HIGHLIGHTED TOPIC | The Physiology and Pathophysiology of the Hyperbaric and Diving Environments

Combined effect of denucleation and denitrogenation on the risk of decompression sickness in rats

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Arieli R, Boaron E, Abramovich A. Combined effect of denucleation and denitrogenation on the risk of decompression sickness in rats. J Appl Physiol 106: 1453–1458, 2009. First published February 19, 2009; doi:10.1152/japplphysiol.91146.2008.—We previously hypothesized that the number of bubbles emerging on decompression from a dive, and the resultant risk of decompression sickness (DCS), may be reduced by a process whereby effective gas micronuclei that might otherwise have formed bubbles on decompression are shrunk and eliminated. In a procedure defined by us as denucleation, exposure to hyperbaric oxygen (HBO) would result in oxygen replacing the resident gas in the micronuclei, to be subsequently consumed by the mitochondria when the oxygen pressure is reduced. Support for the validity of our hypothesis may be found in our previous studies on the transparent prawn and the reduction of DCS in the rat. In all of these studies, HBO pretreatment was given before supersaturation with inert gas at high pressure. The purpose of the present study was to compare DCS outcome in rats that underwent nitrogen washout (denitrogenation) alone (9 min O2 at 507 kPa) after exposure to air at high pressure (33 min at 1,266 kPa), and rats treated by both procedures (denitrogenation + denucleation; 8 min of O2 breathing followed by 5 min air breathing, both at 507 kPa) after high-pressure-air exposure. This was done with the same nitrogen load in both groups before the final decompression (a nitrogen pressure of 467 kPa in fatty and 488 kPa in aqueous tissue). Six of 20 rats in the denitrogenation + denucleation group died, compared with 13 in the denitrogenation group (P < 0.03). Three rats in the denitrogenation + denucleation group suffered mild DCS, recovering completely within 2 h of decompression. The present study indicates an advantage in considering both denitrogenation and denucleation before decompression. This may have practical application before escape from a disabled submarine, when aborting a technical dive, or in the prepartion of aviators for high altitude.

diving; nitrogen load; gas micronuclei; hyperbaric pressure

A rapid reduction in ambient pressure, such as may occur on the abortion of an underwater dive or during high-altitude flight, excursions from a space vehicle, or escape from a disabled submarine, can seriously increase the risk of decompression sickness (DCS). It is widely accepted that DCS is caused by the formation of bubbles in tissues supersaturated with inert gas. Inert gas and that these originate in pre-existing gas micronuclei. We previously hypothesized that exposure to hyperbaric oxygen (HBO) would result in oxygen replacing the resident gas in the micronuclei. When the breathing gas is switched from oxygen to air, at least some of the oxygen in these micronuclei (the amount of oxygen they contain should be very small) will subsequently be consumed by the mitochondria; this may shrink and eliminate effective gas micronuclei. With a reduced number of micronuclei, fewer bubbles will be formed on decompression following the hyperbaric air exposure, and this will reduce the risk of DCS. This procedure is defined by us as denucleation, in contrast to the procedure of denitrogenation, in which the tissue nitrogen resulting from the air exposure is washed out by breathing oxygen before decompression.

Support for the validity of this hypothesis may be found in our previous findings of reduced bubble formation in the transparent prawn (3, 4, 7) and the reduction of DCS in the rat (10, 11). In all of these studies, HBO pretreatment was given before saturation with inert gas at high pressure, and both pretreated and control animals (prawns and rats) had the same inert gas load before the final rapid decompression. These previous experimental protocols were designed to establish the validity of the denucleation procedure for an animal supersaturated with inert gas. To that end, we performed a denucleation procedure using oxygen as distinct from the process of denitrogenation (nitrogen washout), in that the animals were loaded with nitrogen at high pressure after the exposure to pure oxygen. A first attempt to use the denucleation procedure in humans agreed with our animal studies (13).

However, if oxygen is available before decompression, denitrogenation (breathing oxygen to wash out the nitrogen already loaded in the tissues) would also lower the risk of DCS. Nitrogen washout has thus been employed as a prelude to high-altitude flight and space missions (5, 12, 16, 19) and as a preventive measure before escape from a disabled submarine or decompression from a technical dive (6, 8).

Incorporating both denitrogenation and denucleation into a single procedure may therefore make decompression even safer. Denitrogenation and replacement of the resident gas in the micronuclei with oxygen (the first stage of the denucleation procedure) both require exposure to pure oxygen. The second step in denucleation requires a low tissue PO2, which can be achieved by using low oxygen and a high

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The search for a possible denitrogenation procedure with oxygen breathing that would provide only partial protection against DCS during perobaric air exposure with a denitrogenation procedure at the end of decompression results were scored as “No DCS” or “DCS Rec” when the above mentioned symptoms were observed but disappeared later with complete recovery, or “Died” when DCS symptoms culminated in death.

Experimental Protocol

The hyperbaric air exposures lasted for 33 min, which is close to full saturation in the rat (15) and was used in our previous studies (10, 11). Series A and B were conducted in the search for appropriate experimental conditions. Each group in these series consisted of 10 animals. When all of the rats in a particular group came through a test without suffering DCS, that group was used again for another of the tests. Series C was the experimental series, in which denitrogenation alone was compared with denitrogenation + denucleation.

Series A. The search for an appropriate exposure. In this series, we searched for a denitrogenation procedure with oxygen breathing that would not provide complete protection against DCS after hyperbaric air exposure. To accomplish this, we started with 1) a body mass of 250–300 g, air exposure at 1,013 kPa, reduction of the pressure to 507 kPa, and a switch from air to oxygen for 10 min, followed by rapid decompression (Fig. 1). For the subsequent sets of conditions, we increased the risk of DCS by increasing the weight of the rats (2), elevating the air pressure, and reducing the length of the oxygen exposure, namely 2) 250–300 g, 1,114 kPa air, and 8 min in oxygen; 3) 300–350 g, 1,216 kPa air, and 6 min in oxygen; 4) 350–400 g, 1,216 kPa air, and 8 min in oxygen. The pressure-time protocols are shown in Fig. 1, left. Test 4 produced the desired outcome, and Series A was terminated.

Series B. Effect of various segments from the denitrogenation + denucleation procedure. Series B was designed to search for a possible beneficial effect of adding an air period after the oxygen exposure, which thus completes the suggested denucleation protocol. For this series the air pressure chosen was 1,266 kPa, with oxygen exposure similar to that in Test 4 of Series A, followed by 5 min of air breathing. The pressure-time protocols for this series are shown in Fig. 1, right. The procedure was tested in three groups of animals using different segments of the oxygen-air exposure that was to follow the 33 min in air at 1,266 kPa and precede rapid decompression, namely 1) 5 min air at 507 kPa; 2) 13 min air at 507 kPa; and 3) 8 min oxygen at 507 kPa. The hypothesized denitrogenation + denucleation procedure was Test 4, in which the 1,266 kPa air exposure was followed by 8 min in oxygen at 507 kPa, the oxygen was then replaced by air for 5 min at the same pressure of 507 kPa, and this was followed by rapid decompression at 200 kPa/min.

Series C. Comparison of denitrogenation alone with denitrogenation + denucleation. In this series, we used two groups of 20 animals with similar body mass (360–420 g). At the end of the air exposure (1,266 kPa air for 33 min; Fig. 2), two rats at a time from the denitrogenation group (384 ± 11 g) were exposed to 507 kPa oxygen in the exposure cage, which was placed in the hyperbaric chamber. Pressure was increased linearly (at 100 kPa/min) to the desired pressure using air. Before the final decompression, pressure was reduced at 100 kPa/min to 507 kPa for varying times in oxygen, air, or both in sequence. Following this step, the rats were subjected to rapid decompression at 200 kPa/min to ambient pressure. After decompression, the two rats were immediately placed inside the cylindrical cage rotating at ~3 m/min for 30 min. This method of assessing DCS is based on a previously reported study and our own experience (2, 11, 15). The motion pattern of the rat in the cage enabled us to make an early diagnosis of DCS according to the following symptoms: walking difficulties, abnormal breathing patterns, forelimb and/or hindlimb paralysis, rolling in the cage, convulsions, and death. Rats were checked again at 2 and 24 h postdecompression. For the purpose of data analysis, the decompression results were scored as “No DCS” or “DCS Rec” when the above mentioned symptoms were observed but disappeared later with complete recovery, or “Died” when DCS symptoms culminated in death.

METHODS

Animals

One hundred male Sprague-Dawley rats were used. The experimental procedure was approved by the Israel Ministry of Defense Animal Care Committee and the rats were handled in accordance with the principles of laboratory animal care.

Experimental System

Exposures were conducted in a double-walled, thermoregulated metal cage that enables continuous observation of the animal, as described previously (11). The ambient temperature was kept in the range 23–28°C. The exposure cage was placed in a 150-liter hyperbaric chamber (Roberto Galeazzi, La Spezia, Italy). A pneumatically operated cylindrical cage, which could be rotated at a speed of 3 m/min, was used to diagnose DCS by observing the animals’ gait and behavior following the exposure, as described previously (2). The gauges on our chamber’s control panel show the pressure in atmospheres, and these were the readings we took during the hyperbaric exposures. To conform with accepted scientific notation, in the text we have made the conversion to kilopascals, although this may not be absolutely precise at unit level.

Experimental Procedure

Before any exposure, the animals were placed in the rotating cage to ensure a normal motion pattern. Two animals at a time were placed
for 9 min before rapid decompression. After the hyperbaric air exposure, rats from the denitrogenation + denucleation group (386 ± 15 g) were subjected to 8 min oxygen at 507 kPa and then to air at 507 kPa for 5 min before rapid decompression. The nitrogen load in both aqueous and fatty tissues was similar in both groups before the final decompression (the calculation follows). Decompression in both groups was conducted in oxygen.

Calculation of Nitrogen Load in the Tissues

For inert gas load, nitrogen load was also considered to include the argon load. Nitrogen load was calculated for each exposure at the point before the final rapid decompression. This was to help us estimate the risk of DCS and establish equal nitrogen loads in the denitrogenation and denitrogenation + denucleation groups before decompression. The nitrogen load in aqueous tissue was calculated using the time constant obtained by Lillo and Parker (15) for the whole rat: $P_{t0N2} = (P_{ambN2} - P_{t0N2}) \times (1 - e^{-0.068t}) + P_{t0N2}$, where $P_{t0N2}$ is tissue PN$_2$, $P_{ambN2}$ is ambient PN$_2$, $P_{t0N2}$ is tissue PN$_2$ at time 0, $t$ is the time in minutes at the present ambient pressure, and 0.068 is the aqueous time constant in min$^{-1}$.

For calculation of the nitrogen pressure in fatty tissue, we used the same formula with the time constant of 0.0235 min$^{-1}$ suggested by Hyldegaard and Madsen (9).

Data Analysis and Statistics

To find the relationship between denitrogenation or denitrogenation + denucleation and DCS outcome, we used Fisher’s exact test and the $\chi^2$ test.

RESULTS

All cases of DCS occurred within 30 min of decompression, and when obvious signs of DCS were observed the rat was removed from the rotating wheel. No further symptoms of DCS appeared by 2 and 24 h postdecompression. These observation points were therefore used to determine recovery in rats that had symptoms of DCS and recovered. No symptoms of oxygen toxicity were observed in any of the animals following HBO exposure.

Series A

Figure 3 shows the results of the search for a denitrogenation procedure using oxygen at the end of the air compression period with regard to the three outcome categories: no symptoms of DCS, symptoms of DCS followed by recovery, and
symptoms of DCS culminating in death. No DCS was observed after 1,013 kPa air exposure with 10 min exposure to oxygen or after 1,114 kPa air exposure with 8 min of oxygen (Fig. 3, two left columns). Increasing the risk of DCS by elevating the air pressure to 1,216 kPa, increasing the rats' body mass to 300–400 g, and keeping the oxygen exposure short (6–8 min), resulted in symptoms of DCS (Fig. 3, two right columns). It can be seen that DCS occurred at a nitrogen tension in aqueous tissue above 500 kPa but not below 420 kPa, and in fatty tissue above 450 kPa but not below 430 kPa. Six as opposed to 8 min of oxygen may increase the risk of DCS (one rat died), more than 50 g less body mass may lower it. No statistical analysis was performed, because the small sample size of 10 animals was only used for planning the design of the appropriate experimental conditions. However, comparison of the results for 8 min oxygen alone with those for 8 min oxygen followed by 5 min air indicated a possible advantage of the denitrogenation + denucleation procedure. These results helped in the design of the experimental conditions for Series C.

### Series C

Although before the final rapid decompression the nitrogen load was almost the same in the fatty and aqueous tissues of denitrogenation + denucleation and denitrogenation rats, 6 of 20 rats in the denitrogenation + denucleation group died, compared with 13 in the denitrogenation group (Fig. 5). This difference was significant ($P < 0.03$). Three rats in the denitrogenation + denucleation group suffered mild DCS, recovering completely within 2 h of decompression.

### DISCUSSION

The main finding of the present study is that at very similar nitrogen supersaturation, a combination of the denitrogenation and denucleation procedures resulted in decreased severity of decompression sickness compared with denitrogenation alone, 30% mortality compared with 65%, respectively (Fig. 5). The first suggestion of a beneficial effect of denitrogenation + denucleation was noted earlier in the search protocol, as can be seen from the two columns on Fig. 4, right, which show that 50% of the animals died after 8 min oxygen compared with a 10% mortality rate after 8 min oxygen followed by 5 min air.

There may be a denucleation procedure concealed in a number of previous studies and in certain procedures whose purpose is to perform denitrogenation or achieve other beneficial effects of oxygen. In US Navy Treatment Table 6...
the injured diver is compressed to 180 kPa while breathing oxygen. To avoid oxygen toxicity, air breathing intervals are interspersed between the oxygen breathing periods. Thus the inert gas in small bubbles might be replaced by oxygen, and diminution of these activated gas nuclei during air breathing would prevent the bubbles from growing any further.

Latson et al. (14) studied the simulated rescue of submariners from a saturation dive. For denitrogenation, they employed oxygen breathing either during decompression or at pressure and during decompression. The risk of DCS was largely reduced when oxygen was breathed at pressure. For example, after a saturation dive at an equivalent air depth of 250 kPa, 13% of the subjects suffered DCS when oxygen was breathed during 10 h of decompression. However, when oxygen was breathed at the bottom pressure for 4 h and decompression on oxygen lasted only 6 h (the same total oxygen time), no DCS occurred. Air breaks were used during oxygen breathing to avoid oxygen toxicity. Thus during decompression on oxygen, nuclei could have grown, engulfing oxygen and nitrogen, and by the time the air break came round these nuclei might have been too large to be absorbed (18). When the oxygen was switched to air at the bottom pressure, the nuclei remained small and their oxygen could easily have been consumed by the mitochondria. In their conclusions, the authors relate this advantage to “isobaric denitrogenation, or resorption of gas micronuclei.”

An oxygen prebreathe is common practice before altitude exposure and has been extensively studied (12, 17, 19). It may well be that if an oxygen prebreathe at ground level is followed by a period of normoxia or hypoxia, denucleation would be effective before reaching high altitude. The study by Gennser and Blogg (8) of an oxygen prebreathe at 100 kPa for 15 min in goats, before simulated escape from a disabled submarine at 240 msw on air, might also have benefited from the process of denucleation.

Helpful information may be gleaned from the relation between DCS and tissue nitrogen pressure before the final decompression (Fig. 6). These data were taken from the search and other protocols (excluding denucleation protocols). Because the groups were small and there were only five groups in the high weight range (350–420 g), no statistical analysis was carried out. However, a linear relationship does seem to exist between nitrogen tension in the tissues (aqueous or fatty) and the risk of DCS. For rats in the lower weight ranges, the risk function may shift downward. In any additional studies of DCS in rats, it may be possible to define the exposure protocol by calculating the nitrogen load (see calculation of nitrogen load in the methods) and the percentage of DCS from the equations presented in Fig. 6. This may help plan the desired rate of DCS. This should be valid for a decompression rate of 200 kPa/min and a body mass of 350–420 g. Thus our search protocols could save time and animal resources in future investigations. The relationship between tissue nitrogen load and decompression risk supports our suggestion that the difference between the two groups in Series C is related not only to denitrogenation but also to denucleation.

The present study indicates an advantage in considering both denitrogenation and denucleation before decompression. The

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**Fig. 5.** DCS outcome after the denitrogenation + denucleation procedure (right column) compared with denitrogenation alone (left column). No DCS denotes rats with no sign of DCS; DCS-Rec denotes animals that had symptoms of DCS and later recovered; Died denotes animals that had symptoms of DCS that culminated in death. The exposure sequence is given below each column. Calculated nitrogen tensions for fatty (fat) and aqueous (aqua) tissues are given above each column. The animals’ body mass, the number of rats, and the statistical significance for the number of rats that died of DCS are given in the inset.

**Fig. 6.** Percentage of DCS after rapid decompression following high-pressure air exposure and denitrogenation alone, as a function of the nitrogen tension in aqueous (bottom) and fatty (top) tissues. Each symbol represents a group of 10 or 20 rats. Regression lines and equations were derived only for the groups with a body mass of 350–420 g.
procedure we chose is by no means the best. Further studies should define the best procedure for the rat, after which the question should be addressed of an efficient procedure for divers and aviators.

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