RB activation or phagocytic activity of neutrophils quantified by negative controls was less than 5% (RB) and 1% (phagocytic activity) in all samples, respectively. All results are expressed as percentage of RB or phagocytic activity (mean % ± standard deviation, SD) before and after HBO_2 exposure. No significant differences were found between percentage of superoxide anion-producing neutrophils or phagocytic activity following HBO_2, regardless of single or repetitive exposure. Also the % individual variation difference of the RB and phagocytic activity and the respective fluorescence intensities expressed as the mean channels showed no difference before and after HBO_2 exposure (Tables 1 and 2).

Table 1

	Escherichia coli	TNF-α/fMLP	Phagocytosis
before HBO_2	68.1 (9.5)	26.4 (13.9)	79.7 (11.9)
range	48.3-82.6	6.0-47.1	50.7-92.4
after HBO_2	68.2 (11.4)	28.0 (15.9)	84.6 (8.5)
range	45.5-89.7	6.7-60.0	63.5-94.8
difference	*100.3 (11.3)*	*110.7 (37.1)*	*108.1 (18.6)*
range	*77.0-116.7*	*65.1-193.6*	*87.4-177.6*

Percentage of activated neutrophils (mean (SD), range) following *Escherichia coli* or TNF-α/fMLP stimulation, and percentage of PMNs phagocytizing FITC-labelled *Escherichia coli* before and after single HBO_2 exposure (n=20). Additionally, the percentage (= *difference*) of activated PMNs after single HBO_2 was compared to the respective percentage (= 100 %) before HBO_2 exposure.

Table 2

	Escherichia coli	TNF-α/fMLP	Phagocytosis
before HBO_2	65.8 (19.7)	25.9 (12.2)	79.4 (7.8)
range	24.4-97.6	10.4-63.6	62.1-91.6
after HBO_2	64.2 (23.7)	24.6 (12.8)	80.1 (8.9)
range	25.8-99.8	8.0-57.6	58.0-94.7
difference	*97.9 (20.6)*	*98.3 (34.9)*	*101.3 (10.8)*
range	*51.7-127.1*	*42.7-149.1*	*78.7-125.3*

Percentage of activated neutrophils (mean (SD), range) following *Escherichia coli* or TNF-α/fMLP stimulation, and percentage of neutrophils phagocytizing FITC-labelled opsonized *Escherichia coli* before first and after the fifth HBO_2 exposure (n=20). Additionally, the percentage (= *difference*) of activated PMNs after HBO_2 was compared to the respective percentage (= 100 %) before HBO_2 exposure.

DISCUSSION

Neutrophils play a decisive role in non-specific cellular immune defenses in the early stages of bacterial and fungal infections. In addition to adherence, chemotaxis, migration, and phagocytosis, they produce oxygen radicals during the RB to kill microorganisms [9]. Neutrophil function in bacterial killing is directly dependent on local oxygen tension [2, 10, 11]. Stimulation of PMNs triggers a sudden and dramatic increase in their oxygen consumption. During the RB, reactive oxygen species like superoxide (O_2-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), and other products are released [12]. The clinical importance of the formation of reactive oxygen manifests itself clearly in patients with chronic granulomatous disease [13].

Several groups have studied the effects of HBO_2 *in vitro* or in animals, with possible interspecies discrepancies. Using chemiluminescence, Smith and Mohideen found increased free radical production by rat pulmonary alveolar macrophages *in vitro* after a one-hour oxygen exposure (100% O_2) at normal pressure [14]. These results were supported by the work of Kang *et al.*, who demonstrated in mice that HBO_2 increased myeloperoxidase content in the lung, which correlated with increased neutrophil infiltration in lung tissues [15]. In a clinical study, Narkowicz *et al.* found increased amounts of free radicals by low temperature electron spin resonance spectroscopy in persons undergoing a therapeutic regime of HBO_2 exposure and postulated that hyperbaric oxygenation increases ascorbate radical levels in blood [5]. The increased radical production diminished to baseline levels within 10 minutes of cessation of HBO_2 exposure. In accordance Labrouche *et al.* reported an increase in phagocytosis by neutrophils whose intensity paralleled the increase in H_2O_2 production in 10 human volunteers [6]. In contrast to these results, both the production of superoxide radicals during the neutrophil respiratory burst (RB), and phagocytosis measured by flow cytometry remained unchanged after repeated HBO_2 exposure in mice [16]. Thom *et al.* found an inhibited beta-2-integrin dependent adherence of human neutrophils but also no effect on RB in response to phorbol ester [17]. Another clinical study by Kalns *et al.* investigated the effect of HBO_2 on neutrophil functions in healthy human subjects. Mac-1 mediated RB induced by opsonized zymosan was reduced by HBO_2. However, the RB induced by PMA or fMLP and thus differential neutrophil membrane receptors was not affected [18]. Our results are consistent in part with the latter findings since we observed no significant alterations in the percentage of superoxide anion-producing PMNs induced by TNF-α/fMLP. However, Mac-1 mediated RB and phagocytic activity induced by *E. coli* immediately after HBO_2 therapy showed no statistically significant changes.

The conflicting results may be explained by different methodology; our study was performed by flow cytometry, which has been shown to be the most sensitive and specific method available for analyzing changes in oxidative activities of leukocytes, compared to techniques like chemiluminescence and spectrophotometry [19]. Furthermore Smith, Kang and Gadd used animal models [14, 15, 16]. Another explanation for the differences in observations made by others could be that we measured neutrophil function after HBO_2 exposure.

We conclude that hyperbaric oxygenation is unlikely to increase PMN activity in clinical therapy, and suggest that the generation of free radicals *in vivo* reaches its maximum capacity at physiological O_2 tension. Although for some indications (e.g. severe anaerobic infections, non-healing wounds) enhanced activity of PMNs might be desirable, an important therapeutic mechanism of HBO_2 is the elevation of the tissue oxygen tensions. For this reason, the lack of increased RB of PMNs following HBO_2 does not seem likely to represent a major drawback with

regard to therapeutic efficacy. Moreover, our results demonstrate that increased PaO_2 does not impair phagocytic activity.

In summary, the present study demonstrates no detrimental effects of HBO_2 on superoxide anion production during the RB or on phagocytosis by human neutrophils immediately following short- or long-term repetitive HBO_2 exposure. Although an increase in RB after elevation of O_2 tension was not demonstrated, our results suggest that HBO_2 does not lead to toxic side effects in PMN that could impair cellular immune defenses.

ACKNOWLEDGEMENT

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