

## A single air dive induces apoptotic gene regulation but no increase in nucleosomes

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### ABSTRACT

**Background:** Oxidative stress caused by elevated partial pressure of oxygen during diving is a major contributor of inflammation and apoptosis. The underlying molecular mechanisms are poorly understood. The aim of the study was to describe apoptotic gene regulation induced by a single air dive.

**Methods:** 19 healthy volunteers were exposed to a 30-minute dive at 2.8 atmospheres (ATA) absolute in a pressure chamber in ambient air. Blood samples were obtained before, directly after and 24 hours after exposure. Gene expressions of Bcl-2, Bcl-xL and Bax were analyzed in mononuclear cell extracts by real-time polymerase chain reaction (PCR). Circulating nucleosomes were measured in serum before exposure and 24 hours afterward.

**Results:** The pro-apoptotic Bax expression was not significantly increased ( $p=0.74$ ) directly after the dive but was induced ( $2.22 \pm 0.85$ -fold) after 24 hours ( $p<0.01$ ). Bcl-2 expression was not changed significantly directly after ( $p=0.11$ ) but was  $1.88 \pm 1.08$ -fold higher after 24 hours ( $p<0.01$ ). Bcl-xL expression was not elevated significantly ( $p=0.54$ ) but was  $2.04 \pm 1.02$ -fold higher after 24 hours ( $p<0.01$ ). The level of nucleosomes did not change after 24 hours compared to baseline.

**Conclusion:** A single air dive at 2.8 ATA for 30 minutes causes an upregulation of pro- and anti-apoptotic genes but did not elevate circulating nucleosomes. In a single air dive the upregulation of anti-apoptotic Bcl-2 family members may counteract the pro-apoptotic potential of Bax.

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### INTRODUCTION

During scuba diving the human organism is exposed to higher concentrations of oxygen and to high ambient pressure. Breathing 100% oxygen during hyperbaric oxygenation is known to exert multiple immune effects such as DNA damage and downregulation of the T-cell [1–3]. However, even hyperoxia – due to seawater scuba diving with ambient air at 40 meters – induces reactive oxygen species ( $H_2O_2$ ) in lymphocytes and enhances antioxidant enzyme adaptation to avoid oxi-

dativ damage [4]. Increased malondialdehyde levels as a marker of oxidative stress were found after recovery from an immersion dive at 50 meters for 35 minutes [5]

Exposure to hyperbaric oxygen in vitro induces apoptosis [6] via the mitochondrial pathway [7]. While caspase-3 was activated in a caspase-9 dependent mode in vitro [7], caspase-3 activation was not detected after a single sea dive at 40 meters [4]. This intrinsic death pathway is under control of the Bcl-2 family of proteins [8]. Pro-apoptotic members like Bax localized in the

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mitochondrial membrane are required to regulate the mitochondrial outer membrane permeability. This leads to cytochrome c release, apoptosome formation and caspase activation [9]. Bax activity is counterbalanced by anti-apoptotic family members like Bcl-2 and Bcl-xL [8].

Extracellular circulating nucleosomes consist of cell-free DNA wrapped around a central protein octamer containing histones [10]. During apoptotic cell death DNA is typically cleaved at internucleosomal linking sites. Moreover, nucleosomes may also be released during non-apoptotic death forms, when endogenous clearing mechanism are overloaded [11]. Circulating DNA is found during sepsis [12,13] but also in patients with solid tumors [14].

After hyperbaric oxygenation in Navy divers we have observed an increase of circulating nucleosomes [15]. We questioned if exposure to a simulated dive with normal air also increases circulating nucleosomes or if moderate hyperbaric oxygen might induce a counterbalancing mechanism to avoid hyperoxic damage. Secondly, we aimed to investigate whether this single exposure also affects underlying pro- and anti-apoptotic gene expression. Therefore, healthy volunteers underwent a test dive in a pressure chamber breathing ambient air. We hypothesize that hyperbaric exposure to ambient air modulates apoptotic gene regulation and induces circulating nucleosomes. Circulating nucleosomes and mRNA expression of Bax, Bcl-2 and Bcl-xL were analyzed after the dive and compared to findings recorded before the dive.

## MATERIALS AND METHODS

This prospective study was designed to evaluate the effects of a 30-minute single air dive in dry chamber divers. The protocol was approved by the local ethics committee, and participants were recruited after written informed consent. To eliminate other effects (such as physical or mental stress) on the immune system, the dive was performed in a dry pressure chamber.

### Subject recruitment and inclusion criteria

Subjects were recruited in regional swimming and diving clubs and by posting at the local university publishing board. No prior experience in diving was mandatory, but all volunteers were screened for their

**Table 1: Demographic data of all participants single exposure to 2.8 ATA pressure**

	mean $\pm$ SD
n	19
age [a]	33 $\pm$ 9
height [cm]	181 $\pm$ 4
weight [kg]	84 $\pm$ 9
body mass index [BMI]	25.51 $\pm$ 2.53

SD: standard deviation

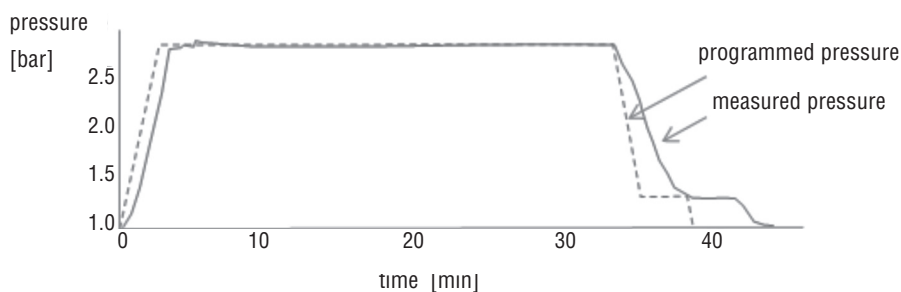
medical diving ability according to the guidelines of the German Society for Diving and Hyperbaric Medicine (GTÜM). Inclusion criteria were: healthy, male, age 18-50, documented diving ability. Exclusion criteria were: intake of any medication, alcohol intake within 24 hours of the test dive, and any acute or chronic diseases, especially infections of the upper airways and otitis. For demographic data see Table 1.

### Diving protocol

Diving simulations in parallel to Weber, et al. [15] were performed in a HAUX Starmed 2200/5,5 chamber (Haux Life Support, Karlsbad-Ittersbach, Germany). Each participant underwent a linear compression phase up to 2.8 atmosphere absolute (ATA) within five minutes of initial compression, at 30 minutes of isopression and during linear decompression, which was once interrupted by a three-minute isopression stop at 1.3 ATA (see Figure 1). Divers were breathing ambient air for the whole time, resulting in moderate transcutaneous monitored peaks of partial pressures of oxygen ( $pO_2$ ) of 300-400 mmHg at the end of 30 minutes of isopression.

### Blood samples, processing and storage

Peripheral venous blood was taken directly before the dive (called 'baseline'), after the dive (called '0 hours post dive') and 24 hours after the dive (called '24 hours post dive'). Isolation of mononuclear cells was described [15]. Ethylenediaminetetraacetic acid (EDTA) was added to the blood samples and centrifuged at 4°C via Ficoll gradient (Ficoll-Paque Plus, Amersham, Little Chalfont, U.K.). Then, mononuclear cells were lysed in a cell lysis buffer for cytometric bead array (Becton Dickinson, Heidelberg, Germany) containing



**Figure 1: Protocol of a typical exposure profile in a dry pressure chamber.**

Programmed and actual pressures were recorded.

Pressure is displayed in bar absolute pressure (equivalent to ATA/atmospheres absolute).

added benzamidine-HCL (16 $\mu$ g/mL), phenanthroline, aprotinin, leupeptin, pepstatin A (all 10  $\mu$ g/mL, Becton Dickinson, Heidelberg, Germany) and 1 mM phenylmethylsulfonyl fluoride (Becton Dickinson, Heidelberg, Germany). Lysates were stored at -80°C for further analysis. For RNA quantification full blood was stored in RNA tubes (Paxgene system, PreAnalytiX, Qiagen, Hilden, Germany).

#### Nucleosome detection

ELISA system was used for detection of mono- and oligonucleosomes according to manufacturer instructions (cell death detection ELISApplus, Roche, Mannheim, Germany) as described [15]. Biotin-labeled antihistone antibodies (clone H11-4) were fixed to a streptavidin-coated microplate. DNA was recognized by a peroxidase-conjugated anti-DNA antibody; the absorbance was detected at 405 nm and quantified using a standard curve. All data were reported in  $\mu$ g DNA/ml at baseline and 24 hours after the single exposition.

#### RNA isolation

For analysis, the PAXgene blood tubes RNA kit (Qiagen, Hilden, Germany) was used according to the manual protocol. After extracting RNA, cDNA was synthesized using a First Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Mannheim, Germany).

We used 8.2  $\mu$ L of total RNA (equivalent to approximately 500 ng), 5mM MgCl 2.1 mM dNTP, 3.2  $\mu$ g 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 buffer

volume of 20  $\mu$ L.

The reaction was incubated at 25°C for 10 minutes, 42°C for 60 minutes, 99°C for five minutes, and 4°C for five minutes.

#### Real-time PCR

Real-time-PCR was performed in total volume of 20  $\mu$ L on a LightCycler instruments employing the LightCycler FastStart DNAPlus SYBR Green I (both from Roche Diagnostics, Mannheim, Germany) as described [15]. Duplicates for target genes were performed for each sample reaction. PCR reactions were performed according to the following protocol: Initial denaturation at 95°C for 10 minutes and following 45 amplification cycles. For Bcl-2: 95°C for 15 seconds, 65°C for 5 seconds, 72°C for five seconds followed by an additional heating to 85°C for melting curve detection. The same protocol was used for Bcl-xL and Bax (modified only regarding annealing temperatures of 64°C respectively 70°C). Melting curve was created for each reaction, and the product was cooled for 30 seconds at 40°C. Housekeeping gene PCR was performed using LightCycler-h-HPRT Housekeeping Gene Set (Roche, Mannheim, Germany). Expression of each target mRNA was calculated relatively to h-HPRT. An external calibrator was added to each run to compensate for inter-run variability. The following primers were used:

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),  
Bax 5'-ACC CGG TGC CTC AGG ATG CGT,

5'-ACC CGG TGC CTC AGG ATG CGT (185 bp) and HPRT 5'-TGACCTTGATTTATTTGCATACC, 5'-CGAGCAAGACGTTTCAGTCCT (Operon, Cologne, Germany).

The crossing point method was used for quantification. Concentrations were calculated with Relative Quantification Software (Roche, Mannheim, Germany) as follows:

### Statistics

The mRNA expression of each gene (i.e., Bcl-2, Bcl-xL and Bax) was measured at three different time points (i.e., baseline, 0 hours post and 24 hours post). We used repeated measures ANOVA with Greenhouse-Geisser Correction and a post hoc Bonferroni's multiple comparisons test;  $p$  was set to  $<0.05$  and the confidence interval to 95%. Data from 'baseline' were compared to data after the dive (called '0 hours post dive') and to data from 24 hours after the dive (called '24 hours post dive').

Nucleosome data were not distributed normally. Therefore, Mann-Whitney testing was applied. The level significance was set to  $p<0.05$ . Analysis was carried out with GraphPad for Windows (Version 6.07, San Diego, California, U.S.). Values were given as mean and standard error of the mean if not indicated differently.

### RESULTS

A total of 22 persons underwent screening after informed consent. Three persons had to be excluded before exposure because of screening failures (i.e., two otitis media and one Wolff-Parkinson-White syndrome). For demographic data see Table 1.

Gene expression of Bcl-2, Bcl-xL, and Bax was analyzed by real-time PCR. Bcl-2 mRNA expression (Figure 2a) was elevated from baseline level of  $3.05 \pm 1.56$  (normalized ratio) to  $4.82 \pm 2.57$  after the exposure (0 hours post). This change was not significant ( $p=0.107$ ; 95% CI -3.758 to 0.2208). In contrast, Bcl-2 gene expression was elevated to  $4.81 \pm 2.05$  after 24 hours. This change was significant ( $p<0.05$ ; 95% CI -2.746 to -0.7837).

Bcl-xL mRNA gene expression (Figure 2b) was elevated from baseline level of  $132.05 \pm 87.38$  to  $169.8 \pm 130.13$  direct after the dive (0 hours post). This change was not significant ( $p=0.54$ ; 95% CI -106.8

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

to 31.31). Instead, the Bcl-xL gene expression after 24 hours was elevated to  $239.59 \pm 164.28$  (24 hours post). The expression in Bcl-xL increased between 'baseline' and '24 hours post' significantly ( $p<0.05$ ; 95% CI -170.8 to -44.26); furthermore, there was a non-significant increase between '0 hours post' and '24 hours post' ( $p=0.06$ ; 95% CI -139.5 to -0.07659).

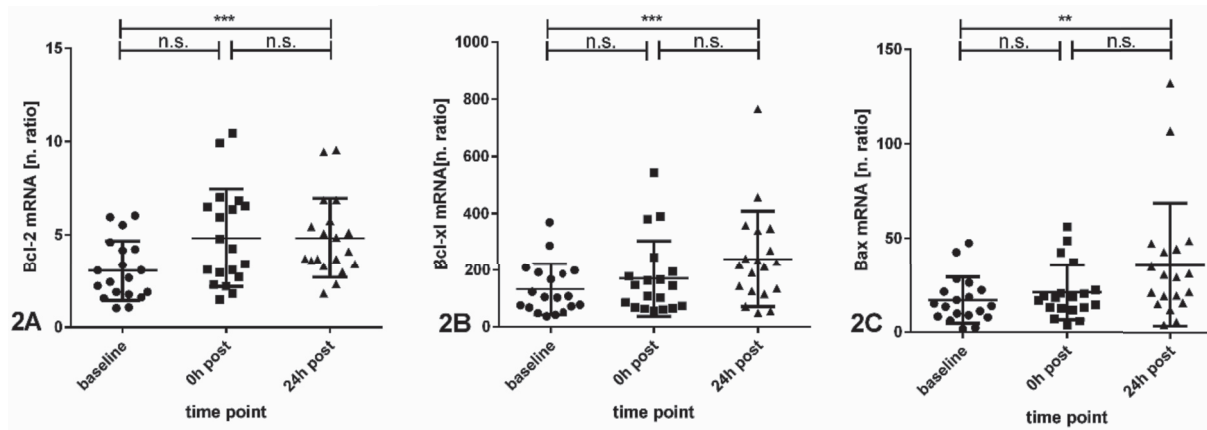
Bax mRNA gene expression (Figure 2c) expression was elevated from baseline level of  $16.89 \pm 12.04$  to  $21.08 \pm 14.21$  direct after the dive (0 hours post). This change was not significant ( $p=0.743$ ; 95% CI was -13.14 to 4.763). However, Bax gene expression was elevated to  $35.92 \pm 31.63$  after 24 hours. This change reached the level of significance ( $p<0.05$ ; 95% CI -32.32 to -5.739).

As a marker of cell death the concentration of circulating mono- and oligonucleosomes was determined. Circulating nucleosomes were not significantly changed ( $0.21 \pm 0.29$  AU to  $0.08 \pm 0.09$  AU) (Figure 3).

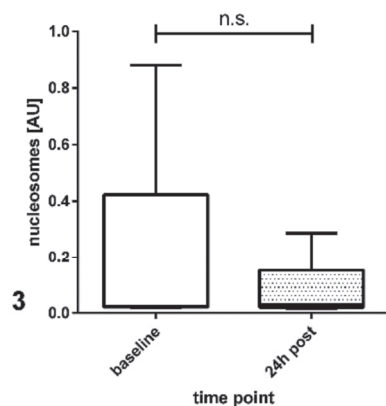
### DISCUSSION

The goal of this study was to study the effects of a single air dive to the apoptotic gene expression and circulating nucleosomes. We demonstrated that a single dive at 2.8 atm absolute for 30 minutes in a pressure chamber with ambient air alters gene expression of Bcl-2 family members. The gene expression of the mitochondrial pro-apoptotic protein Bax was increased. In parallel, the anti-apoptotic proteins Bcl-2 and Bcl-xL were elevated in gene expression as well. However, the exposure did not increase circulating nucleosomes as an indicator for cell death.

The regulatory responses to hyperoxia and hyperbaric oxygenation may initiate gene expression. For example, in vitro exposure to hyperoxia induces apoptotic cell death that is regulated by Bcl-2 family members [16]. In alveolar epithelia cells, reactive oxygen species (ROS) are required for hyperoxia-induced Bax activation [17]. The pro-apoptotic activity of Bax is counterbalanced by anti-apoptotic members of the Bcl-2 family such as Bcl-2 and Bcl-xL [8]. In patients with severe sepsis Bcl-2 and Bcl-xL were markedly



**Figure 2: Relative mRNA expression (normalized ratio) in peripheral blood mononuclear leukocytes under hyperbaric conditions (2.8 ATA, 30 minutes).** mRNA was quantified by real-time PCR. The baseline time points indicate samples before hyperbaric condition. The other time points were directly after exposure (0 hours post) and 24 hours after (24 hours post). Scatter dot plot with mean and standard deviation. (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ ; n.s. = not significant)



**Figure 3: Changes of circulating nucleosomes under hyperbaric conditions (2.8 ATA for 30 minutes isopression) before and 24 hours after exposure (n.s. = not significant).** Circulating nucleosomes in serum were quantified by ELISA. The concentrations are displayed in arbitrary units [AU].

decreased in lymphocytes while the mobile pro-apoptotic Bcl-2 family members Bim and Bid were upregulated [18]. In contrast, overexpression of Bcl-2 protected against apoptosis in hyperoxia-induced stress [16].

In a cell culture, exposure to hyperoxia under hyperbaric conditions induces both spontaneous and stimulus-induced apoptosis after a long-time exposure of

12 hours [19], at least in part, by intracellular accumulation of hydrogen peroxide. On the other hand, even a short exposure of 30 minutes caused apoptosis [7]. Apoptosis was demonstrated to be transmitted via the mitochondrial pathway [7] and involves a loss of mitochondrial membrane potential and activation of caspase-9. Bcl-2 was upregulated in this setting and may have acted as a cellular mechanism to counteract apoptosis.

To investigate the effects of hyperbaric oxygen on gene expression in vivo we previously investigated divers of the German Navy [15]. In this group of extremely physically fit divers, no gene regulation of Bcl-2 family members could be observed. This is in contrast to the findings of the current study. It could be speculated that these differences may be caused by differences in age (mean age of navy divers  $23 \pm 3$ ; mean age of voluntary scuba divers  $33 \pm 9$ ) and training status of the two groups. Whereas divers from the federal army were regularly tested for physical fitness, the subjects in this study were recruited via local announcements, with no mandatory experience in diving. In a gene expression study it was shown that a history of regular diving activity alters gene expression profiles as compared to non-divers already without an acute challenge [20]. Interestingly changes in gene expres-

sion due to oxidative stress during scuba diving are linked to neutrophil activation, subsequent vascular injuries and microparticle formation [21]. The production of microparticle and neutrophil activation exhibits a strong association with decompression sickness [22]. Frequent and highly intense dive training may have already led to longer-lasting adaptation processes on the level of gene transcription. Thus, responses to yet another hyperoxic stress may have been less pronounced due to previous adaptation of baseline expression levels.

The mitochondrial pathway of apoptosis in cases of hypoxia is enhanced via inhibition of Akt (a serine/threonine kinase), which results in a decreased phosphorylation of transcription factor FoxO3a [23]. Translocation of FoxO3a to the nucleus also leads to elevated ROS accumulation. But ROS formation is further directly stimulated by the hypoxia-inducible factor (HIF)-1 $\alpha$ . It is well known that both pathways lead to apoptosis. Paradoxically, in a recent study the opposite condition – i.e., hyperoxia – induces HIF-1 $\alpha$  at levels twice higher than normoxia and hypoxia do [24]. Thus, elevated nucleosomes in our recent study of Navy divers may have been induced not by imbalance of the Bcl-2 family in mononuclear cells but via an alternative ROS formation pathway, or another origin of the nucleosomes – e.g., other tissues.

Nucleosomes as markers of DNA degradation and apoptosis [13,25] were found by our group after a single exposure to hyperbaric oxygen [15]. In the current study we observed an upregulation of anti-apoptotic (i.e., Bcl-2 and Bcl-xL) and pro-apoptotic (Bax) mRNA expression, but no increase in nucleosomes. Changes in pro- and anti-apoptotic gene expression were seen immediately after the dive and were even more pronounced after 24 hours. There was no indication of increased cell death due to a single air dive. An increase in oxidative stress after diving was found at an exposure to increased pressure at 40 meters for 25 minutes [4]. In our study the exposure was carried out at 18 meters, which may have evoked less oxidative stress.

We could confirm our hypothesis that a single air dive modulates apoptotic gene expression. Contrary to our expectations, it did not increase circulating nucleosomes. Therefore, it might be discussed that the upregulation of both (pro- and anti-apoptotic genes) sides

might counterbalance oxidative stress in a single air dive and that the stress caused by moderate oxygen levels is not high enough to stimulate HIF-1 $\alpha$ -mediated direct apoptosis.

## CONCLUSION

In conclusion, a single air dive at 2.8 ATA for 30 minutes caused an upregulation of pro- and anti-apoptotic genes but no signs of increased cell death. The upregulation of anti-apoptotic Bcl-2 family members may sufficiently counteract the pro-apoptotic activity of Bax in this setting. Future studies should be directed toward the analysis of apoptotic gene regulation in the leukocyte subpopulation and the need to control for training and adaptation effects.

## LIMITATIONS

Exposure to elevated pressure in a dry diving chamber differs from free water diving. Thus results may not completely reflect the situation of a free water dive. Due to missing intermediate time points (i.e., six hours and 12 hours after the initial dive) no statement could be made about the dynamic effects of gene expression after a single air dive. Due to missing physical pre-tests, actual fitness levels of the subjects could only be estimated. Gene expression analyses in this study were also performed in mononuclear cells, which comprise functionally different leukocyte subpopulations. Nucleosome detection in serum does not specify the nucleosome-releasing cell type and was not performed directly after the dive. Thus, no conclusion can be drawn about gene regulation or cell death status in specific different leukocyte subpopulations.

## Acknowledgments

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## Conflict of interest

All authors have no conflicts of interest.

## Ethical standard

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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