A single exposure to hyperbaric oxygen increases levels of circulating nucleosomes but does not induce mononuclear cell apoptosis in divers.

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Weber SU, Koch A, Siekmann U, Neitzel C, Stüber F, Hoeft A, Schröder S. A single exposure to hyperbaric oxygen increases levels of circulating nucleosomes but does not induce mononuclear cell apoptosis in divers. Undersea Hyperb Med 2009; 36(2):117-125. Recent reports that hyperbaric oxygenation (HBO₂) induced apoptosis in T-cell lines raised concern about a possible immunosuppressive effect of HBO₂. Nucleosomes, DNA fragments wrapped around a histone core, have been observed in the circulation in diseases with increased cell death such as sepsis. Our aim was to investigate, whether HBO₂ increases circulating nucleosomes as a marker of cell death and induces apoptosis of peripheral blood mononuclear cells in vivo. After informed consent 29 healthy volunteers were exposed to a 30 minute dive at 2.8 atmospheres absolute in a pressure chamber under resting conditions, while breathing 100% oxygen. Samples were obtained before and 24 hours after exposure. Circulating nucleosomes were measured in serum. Caspase-3 activation, Bcl-2 expression and mRNA of Bcl-2, Bcl-xl and Bax were analyzed in mononuclear cell extracts. Nucleosomes were elevated markedly 24h after exposure (p<0.01), while caspase-3 was not activated significantly. mRNA levels of Bcl-2, Bcl-xl and Bax were not altered. In conclusion, while evidence of elevated levels of circulating nucleosomes was found, mononuclear cell apoptosis was not affected by a single exposure to hyperbaric oxygen.

INTRODUCTION

During treatment with hyperbaric oxygenation (HBO₂) or diving with rebreathing systems individuals are exposed to high oxygen concentrations. HBO₂ is known to exert pleiotropic immune effects. Cellular effects include the inhibition of interferon-γ (1), interleukin-1β and tumor necrosis factor α release (2). This effect was seen after an exposure to oxygen at 2.4 atmospheres absolute (ATA) for 90 minutes, while the effect was lost after exposure intervals of more than 3h hours. Also, a temporary drop in the CD4:CD8 lymphocyte ratio (3), upregulation of the interleukin-2 receptor, downregulation of the αβ T-cell receptor (4) and induction of DNA damage (5-7) were observed. Especially, after repetitive exposure, compensatory heme oxygenase-1 upregulation occurred (8, 9). Furthermore, decrease of lymphocyte proliferation (10) and inhibition of human mammary epithelial proliferation (11) may be observed. In rodents, hyperbaric oxygenation...
delays allograft rejection (12-14).

High partial oxygen pressure due to HBO$_2$ or scuba diving causes systemic oxidative stress in vivo (7, 15, 16) which may, in principle, cause necrotic or apoptotic cell death. Occurrence of circulating nucleosomes in serum is regarded as an indicator of cell death (17). Nucleosomes mainly consist of cell free DNA (146 bp) wrapped around a core particle of histones, which protects the DNA against rapid digestion by endonucleases (18). They are found to be increased in pathologic conditions such as sepsis (19) when endogenous clearance mechanisms are overwhelmed. Also, nucleosomes can be detected in ischemic disorders (20) and are regarded as new prognostic marker in early cerebral stroke (21). Furthermore, they occur in autoimmune disease (22) and trauma (18). Finally, circulating DNA is found in the serum of patients with solid tumors (23). During chemotherapy the concentration of serum nucleosomes increases early on (24) and may be useful to assess an adequate response to chemotherapy of advanced non-small cell lung cancer (25) and acute myeloid leukemia (26).

On the one hand HBO$_2$ may act anti-apoptotic when oxygen supply is limited by ischemia. A single exposure to hyperbaric oxygen at 2.5 ATA for 2h may inhibit apoptosis in models of ischemic brain injury (27) and repeated exposure to oxygen at 2.4 ATA for 90 minutes each attenuated cell apoptosis in ischemic wound healing (28). On the other hand HBO$_2$ induced apoptosis in several cell culture models. While a 30-60 minute exposure at 2.5 ATA raised the proliferation rate of mouse fibroblasts, an exposure time of 2h enhanced apoptosis (29). Jurkat-T-cells and murine thymocytes underwent accelerated apoptosis in response to oxygen at 2.4 ATA for time intervals of 6-12h (30).

Removal of lymphocytes or antigen presenting cells may limit the capacity to initialize an adequate immune response. Moreover, phagocytosis of apoptotic bodies provokes an anti-inflammatory signaling cascade inhibiting the immune system (31). In both ways accelerated apoptosis of lymphocytes and other circulating immune cells may inhibit immune functions and may, in principle, be of relevance for divers as well as for patients treated with HBO$_2$. Immunosuppression by hyperbaric oxygen may limit deployment time. It also may limit the use of HBO$_2$ for treatment of already immune compromised patients such as patients with severe sepsis.

The death program may be initiated via the extrinsic death receptor pathway or the mitochondrial intrinsic pathway. In the mitochondrion, pro- and antiapoptotic members of the Bcl-2 family of proteins control the release of cytochrome c, APAF-1 and caspase-9 (32). While Bcl-2 and Bcl-xl represent classical survival proteins, Bax clearly exerts a pro-apoptotic activity. The apoptosome formed by components released from the mitochondrial intermembrane space in turn activates caspase-3 (33, 34), which degrades multiple targets leading to the common phenotype of the apoptotic cell (33).

Since exposure to high levels of oxygen under hyperbaric conditions causes oxidative stress in vivo and induces cell death in vitro we hypothesized that it also leads to cell death in divers. The aim of the current study was to investigate if exposure to hyperbaric oxygenation induces cell death, measured as the occurrence of circulating nucleosomes. Furthermore, we aimed to investigate whether HBO$_2$ specifically induces apoptosis in mononuclear cells.

Therefore, healthy volunteer divers were exposed to hyperbaric environments in pressure chambers breathing 100% oxygen. Before and 24h post exposure, we measured the concentration of circulating nucleosomes in serum. We also assessed the activation of caspase-3, the key executioner of the classical
apoptotic pathway in mononuclear cells. Since hyperbaric oxygenation regulates Bcl-2 protein expression in vitro (35) we quantified Bcl-2 protein expression and analyzed mRNA expression of Bcl-2, Bcl-xl and Bax.

MATERIALS AND METHODS

Study population

We prospectively analyzed healthy test divers 24h after a single exposure to a simulated dive in a pressure chamber while breathing 100% oxygen compared to intra-individual controls immediately before exposure. The protocol was approved by the local ethics committee and participants were recruited after written informed consent. The study population was recruited from soldiers of the federal army, who were individuals with a high degree of physical fitness. As a part of their diver training they voluntarily underwent an exposure in the pressure chamber as routine evaluation of their adaptive capabilities under hyperbaric and hyperoxic conditions.

Inclusion criteria were male sex and documented diving ability. Exclusion criteria were acute or chronic diseases, especially infections of the upper airways, otitis, intake of any medication, and alcohol intake within 24h before test dive. Prior to inclusion all test person needed to undergo a screening of physical fitness. Their medical diving ability was examined according to the guidelines of the federal navy.

Pressure chamber and hyperbaric oxygenation

The study population was exposed in a diving simulation chamber Hydra 2000 (Haux Life Support, Karlsbad-Ittersbach, Germany). During a linear compression phase total pressure reached 2.8 atmospheres absolute (ATA) within 5 minutes, followed by an isopression phase of 30 minutes. The decompression phase was linear but was interrupted by a 3 minutes isopression stop at 1.3 ATA. All participants underwent a safety training immediately prior to the test dive. During 30 minutes of isopression test divers were breathing 100% O₂ through a face mask. Using transcutaneous pO₂ monitoring breathing ambient air at 2.8 ATA results in a pO₂ of 300-400 mmHg while breathing 100% O₂ increases pO₂ to about 1600-2000 mmHg.

Fig. 1. Protocol of a typical exposure profile in a pressure chamber. Programmed pressure, actual pressure, temperature as well as fraction of oxygen (FO₂) in main and antechamber (which is separated from the main chamber by air lock) were recorded. Recordings are labeled in the figure. Pressure is displayed as in bar absolute pressure (equivalent to ATA), FO₂ in % of total gas and temperature in °C.

Sample acquisition, processing and storage

Peripheral venous blood was obtained before the test dives and 24h after the dive. EDTA blood was collected for routine white blood cell counts. Mononuclear cells were isolated from full blood anticoagulated with EDTA employing a Ficoll gradient centrifugation (Ficoll-Paque Plus, Amersham, Little Chalfont, UK). Mononuclear cells were lysed in a Cell Lysis Buffer for Cytomtric
Bead Array (Becton Dickinson Heidelberg, Germany) containing added benzamidin-HCL (16μg/mL), phenantrolin, aprotinin, leupeptin, pepstatin A (all 10 μg/mL, Becton Dickinson, Heidelberg, Germany) and 1 mM phenylmethyl sulfonylfluoride (Becton Dickinson, Heidelberg, Germany) at 4°C. The lysates were frozen immediately and stored at -80°C until staining. 2.5 ml whole blood for RNA quantification were collected using Blood RNA tubes (Paxgene system, PreAnalytiX, Qiagen, Hilden, Germany) and stored at -80°C until extraction. This method stabilized the RNA until further analysis.

**Nucleosome Detection**

Mono- and oligo-nucleosomes were detected using an ELISA-system according to manufacturer’s instructions (cell death detection ELISAplus, Roche, Mannheim, Germany). In brief, biotin-labelled anti-histone antibodies (clone H11-4) were fixed to a streptavidin-coated microplate. After binding of histone-DNA complexes, DNA was recognized by a peroxidase-conjugated anti-DNA-antibody and the absorbance was detected at 405 nm and reported in relation to a positive control with an arbitrary unit [AU].

**Caspase-3 activation and Bcl-2 protein expression**

Caspase-3 activation and Bcl-2 expression in the lysates of mononuclear cells were quantified using a modified cytometric bead array (CBA Human Apoptosis Kit, Becton Dickinson, Heidelberg, Germany). Briefly, 50 μL of lysates were mixed with 50 μL of beads linked to capture antibodies for caspase-3 or Bcl-2 followed by one hour incubation at room temperature. After a washing step, captured proteins were detected using an antibody against activated caspase-3 linked to phycoerythrin (PE) and an anti-Bcl-2-PE antibody during another one hour incubation time followed by washing. A flow cytometer (FacsCalibur, Becton Dickinson, Heidelberg, Germany) was set up using the Qualibrite sytem (Becton Dickinson, Heidelberg, Germany). Measurements of 300 capture beads were acquired per sample and mean fluorescence on the PE channel were recorded. Caspase-3 and Bcl-2 containing beads were identified on the basis of their fluorescence intensities on channel 3. 12 step calibration curves were obtained by serial standards and data were expressed as units per mL.

**RNA isolation and cDNA synthesis**

Using PAXgene Blood RNA kit (Qiagen, Hilden, Germany) according to the manual instruction total RNA was extracted from whole blood. cDNA was synthesized using a 1st Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Mannheim, Germany). The reaction mixture contained 8.2 μL of total RNA (equivalent to approximately 500 ng), 5mM MgCl 2, 1 mM dNTP, 3.2 μg of random primer pdN), 6.50 units of RNase inhibitor, 20 units of avian myeloblastosis virus reverse transcriptase and 1x reaction buffer in a total volume of 20 μL. The reaction was incubated as follows: 25°C for 10 minutes, 42°C for 60 minutes, 99°C for 5 minutes and 4°C for 5 minutes.

**Real-time PCR**

Real-time-PCR was performed according to the supplier’s manual in total volume of 20 μL on a LightCycler instrument employing the Light-Cycler FastStart DNA Plus SYBR Green I (both from Roche Diagnostics, Mannheim, Germany). For each sample reactions were performed in duplicates for target genes. Fluorescence was monitored at the end of the second segment of each cycle. PCR reactions were performed under the following conditions: The reactions started with an initial denaturation at 95°C for 10 minutes followed
by 45 amplification cycles. Bcl-2: 95°C for 15 seconds, 65°C for 5 seconds, 72°C for 5 seconds followed by an additional heating to 85°C for melting curve detection. The conditions for Bel-xl and Bax were only different regarding their annealing temperatures of 64°C respectively 70°C. Then, a melting curve was created for each reaction, and the product was cooled at 40°C for 30 seconds. To calculate the amounts of transcripts relative to the housekeeping gene h-HPRT, housekeeping gene PCR was performed using the Light-Cycler-h-HPRT Housekeeping Gene Set (Roche, Mannheim, Germany) according to the manual instruction. For relative quantification analysed target mRNA-expression in each sample was calculated relative to the housekeeping gene using the LightCycler quantification software (Roche, Mannheim, Germany). An external calibrator was added in duplicates to each run to compensate for inter-run variability. PCR products were cloned into Jm109 plasmids (Promega, Mannheim, Germany) according to the manufactures instructions.

For specific genes the following primers were purchased:

HPRT 5’-TGACCTTGATTTATTTTGCAT
ACC, 5’-CGAGCAAGACGTTCAGTCTCCT
(Operon, Cologne, Germany), Bcl-2 5’-GCC
AGC TGC ACC TGA CGC CCT TC, 5’-CCG
CAT GCT GGG GCC GTA CAG TT (271 bp),
Bcl-xl 5’-CAC AGT CAT GCC CGT CAG G,
5’TGA ATG AAC TCT TCC GGG ATG (281 bp) and Bax 5’-ACC CGG TGC CTC AGG
ATG CGT, 5’-ACC CGG TGC CTC AGG
ATG CGT (185 bp).

Dilution curves of PCR quantifications of cloned PCR products were created for initial calibration of the software. For quantification the crossing point method was used. Concentrations were calculated as “normalized ratio” with Relative Quantification Software (Roche, Mannheim, Germany).

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\text{normalized ratio} = \frac{\text{conc. target(sample)}}{\text{conc. reference(sample)}} : \frac{\text{conc. target(calibrator)}}{\text{conc. reference(calibrator)}}
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**Statistics**

Normally distributed data at 24h post exposure were related to baseline values before exposure using paired t-testing. Nucleosome data were not distributed normally. Therefore, Mann-Whitney testing was applied. The global level significance was set to p<0.05. Using Bonferroni correction for multiple testing, significance for an individual test was accepted at p<0.01. Analysis was carried out with GraphPad for Windows (Version 3.02, San Diego, Ca, USA). Values were given as mean and standard error of the mean if not indicated differently.

**RESULTS**

29 male divers (mean age 23±3) entered and all completed the study. WBC counts before exposure were 6.66±0.366 and did not change significantly after exposure. As a marker of cell death the concentration of circulating mono- and oligo-nucleosomes was determined. Circulating nucleosomes were elevated from 0.118 AU (median) to 0.263 AU (median, Figure 2).
Gene expression of Bcl-2, Bcl-xl, and Bax was analyzed by real-time PCR. mRNA expression of Bcl-2 was 4.73±0.39 (normalized ratio) and did not change significantly in response to exposure (4.43±0.29, Figure 4a). In analogy, the intracellular content of mononuclear Bcl-2 protein was not affected by the test dive (906.0±221.2 vs. 847.6±203.8 U/mL, Figure 4b).

Caspase-3 increased from 31.93±7.16 U/mL before to 40.71±10.26 U/ml 24h hours after exposure. However, these changes did not reach significance within the sample size (Fig. 3).

Fig. 2. Circulating nucleosomes under hyperbaric conditions (2.8 ATA for 30 minutes, 100% O₂ during isopression). Circulating nucleosomes in serum were quantified by ELISA. The concentrations are displayed in arbitrary units [AU]. **p<0.01.

Fig. 3. Activation of caspase-3 in peripheral blood mononuclear leukocytes under hyperbaric conditions (2.8 ATA for 30 minutes, 100% O₂ ) before and 24h after exposure. Caspase-3 activation was measured by flow cytometry.
**DISCUSSION**

The study showed that a single test dive at 2.8 ATA for 30 minutes with 100% O₂ clearly increased circulating levels of mono- and oligo- nucleosomes as an unspecific marker of cell death. However, it did not induce caspase-3 activation in mononuclear cells. Neither did it affect the gene expression of the apoptosis-related genes Bcl-2, Bcl-xl and Bax.

Circulating nucleosomes are found in severe disorders with a considerable amount of cell death involved (18). They have been observed in sepsis and correlated with the severity of disease (19). The increase of circulating cell free DNA after exposure to hyperbaric oxygen indicates cell death in the organism. The specific type or location of cell death remains unknown.

Induction of cell death may be explained by oxidative stress due to hyperbaric oxygen. Exposure to 100% O₂ at 240 kPa depleted plasma vitamin C and increased lipid peroxidation (15). Diving at 40 m for 25 minutes as well as hyperbaric oxygenation increased lymphocyte hydrogen peroxide content (16). HBO₂ caused DNA damage in vitro and in vivo (8, 36). An orally effective superoxide dismutase preparation was able to reduce oxidative stress, indicated by F2-isoprostane levels and inhibited DNA damage (7).

In mononuclear cells we could not detect significant activation of caspase-3, regulation of Bcl-2 expression or changes in mRNA levels of Bcl-2, Bcl-xl and Bax. However, mononuclear cells contain several important T- and B-cell subpopulations that may not be high in proportion but nevertheless functionally important. Our study cannot exclude apoptosis in a small subpopulation of cells that would not register when analyzing the mononuclear cell pool as a whole. Furthermore, the test subjects were highly trained individuals. Thus, at this point it is unknown, if the observed results...
would be true in the case of untrained divers or sick patients. Also, experiments were carried out at rest. In reality, however, divers are not only exposed to hyperbaric conditions but also work under considerable stress due to physical activity. Hyperbaric oxygenation during increased oxygen metabolism under work out conditions may impact apoptosis and cell death more than at rest.

In vitro, several groups have shown that exposure to hyperbaric oxygen induces apoptosis in lymphoid cells, namely Jurkat-T-cell cultures (30, 37), HL-60 cells (30), NCI-H929 cells (37), and murine thymocytes (30). These findings could not be transferred into an in vivo situation in our study. Several factors may explain these discrepancies. The dose used in the in vitro experiments was higher than human volunteers could be exposed to. The group of Chen (37) used a minimal time pressure of 2.5 ATA, however for at least 2h. Ganguly et al. exposed cells to oxygen at 2.4 ATA for time periods of 6 to 12 hours (30). Another factor may be the type of cells used. Leukemic cells investigated in the cell culture studies (30, 37) may behave differently in response to HBO2 than primary mononuclear cells of healthy donors do. Furthermore, the cells in these studies were incubated in medium (30, 37), which may cause artifacts (38). Serum instead contains both small molecule and enzymatic antioxidants (39), which may protect cells in serum from the toxic effects of hyperbaric oxygen in vivo.

The design of the study does not allow differentiating between effects of pressure alone and increased oxygen partial pressure, since a combination of both was used for exposure. In vitro data at least, however, indicates that increased pressure alone without elevated oxygen partial pressure does not even induce necrotic or apoptotic cell death in cell cultures (30, 37). Thus it is not very likely that a pressure of 2-3 ATA under conditions of normoxia would induce relevant cell death.

In conclusion, a single exposure to hyperbaric oxygen at 2.8 ATA for 30 minutes at resting conditions does not induce mononuclear cell apoptosis in healthy divers, when analyzing the mononuclear cell pool as a whole. However, elevated levels of circulating mono- and oligo-nucleosomes indicate a certain degree of cell death due to hyperbaric oxygen, although in this study neither the origin of nucleosomes nor the mode of underlying cell death can be determined. Further studies should investigate potential cell death of functionally important mononuclear cell subpopulations during physical exercise under conditions of hyperbaric oxygenation.

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