The effect of two consecutive dives on bubble formation and endothelial function in rats

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Key words
Decompression sickness, endothelium, bubbles, hyperbaric research

Abstract
(Havnes MB, Møllerløkken A, Brubakk AO. The effect of two consecutive dives on bubble formation and endothelial function in rats. *Diving and Hyperbaric Medicine*. 2008; 38: 29-32.)
Introduction: Gas bubble formation during and after decompression is considered to be the main initiator of decompression sickness (DCS). Compressed-air workers have been reported to acclimatise to the working environment and hence have a reduced risk of DCS, but the exact nature of the adaptation is not known. In the present study, we investigated the effect of two consecutive dives, separated by a 24-hour surface interval, on bubble formation and endothelial damage in rats.
Methods: A total of 30 rats were divided into four groups, one control group and three dive groups with different dive profiles, of which two of the groups had two dives. The amount of bubbles in the pulmonary artery was estimated by ultrasound for one hour after surfacing, and tension measurements were performed *in vitro* on segments of the abdominal aorta following sacrifice of the animals.
Results: No significant differences between the groups were found in endothelial function or bubble grade. However, animals that died immediately after the dive, irrespective of grouping order, had lower acetylcholine-induced dilatory responses in the aorta than surviving rats.
Conclusion: Bubble formation and endothelial function among rats were not significantly affected by exposure to consecutive dives 24 hours apart. An adaptive, protective effect of repeated dives was hence not seen in this animal model.

Introduction

Injuries to the organism related to decompression sickness (DCS) are caused by gas bubbles, which are believed to originate from pre-formed bubble-nuclei during decompression.[1] Gas bubbles formed during decompression can lead to mechanical damage of the endothelium or even stripping of endothelial cells.[2]

Repeated dives have generally been associated with increased risk of DCS, due to cumulative upload of nitrogen (N_2).[3,4] There are different opinions on the effects of repetitive diving and how to minimize the risk of DCS in such dives. Divers and caisson workers have reported increased tolerance to DCS with daily pressure exposures, which decreases after a few weeks' layoff. This observation has been confirmed in controlled studies.[5, 6]

Different explanations for adaptation or acclimatization to hyperbaric conditions range from increased resistance to DCS through repeated exposures, to different populations of gas nuclei being eliminated at specific pressures and loss of adaptation when nuclei re-accumulate.[4–7] The exact nature of adaptation or acclimatization is not yet known. Hence, the present study was initiated to determine in rats the effect of two consecutive dives, separated by a 24-hour surface interval, on bubble formation and on endothelial function.

Methods

A total of 32 female Sprague-Dawley albino rats (Kirkeby, Sweden) were selected, but two were excluded for technical reasons; the remaining 30 animals, weighing 321.1 ± 21.8 g, were used in the experiment. All experimental procedures and the care of the experimental animals conformed to the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the protocol was approved by the Norwegian Council for Animal Research.

PRESSURE PROFILE

Following one week of acclimatization, the rats were randomly assigned to one of four groups (A, B, C or D), where three of the groups (B, C & D) were exposed to different compression profiles (Table 1). The compressions were performed in a 20 L hyperbaric chamber with continuous air supply. Group A was a non-diving control group. Group B was observed for one hour with ultrasound after surfacing to detect bubbles and then sacrificed. Groups C and D underwent the first of two compressions, were observed for one hour and then rested for 24 hours before undergoing a second compression. After the second dive, the rats in groups C and D that did not die were again observed for one hour with ultrasound, before they were sacrificed.

BUBBLE DETECTION

Immediately after surfacing, the rats were anaesthetised with a 2 ml per kg bodyweight injection of haloperidol (0.7 $mg.ml^{-1}$), fentanyl (0.01 $mg.ml^{-1}$) and midazolam (1.07 $mg.ml^{-1}$) s.c. The pulmonary artery was monitored for gas bubbles using a 10 MHz transducer connected to a GE Vingmed System Five ultrasound scanner (GE Vingmed,

Table 1
The compression and decompression rates and depth of the dive profiles together with the observation period. The two dives in groups C and D were separated by a 24 hr surface interval. *The rats that died after the dive did not have an hour observation period

Group (n)	Compression rate kPa.min^{-1}	Decompression rate kPa.min^{-1}	Dive depth kPa	Observation period hr*
Group A (8)	0	0	0	0
Group B (7)	200	700	50	1
Group C (8)	200	400 + 700	50	1
Group D (7)	200	550 + 700	50	1

Horten, Norway). Bubbles were seen as bright spots, and verified with Doppler. The amount of bubbles was graded using a six-level grading scale described previously.[8]

TENSION MEASUREMENTS

Following the observation period, each live rat was sacrificed, or if the rat died early post-dive, the abdominal aorta was carefully dissected out and placed in an aerated (5% CO_2, 95% O_2) sodium-potassium buffer (139 mM) of the following composition: Na^+ 139 mM, K^+ 4.6 mM, Mg^{2+} 1.2 mM, Cl^- 134 mM, HCO_3^- 15 mM, $H_2PO_4^-$ 1.2 mM, Ca^{2+} 1.5 mM and glucose 11 mM (Sigma-Aldrich).

The tension in the abdominal aorta was measured using a myograph and PowerLab™ data system (Danish Myo Technology, Chart™ software, ADInstruments, Oxfordshire, England) as described previously.[9] The myograph measures the force (in milliNewton, mN) generated in the muscles during contraction caused by exposure to agonists. The incoming signals were digitised and displayed real-time on computer. After calibration of the myograph, three cylindrical segments (1.5–2.5 mm) of the abdominal aorta were mounted on two parallel L-shaped metal prongs in organ baths filled with the sodium-potassium buffer. A tension of 0.7–0.8 gram was gradually applied to the segments before they were allowed to stabilise for half an hour.

The contractile capacity of each vessel was examined by alternate exposure to a potassium-rich (60 mM) buffer solution (Na^+ 84 mM, K^+ 60 mM, Mg^{2+} 1.2 mM, Cl^- 133 mM, HCO_3^- 15 mM, $H_2PO_4^-$ 1.2 mM, Ca^{2+} 1.5 mM and glucose 11 mM) and to a sodium-potassium buffer. The vessels were precontracted in the bath with cumulative doses of nor-adrenaline (NA) until a stable level (70–100% of the response to potassium) was reached. After 30 minutes, cumulative doses of acetylcholine (ACh) were added in increments (10^{-8}–10^{-3} M, Sigma-Aldrich). The relaxation response that followed was assumed to depend on how much the endothelium function was affected by the bubbles. The relaxation response was also examined with cumulative doses of substance P (SP) (10^{-11}–10^{-6} M; Sigma-Aldrich) after a new precontraction of NA. The performance of the smooth muscle layer was examined with cumulative doses of sodium nitroprusside (SNP, 10^{-8}–10^{-4} M). Dose-response curves with all agonists were obtained. The resultant values are relative relaxation percentages, calculated using the baseline tension and the precontraction values as reference points and are presented with the maximal relaxation response to the agonists (Imax). In addition, EC_{50} was calculated, which is defined as the concentration of the agonist that leads to 50% of the total relaxation.

STATISTICAL ANALYSIS

The results are presented as means ± SD. Non-parametric statistical methods were used due to the small number of animals in each group. Kruskall-Wallis test was performed to assess differences between all the groups. Further investigation of differences between the groups was achieved using Mann-Whitney and the Wilcoxon signed-rank tests for unpaired data. The relationship between death/survival and Imax of ACh was also calculated using Mann-Whitney and the Wilcoxon signed-rank tests. The level of statistical significance was set at $P < 0.05$. All statistical analyses were performed using SPSS 13.0.

Results

The survival rate of the rats varied between the groups (Table 2), but the differences were not statistically significant. No significant correlation was found between survival and body weight.

Table 2
The numbers of animals that died or survived; all animals in Groups C and D survived the first dive. Imax of acetylcholine (Ach) presented as mean ± SD; *one animal in each of groups B and D excluded for technical reasons; † P = 0.048

Group (n)	Dive outcome	
	Died	Survived
Group A (8)	n/a	n/a
Group B (7)*	4	3
Group C (8)	5	3
Group D (7)*	2	5
Imax (ACh)†	43.33 ± 21.86	65.52 ± 23.11

Table 3
Dilatation response in abdominal aorta presented as relative percentages as defined by precontraction triggered by NA and baseline tension (Imax). In addition EC_{50} values are presented. Agonists used to trigger relaxation were acetylcholine (ACh), substance P (SP) and sodium nitroprusside (SNP). Data presented as mean ± SD. EC_{50} values are the concentrations ($^{-log}$M) of the agonists that lead to 50% of the total relaxation

Group (n)	ACh		SP		SNP	
	Imax (%)	EC_{50} ($^{-log}$M)	Imax (%)	EC_{50} ($^{-log}$M)	Imax (%)	EC_{50}($^{-log}$M)
Group A (8)	47.15 ± 18.66	6.08 ± 0.61	9.70 ± 13.17	7.88 ± 1.20	80.50 ± 21.77	5.17± 0.23
Group B (7)	57.61 ± 22.26	5.73 ± 0.42	22.72 ± 21.10	8.28 ± 1.86	73.48 ± 41.63	4.84 ± 0.20
Group C (8)	54.08 ± 28.53	6.23 ± 0.41	13.61 ± 9.34	9.38 ± 0.82	100.39 ± 67.22	4.92 ± 0.19
Group D (7)	51.63 ± 25.91	5.99 ± 0.42	14.15 ± 22.40	9.54 ± 0.76	84.44 ± 22.31	5.04 ± 0.28

BUBBLE DETECTION

The bubble grade varied from 0 to 5 within all the dive groups. In group D, two out of seven animals had bubble grade 5, while in group B four out of seven had grade 5 and in group C, five out of eight. This difference was not significant at the 5% level. All the rats that died immediately after the dive had bubble grade 5. In the present study there were no significant differences in bubble formation related to weight (P = 0.207).

TENSION MEASUREMENTS

There were no significant differences in the *in vitro* relaxation response of the abdominal aorta between the four groups (Table 3). However, animals in groups B, C and D that survived the observation period had a significantly higher maximal dilatory response in the abdominal aorta induced by ACh (Imax (ACh)) compared with animals that died immediately after the dive (P = 0.04, Table 2). Sensitivity to the agonists was tested by calculation of EC_{50} values for the agonists. There were no significant changes in sensitivity to the agonists in any of the groups.

Discussion

An impaired endothelial response (Imax) to ACh was found in animals that died immediately following a dive compared with animals that survived, but no differences were found between the four groups in bubble formation and endothelial function. Thus, two consecutive dives separated by 24 hours did not lead to any adaptation regarding tolerance to decompression stress. However, a higher bubble grade was observed in non-survivors compared with those who survived the entire observation period. Nishi found an increased risk of developing serious DCS when a large number of bubbles were detected in the vascular system of humans.[10] Previous research at our laboratory has shown a relationship between gas bubbles and mechanical endothelial damage and that the damage seems to be related to the amount of bubbles and not to the duration of exposure.[11] This is in accordance with the results of the present study.

The degree of relaxation varied within the groups, but the response to the endothelium-independent agonist SNP seemed unaffected by the dive and the vascular bubbles. Thus, this result confirms that the change in vasoactive response is related only to endothelial function and not to the function in the vascular smooth muscle layer.

It might be that the difference found in endothelial function is somehow influenced by the survival rate itself, due to severe hypoxia. However, from the experimental design of this study, this remains as speculation. In all of the animals that did not survive the entire observation period, the abdominal aorta was dissected out within 10 minutes. The endothelial measurements were also performed in isolated organ baths allowing for the exclusion of any influence from higher regulatory systems. Although possible, we consider death in itself as unlikely to be the cause of the difference in endothelial measurements.

The survival rate of the rats varied between the groups, but the differences were not statistically significant. It is well known that there is a significant variability in bubble formation among individuals. While the mechanisms that cause individual differences in susceptibility to DCS are unknown, body weight has been regarded as a predisposing factor for bubble formation.[12] Broome et al, however, state that weight is not a risk factor for DCS, but rather a supplementary factor in sedentary animals.[13] They found no reduction in DCS incidence in lighter animals compared with heavier animals. A study by Carturan supports these findings.[14] In the present study weight was significantly related neither to survival (P = 0.14) nor bubble formation (P = 0.207).

Although not significantly different, there was a trend towards reduced bubble production in group D compared with the other dive groups. Group D had two rats with bubble grade 5, while group B had four and group C five rats with the same bubble grade. Group B had only a single dive, unlike groups C and D. The pressure difference in the first dive between group C and D was 150 kPa with the same bottom time. Although too early to draw any conclusions from

this observation, it is tempting to speculate that if previous exposure to pressure does have any 'protective' effect, the pressure has to be above a certain level.

The present study examined if a prior dive had any effect on bubble formation and endothelial function in a second dive performed 24 hours later. There were no significant differences between the groups with regard to either of the two parameters, but an impaired endothelial response to ACh was found in the animals that died compared with the ones that survived.

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