Hyperbaric oxygen preconditioning reduces the incidence of decompression sickness in rats via nitric oxide

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ABSTRACT
Divers are at risk of decompression sickness (DCS) when the ambient pressure decrease exceeds a critical threshold. Hyperbaric oxygen (HBO₂) preconditioning has been used to prevent various injuries, but the protective effect on DCS has not been well explored. To investigate the prophylactic effect of HBO₂ on DCS, rats were pretreated with HBO₂ (250 kPa-60 minutes) (all the pressures described here are absolute pressure) for 18 hours before a simulated air dive (700 kPa-100 minutes) with fast decompression to the surface at the rate of 200 kPa/min (n=33). During the following 30 minutes, the rats walked in a 3 m/minute rotating cage and were monitored for signs of DCS. The control rats were pretreated with normobaric air (n=30), normoxic hyperbaric nitrox (250 kPa, 8.4% O₂) (n=13), or NG-nitro-L-arginine methyl ester (L-NAME) 30 minutes before HBO₂ exposure (n=13). Nitric oxide (NO) levels were recorded immediately and 18 hours after HBO₂ exposure in the brain and spinal cord. The incidence of DCS in rats pretreated with HBO₂ was 30.3%, which was significantly lower than those treated with normobaric air (63.3%) (p<0.05) or hyperbaric nitrox (61.5%) (p<0.05). The onset time of DCS of the rats pretreated with HBO₂ was significantly delayed compared with those treated with air (p<0.05). L-NAME nullified the HBO₂ preconditioning effect. HBO₂ increased NO level in the rat brain and spinal cord right after exposure; this effect was inhibited by L-NAME. Taken together, HBO₂ preconditioning reduced the incidence of DCS in rats, and NO was involved in the prophylactic effect.

INTRODUCTION
It is widely accepted that decompression sickness (DCS) is caused by bubble formation, from pre-existing gas micronuclei, in tissues supersaturated with inert gas (1). Symptoms of DCS range from minor joint pain to cardiorespiratory complications, paralysis and even death. Mechanism studies suggest that hemodynamic and biochemical changes associated with venous bubbles can damage microvasculature of the lung and nerve system (2, 3), and initiate an inflammatory response due to recruitment of neutrophils (4). Hyperbaric oxygen (HBO₂) is one of the main DCS treatment modalities; its efficacy has been validated by extensive clinical experience and scientific studies (5). HBO₂ has been used to prevent DCS, but this has been routinely performed just prior to hyperbaric exposure (6). Regardless of the proposed mechanism of action, such as denitrogenation or denucleation, the preventative effect of HBO₂ on DCS is thought to be short-lived. Butler, et al. (7) are the only group to report the effect of HBO₂ administered substantially before diving on DCS. They demonstrated that HBO₂ pretreatment up to
18 hours ahead of exposure provided significant protection from inflammatory DCS symptoms in rats. Recently, our laboratory (8-11) and others (12) found that HBO2 preconditioning (HBO2-PC) had protective effects against ischemic injury (one of the etiologies of DCS) in many organs of different species. We postulate that HBO2-PC has the potential to protect against DCS. The purpose of this study was to confirm potential prophylactic effects of HBO2 on DCS and explore the underlying preliminary mechanisms.

METHODS

Animal housing and care
All procedures were approved by the Ethics Committee for Animal Experimentation and were conducted according to the Guidelines for Animal Experimentation of the university. One hundred and forty-five male Sprague-Dawley rats weighing 300-310 g were obtained from Shanghai Slac Laboratory Animal Co. Ltd. Animal weight may have a significant effect on the decompression outcome: a remarkable increase of 1% in DCS risk was estimated for each gram increase of rat weight (13). It has been shown that rats weighing more than 300 g had more vascular bubbles formation than leaner rats (less than 300 g) (14), so we use the rats with a fixed weight to stabilize the model. Rats were housed with free access to a pelleted rodent diet and water. A controlled light cycle included a 12-hour dark and 12-hour light phase. Temperature and relative humidity were maintained at 23-25°C and 50.0-56.0% respectively.

Pre-exposure
All 145 rats were randomly assigned to four groups:
(1) HBO2 (n=54);
(2) air (n=44);
(3) nitrox (n=13);
(4) L-NAME+HBO2 (n=34).
The rats in the former three groups were treated with 250 kPa HBO2, normobaric air and 250 kPa nitrox (containing 8.4% oxygen) for 60 minutes, respectively. All the pressures described in this text are absolute pressure. The rats in the last group received 40 mg/kg NG-nitro-L-arginine methyl ester (L-NAME) [a non-specific nitric oxide synthase (NOS) inhibitor] intraperitoneally 30 minutes before HBO2 exposure. The exposures were performed in a 4 L transparent hyperbaric rodent chamber (Type DWC150, Institute No. 701, Shanghai, China), which was continuously ventilated with the respective gas to avoid carbon dioxide retention.

Simulated air diving
Eighteen hours after pre-treatment, 89 rats (33, 30, 13, 13 rats for the above four groups, respectively) were subjected to a simulated air dive with the protocol of 700 kPa for 100 minutes, and then decompressed to surface at 200 kPa/minute. The rats were compressed over five minutes, which began at a low rate (50 kPa/minute) to minimize possible middle ear squeeze. The chamber and the ventilation were the same as above.

DCS symptoms observation
Three minutes after decompression, the rats were subjected to walking inside an electrically controlled cylindrical cage rotating at a perimeter speed of 3 m/minute for 30 minutes to standardize the activity level and facilitate DCS scoring. According to the experiences of our own and others (15), 30 minutes of observation was sufficient for all cases of DCS to become evident. The DCS diagnosis was based on observation any of the following symptoms:
• walking difficulties
• abnormal breathing patterns
• forelimb and/or hindlimb paralysis
• rolling in the rotating wheel
• convulsions or
• death.
Animals were scored as having DCS only when one or more of these symptoms developed. Outcome was divided into two categories: “No DCS” and “DCS” when the above-mentioned symptoms (including death—when DCS symptoms culminated in death) were observed.
Determination of nitric oxide

Immediately following exposure, 14 rats in each of the HBO₂, air and L-NAME+HBO₂ treated groups were killed with chloral hydrate anesthesia. Another seven rats each in the HBO₂ and L-NAME+HBO₂ groups were killed 18 hours after exposure. The brains and spinal cords were quickly removed and weighed, then homogenized in ninefold-volume cooling saline. All the procedures were performed on the ice. After centrifugation (3000 rpm, 10 minutes at 4°C), the supernatants were immediately frozen at -80°C until the determination of protein and NO by chemical colorimetry using commercial assay kits (Jiancheng Bioengineering Institute, Nanjing, China). The Protein Assay, based on the method of Bradford, was a simple and accurate procedure for determining concentration of solubilized protein. It involves the addition of an acidic dye to a protein solution, and subsequent measurement at 595 nm with a spectrophotometer. Comparison to a standard curve provided a relative measurement of protein concentration. The NO Assay Kit measured total nitrate/nitrite concentration in a simple process as below. First, nitrate was converted to nitrite utilizing nitrate reductase. Then, the Griess reagents were added to convert nitrite into a deep purple azo compound. Photometric measurement of the absorbance due to this azo chromophore accurately determined NO₂⁻ concentration. The results of NO concentration were expressed in μmol/mg protein.

Statistical analysis

Incidences of DCS of different groups were compared by means of a Chi-square test. Log-rank testing was used to analyze for significant differences in symptom onset time and time of death between the HBO₂ and air groups. To examine whether the difference between two mean values of NO level were different from zero, a test for normality by means of ANOVA was performed. The difference between mean values of the various treatment groups was then analyzed by the Student Newman-Keuls procedure for multiple comparisons of means between groups: \( p < 0.05 \) was considered statistically significant.

RESULTS

No symptoms of oxygen toxicity were observed in any of the animals during or following HBO₂-PC.

Effects of HBO₂-PC on the incidence of DCS

The rats in the four groups were divided into two subgroups based on the outcome after decompression: no DCS symptoms and DCS including death (Table 1, above). The percentage of DCS in rats pretreated with HBO₂ 18 hour prior to testing (30.3%) was significant lower (\( p < 0.05 \)) than that of the air control (63.3%). Although the difference in the percentage of mortality between the HBO₂ and air groups was not statistically significant, it showed a decreasing trend (from 30% to 15.2%).
The DCS onset time of in the HBO2 group was significantly delayed compared with the air group \((p<0.05)\), but the survival time of rats between the two groups was not significant \((p=0.495)\) (Figure 1, above.). Normoxic hyperbaric nitrox pre-exposure had no effect on the incidence of DCS. All the dead rats convulsed before death and were postulated to have died as a result of CNS damage.

**NO levels after HBO2 exposure**

The NO levels in both brain and spinal cord increased significantly \((p<0.05)\) immediately after HBO2 exposure, but decreased to control level 18 hours later. Because nitrox exposure did not affect the outcome of DCS, the NO level in the rats that were pretreated with nitrox was not determined.

**The effects of L-NAME on the HBO2 preconditioning on DCS and NO level**

The morbidity and mortality of DCS in rats treated with L-NAME before HBO2 were significantly higher than those treated with HBO2, indicating that the NOS inhibitor abolished the prophylactic effect of HBO2 \((p<0.05, Table 1)\). And the increase of NO after HBO2 exposure was also totally blocked by L-NAME \((p<0.05, Figure 2, facing page)\). L-NAME did not affect the NO level 18 hours after HBO2 exposure (Figure 2).

**DISCUSSION**

DCS is a significant concern for scuba divers, pilots and astronauts when a sudden or excessively rapid reduction in the ambient pressure occurs,
such as when aborting an underwater dive, during “blow-up” in diving accidents, during high-altitude flights or extravehicular activities in space. Recently, investigations into DCS preventive measures have been focused on exercise (16), NO (17), heat stress (18, 19), oxygen pre-breathing (20) and vibration (21). The use of HBO2 pre-exposure several hours before hyper- or hypobaric exposure as a DCS prophylactic measure has not been well documented. If effective, this preventative method may have practical value for professional divers. Because most commercial or military diving units are equipped with hyperbaric chambers, it is a convenient arrangement for the divers to receive HBO2 at around 18 hours prior to diving.

Preconditioning of animals using HBO2 in various models has been reported to show injury tolerance (8-12). The present study demonstrated that HBO2-PC 18 hours before diving significantly reduced the incidence of DCS in rats. Preconditioning with normoxic hyperbaric nitrox – which had the same pressure HBO2 administered and oxygen partial pressure as normobaric air – did not change the outcome, suggesting that the prophylactic effect was not exerted by the pressure per se but by the elevated oxygen partial pressure. Pressure applied before diving might crush the nuclei in tissue and also might induce stress reaction (22). In this study, the pressure (2.5 ATA) did not affect the DCS outcome, possibly because the pressure was not enough to exert effect.

Because the time interval between HBO2 exposure and subsequent insults was relatively long, physical effects of HBO2, such as increase in the tissues’ oxygen partial pressure and denitrogenation, have already disappeared (6, 23).

It is generally accepted that gas bubbles are formed by the expansion and growth of bubble nuclei universally existing in body tissues and blood vessels (1). HBO2 pre-breathed shortly before hyperbaric exposure can decrease the bubble formation or alleviate DCS symptoms, and the effect was thought to be exhibited through denucleation (6, 20). HBO2 pretreatment before diving has been proven effective to replace the resident gas in the micronuclei by oxygen. When the breathing gas

**FIGURE 2 – Effects of HBO2 on the levels of NO in brain and spinal cord in rats (n=14 for Air, five minutes after HBO2 or HBO2+L-NAME groups; n=7 for 18 hours after HBO2 or HBO2+L-NAME group).**

The mean level of NO in the rats treated with HBO2 was significantly higher than that of the air control immediately after exposure, and the increase was blocked by L-NAME. 18 hours after HBO2, NO decreased to the control level.

NO – nitric oxide
HBO2 – hyperbaric oxygen
L-NAME – N^G^-nitro-L-arginine methyl ester

Significance of difference: * p<0.05 vs. Air group; # p<0.05 vs. HBO2 group
is switched from oxygen to air, at least some of the oxygen in these micronuclei would subsequently be consumed by the mitochondria, which results in a reduced volume and number of micronuclei. Then fewer bubbles would be formed during or after the following decompression from hyperbaric exposure, and the risk of DCS is decreased (1). In the present study, although the HBO₂ pre-breathe was administered many hours prior to the simulated diving, it is still possible that the denucleation effect might be involved in the prophylactic effect observed, for it should take 10-100 hours to regenerate a depleted nuclei population (24). Nevertheless, Martin et al. found the preventive effect of HBO₂ against decompression-induced ambulatory dysfunction did not modify the bubble formation, but that the inhibition of leukocyte sequestration was considered the underlying mechanism (25). It is possible to share the same mechanism in this study, for the inhibitory effect of HBO₂ on neutrophil adhesion lasted at least 24 hours in rats (26).

Previously, Wisløff et al. (27) have shown that administration of an NO donor before a dive protects against bubble formation. NO can maintain the integrity of vascular endothelium, inhibit platelet aggregation and adhesion, and inhibit leukocyte activation and adhesion (28-30). So, an increase in NO will reduce the hydrophobicity of the endothelial wall, reducing the number of nuclei adhering to the surface (31). All these properties could conceivably reduce bubble formation and the severity of tissue injuries (14, 17).

In this study, we confirmed previous findings (32) that HBO₂ exposure significantly increased the NO synthesis. L-NAME, a non-specific inhibitor of NO, inhibited the prophylactic effect of HBO₂ on DCS, and additionally the NO increase. L-NAME was given 20 hours 10 minutes before decompression; its direct effect on DCS was supposed to be nonexistent (33). These results suggest the prophylactic effect of HBO₂ on DCS was via NO induction. However, the increase of NO induced by HBO₂ diminished quickly following the stoppage of HBO₂ exposure (32), and in this study, the NO level at 18 hours post-HBO₂ had decreased to the control level.

How can NO exert its beneficial effect on DCS 18 hours later? Other molecules or proteins which could be activated by NO, such as the heat shock proteins, might have participated in this. More detailed studies are currently in progress.

In conclusion, HBO₂ preconditioning 18 hours before diving effectively reduced the incidence of DCS in rats; this might be mediated by NO. Further studies are needed to clarify the precise mechanism.

ACKNOWLEDGMENTS
This work was supported by the Science & Technology Program of the Chinese Liberation Army No. 08G064. We gratefully acknowledge the assistance of JM Cai for his helpful comments about the manuscript and XF Ye for his professional assistance in the statistical analysis.

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