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Pre-treatment with hyperbaric oxygenation reduces bubble formation and platelet activation

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Abstract Bubble formation and platelet activation are major factors contributing to decompression sickness. We hypothesized that pretreatment with hyperbaric oxygen immediately before a dive may reduce bubble formation and platelet activation in humans. Five healthy volunteer subjects (1 female and 4 males; age, 33.6±2.9 years; height, 170±3 cm; weight, 71±8 kg, body mass index, 24.5±22.0 kg/m^2) participated in this study with a 4-day protocol. On day 1, a multiplace hyperbaric chamber was used to compress all subjects with air to 4 atmosphere absolute (ATA) for 25 minutes; they were then decompressed to surface pressure at a rate of 10 m/min. Once surface pressure was reached, they were monitored with precordial ultrasonic Doppler at 20 min, 50 min and 80 min. Venous blood samples were obtained immediately before and after pressure exposure. On day 2, all subjects were compressed at 1.6 ATA for 45 min with 100% oxygen; they were then decompressed to surface pressure at a rate of 10 m/min. As soon as they reached surface pressure, they were immediately exposed to the same compression-decompression protocol as day 1; blood samples were taken after the second pressure exposure. Platelet activation was examined before and after exposure. On days 3 and 4, we inverted the protocol to minimize the influence of the first immersion on bubble formation. In comparison to the standard compression protocol, compression after hyperbaric oxygenation led to significantly reduced bubble numbers and platelet activation (11.4%±0.7% vs. 5.4%±0.5%, $p<0.05$). This study shows that hyperbaric oxygenation pretreatment significantly reduces decompression-induced bubble formation and platelet activation. Hyperbaric oxygenation pretreatment may reduce the risk of decompression sickness in at-risk activities.

Key words Bubbles • Platelet activation • Hyperbaric oxygenation • Doppler • Diving • Decompression

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Introduction

Decompression sickness generally occurs in occupational groups such as compressed air workers, divers, aviators, and astronauts. Patients may manifest a spectrum of symptoms

ranging from minimal disease to neurological consequences including cognitive impairment, sensorimotor dysfunction, and death. Decompression sickness is considered a pathological event caused by intravascular [1] and extravascular [2] gas bubbles. These circulating microbubbles are produced during acute changes of hydrostatic pressure [3, 4]. One mechanism by which microbubbles induce decompression sickness may be through the activation of platelets [5, 6], resulting in the stimulation of prothrombotic and pro-inflammatory processes [7].

Conditions under which gas leaves solution and forms bubbles are not well understood. A 1969 study suggested the existence of micronuclei within living tissue under normal conditions [8]. Now it is widely accepted that gas bubbles are formed by the expansion and growth of micronuclei in tissue [9]. Therefore, the reduction or elimination of micronuclei may be beneficial in preventing the formation of gas bubbles and consequently the risk of decompression sickness. The growth of a nucleus into a bubble can be retarded by the action of surface tension [10]. Pre-exposure of animals to high pressure oxygenation significantly reduced decompression-induced bubble formation, hypothesized to be due to the elimination of micronuclei [11–13].

The inert gas of micronuclei could change with the inert gas dissolved in tissues following Fick's diffusion principle. Therefore, change of nitrogen with oxygen in the breathing mixture would replace the nitrogen in micronuclei. Metabolic processes would eliminate the oxygen and liquid would then replace the consumed oxygen, abolishing the condition required to form a gas nucleus. A recent study [11] suggested that hyperbaric oxygenation may eliminate most of the gas nuclei in decompressed prawns, therefore reducing the number and size of developing bubbles, consequently reducing the risk of decompression sickness. The authors suggested that the effectiveness of hyberbaric oxygen exposure warrants further studies in mammals. In the present study, we examined our hypothesis that pretreatment with hyperbaric oxygen may reduce the formation of gas bubbles in simulated dives in 5 healthy subjects. This study also examined the effects of hyperbaric oxygenation on platelet activation during simulated diving.

Subjects and methods

Five healthy volunteer subjects participated in this study. Each participant from Salerno had a valid medical certificate for diving. All experimental procedures were conducted in accordance with the Declaration of Helsinki. Each method and its potential risks were explained to the participants and they gave written informed consent before participating in the experiment. All subjects were instructed not to take any medications, nor to dive or fly 48 hours prior to the study.

Experimental protocol

The study was carried out over 4 days in a multiplace hyperbaric chamber of the Hyperbaric Medicine Institute (Salerno, Italy). Diving was simulated according to 2 conditions of compression and decompression. Before compression, venous blood samples were collected and a precordial 2 MHz ultrasonic Doppler probe was placed on all subjects. On day 1, subjects were compressed at 4 atmosphere absolute (ATA) for 25 minutes and then decompressed at a rate of 10 m/min (Condition A). Once the surface pressure was reached, they underwent another venous blood sampling and were monitored ultrasonographically at 20, 50 and 80 min. The day after, subjects were compressed at 1.6 ATA for 45 min with 100% oxygen (hyperbaric oxygenation) and then decompressed to surface pressure at a rate of 10 m/min (always breathing oxygen); as soon as surface pressure was reached, they were immediately recompressed at 4 ATA for 25 minutes and decompressed at 10 m/min (Condition B). When surface pressure was reached, blood samples were taken immediately and subjects were monitored ultrasonographically at 20, 50 and 80 min.

After three months, on day 3 and 4, simulated diving was carried out according to Condition B and then Condition A, respectively, to minimize the influence of immersion on bubble formation.

Doppler ultrasound analysis of bubble sounds

A technician was trained in the use of Doppler equipment for detecting air bubbles. Doppler signals were obtained using a Hadeco SonoMate 300 G-2 MHz probe instrument and dual-ear headphones. Pre-cordial Doppler signals were recorded on digital apparatus (Panasonic IC Recorder RR-US360) to achieve high quality echo and stored with USB exit for PC connection. Before simulating diving, one minute precordial Doppler signals were recorded for each subject as examples of bubble-free heart sound. These served as baseline for comparisons during subsequent signal grading after the completion of simulated diving. The Doppler probe was placed at the left sternal border and manipulated until the flow sounds were strong with valve sounds audible in the background. Doppler monitoring was performed during simulated diving at rest and after movement (two deep knee bends) in each situation for about 30-40 s. Bubble sounds were analyzed using *WavePurity Professional* software (Berlin, Germany) that allows graphic representation of the Doppler signal.

The auditory output from the bubble detector was categorized using Kisman-Masurel (KM) code [14]. The 3-digit KM code (each digit ranging from 0 to 4) represents the three components of bubble signals (Table 1): the *frequency* (number of bubbles per cardiac period); the percentage/*duration*, defined at rest as the percentage of cardiac cycles with bubbles and defined after a specified movement as the number of cardiac cycles with elevated bubble sound); and the *amplitude* of the bubble signal relative to normal cardiac background sounds. The KM codes (in format "fpA" for frequency, percentage and amplitude) were subsequently converted to a bubble grade (BG) from 0 to IV based on a 12-point ordinal scale according to Nishi et al. [15] (Table 2).

Table 1 Parameters of the 3-digit Kisman-Masurel code [14]

Code	Definition and values
First digit	Frequency (f)
1	1-2
2	Several (3-8)
3	Rolling drumbeat (>9)
4	Continuous sound
Second digit at rest	Percentage (p) of cardiac periods
0	0
1	1-10
2	10-50
3	50-99
4	100
Second digit after movement	Duration (d) of cardiac cycles
0	0
1	1-2
2	3-5
3	6-10
4	>10
Third digit	Amplitude (A)
0	No bubbles detected
1	Barely perceptible, A(b)<<A(c)
2	Moderate amplitude, A(b)<A(c)
3	Loud, A(b) ≈ A(c)
4	Maximal, A(b)>A(c)

Assessment of platelet activation

Blood samples were drawn from an antecubital vein through a 20-gauge needle by staff trained to minimize artificial platelet activation. Samples were injected into vacutainer tubes (Becton Dickinson) containing 3.2% trisodium citrate and centrifuged at room temperature for 10 min at 200 × g. Platelet-rich plasma was obtained with a pasteur pipet and then fixed by adding an equal volume of 2% paraformaldehyde (v/v) in phosphate-buffered saline (PBS). Samples were washed twice with 1 ml of PBS.

The fixed platelets were placed in polypropylene tubes containing 10 μl anti-human CD42a labeled with fluorescein isothiocyanate (FITC), 10 μl anti-human CD62p labeled with phycoerythin (PE) and 10 μl anti-human CD63 labeled with PE. As a negative control, 10 μl mouse IgG1 labeled with PE was added in place of anti-human CD63 and anti-human CD62p, and 10 μl mouse IgG2a labeled with FITC was added in place of anti-CD42a. All samples were immediately placed on ice for 30 min in the dark. The stained cells were washed twice and the pellet was finally resuspensed in 500 μl PBS and analyzed by flow cytometry.

Flow cytometric analysis was performed with a Coulter Elite flow cytometer (Coulter Electronics, Miami, USA) equipped with a 488-nm helium-neon laser. The flow cytometer was calibrated with microbeads (Coulter) to verify the reproducibility of light scatter and fluorescence signal. At least 10 000 cells were analyzed for each sample. Each region of interest (platelet population) was memorized by computer, to analyze each platelet sample every time with the same parameters. The platelet population was identified by gating the CD42a-positive cells. The negative and positive delineators were determined by gating 2% background staining on the isotype control fluorescence. The percentage of PE-positive (CD62p and CD63) events in the population was then determined. A separate linear gate was used to determine mean PE fluorescence in arbitrary units on a log scale.

Statistical analysis

ANOVA was used to analyze the time-course data within the groups, while Student's paired *t* test was used in the comparison of

Table 2 Conversion fo KM codes to KM bubble grades (BG), according to Nishi et al. [15]. The term fpA indicates the 3 digits of KM code (frequency, percentage, amplitude)

fpA	BG	fpA	BG	fpA	BG	fpA	BG
111	I-	211	I-	311	I	411	II-
112	I	212	I	312	II-	412	II
113	I	213	I+	313	II	413	II+
114	I	214	II-	314	II	414	III-
121	I+	221	II-	321	II	421	III-
122	II	222	II	322	II+	422	III
123	II	223	II+	323	III-	423	III
124	II	224	II+	324	III	424	III+
131	II	231	II	331	III-	431	III
132	II	232	III-	332	III	432	III+
133	III-	233	III	333	III	433	IV-
134	III-	234	III	334	III+	434	IV
141	II	241	III-	341	III	441	III+
142	III-	242	III	342	III+	442	IV
143	III	243	III	343	III+	443	IV
144	III	244	III+	344	IV-	444	IV

different groups (Statview 4.0; Abacus Concepts, USA). Differences with a p value <0.05 were considered statistically significant.

Results

We studied the effect of hyperbaric oxygenation on parameters related to decompression sickness in 5 healthy individuals (29-45 years of age) during simulated diving (Table 3). Pre-treatment with hyperbaric oxygen significantly reduced the bubble grade detected by precordial Doppler ultrasonography at 20 min. Thereafter, the bubble grade greatly decreased either at 50 or at 80 min, showing no significant difference between the two groups (Table 4). There was no difference in precordial Doppler signals between those measured at rest and after movement (data not shown).

Simulated diving (Condition A) significantly increased platelet activation from a mean of 2.6% (SE=0.53%) to 11.4% (SE=0.7%), p<0.01 (Table 5). Pretreatment with hyperbaric oxygen (Condition B) significantly attenuated platelet activation compared to Condition A.

There was no influence of the first immersion on the bubble formation of the second immersion (data not shown).

Table 3 Physical characteristic of study subjects

Subject	Sex	Age, years	Height, cm	Weight, kg	BMI, kg m^{-2}
1	F	32	164	50	18.5
2	M	30	167	60	21.5
3	M	32	182	95	28.6
4	M	45	170	84	29.1
5	M	29	165	68	24.9

BMI, body mass index

Table 4 Bubble grade detected at Doppler ultrasound in 5 subjects in 3 moments after simulated diving in a hyperbaric chamber, by standard protocol Condition A and with hyperbaric oxygen pretreatment (Condition B). Bubble grade was calculated from KM code according to Nishi et al. [15]

Subject	20 min		50 min		80 min	
	A	B	A	B	A	B
1	I+	I-	I	I	I-	I-
2	II-	I+	I	I	I-	I-
3	I	I	II-	I+	I-	I-
4	II	I+	I+	I	I-	I-
5	II	I+	I-	I-	I-	I-

Table 5 Platelet activation before and immediately after simulated diving by standard protocol (Condition A) and with hyperbaric oxygen pretreatment (Condition B)

		After simulated diving	
Subject	Before	Condition A	Condition B
1	2.6%	11.7%	4.0%
2	2.0%	9.5%	6.1%
3	3.0%	12.3%	7.0%
4	3.1%	13.4%	5.0%
5	2.1%	9.8%	4.8%
Mean (SE)	2.6% (?%)	11.4% (0.7%)*	5.4% (0.5%)$^{\#}$

* p<0.05 vs. value before simulated diving; $^{\#}$ p<0.05 vs. Condition A

Discussion

Decompression sickness is still a threat to some occupational groups such as compressed air workers, divers, aviators, and astronauts. Bubble formation and platelet activation are believed to be involved in the development of this condition. Reduction or elimination of bubble formation and platelet activation during at-risk activities may have preventive effects. The main findings of the present study are that pretreatment with hyperbaric oxygen significantly reduced bubble formation and platelet activation in simulated dives.

Role of pretreatment with hyperbaric oxygen in decompression-induced bubble formation

The conditions under which gas leaves solution and forms bubbles are not fully understood. An early study suggested that the micronuclei may exist within living tissue under normal conditions [8]. The origin of these micronuclei is not clear. According to tribonucleation theory, large local mechanical supersaturations are generated when two closely opposed surfaces are separated in a liquid. Tribonucleation may occur within the body in joints, in blood vessels which collapse and separate, or at sites where tendons and muscles slide across bone. This hypothesis is supported by the observation that skeletal muscle exercise, in particular high-intensity exercise immediately before decompression, increases the risk of deconpression sickness, presumably due to gas micronuclei formation [16, 17]. Now it is widely accepted that these micronuclei expand and grow to form gas bubbles [4] during decompression. The nucleus may be stabilized when the collapsing force of negative supersaturation is balanced by the elastic resistance of the tissue [17]. The resident gas in the nucleus can be exchanged with a gas in the tissue by diffusion. Because oxygen has a greater permeation coefficient than nitrogen,

oxygen permeates rapidly into the bubble, and simultaneously nitrogen exits the bubble to the surrounding tissue. Then, oxygen is rapidly consumed by the cellular metabolism, resulting in a rapid bubble decay. Theoretically, pretreatment with hyperbaric oxygen reduces micronuclei, and consequently reduces bubble formation during decompression. Some studies have demonstrated that pre-treatment with oxygen inhalation provides beneficial effects in alleviating decompression sickness [9, 18]. A recent study provided evidence that pretreatment with hyperbaric oxygen significantly reduced gas bubbles in decompressed prawns [11]. The authors speculated that reduced gas bubbles may be due to elimination of micronuclei. The present study provides new evidence that hyperbaric oxygen pretreatment significantly reduces decompression-induced air bubble formation in humans. In the present study, breathing pure oxygen under hyperbaric conditions led to an higher arterial oxygen content achieved by increasing dissolved oxygen. This increased oxygen content rapidly diffuses into the micronuclei to exchange for nitrogen which rapidly diffuses out. The oxygen then is absorbed by the surrounding tissue to cause rapid micronuclei decay. Although the numbers of micronuclei are not known before and after decompression, we speculate that the decreased micronuclei number may be responsible for reduced decompression-induced bubble formation. In the present study, the numbers of decompression-induced air bubbles were not significantly different between the two compression conditions 80 minutes after simulating diving. This is probably due to the human body's ability to gradually remove these air bubbles. Our study suggests that hyperbaric oxygen pretreatment significantly enhances the human body's ability to remove the air bubbles.

The role of denitrogenation and estimation of dissolved N_2 tension in tissues

The beneficial effect of hyperbaric oxygenation may also be due to effective denitrogenation, even though inert gas washout is controlled by gas partial pressure gradient during pre-oxygenation and it is independent of depth [19]. Furthermore, breathing oxygen at a deeper depth could have the advantage of a greater hydrostatic pressure to reduce bubble dimension in accordance with LaPlace's law. Assuming a perfusion-limited system, an approximation of the dissolved inert gas (N_2) partial pressure in the tissue during any partial pressure change of the same gas in the breathing medium, is provided by the classic exponential Heller-Mager-Von Schrotter equation:

$$P_t = P_0 + [P_i - P_0] \times [1 - e^{-0.693 \times t/T(1/2)}]$$

where P_0 is the pressure of inert gas in the tissue at the start of exposure, Pi is the pressure of inert gas in the breathing mix, $T_{(1/2)}$ represents the half-time compartment and t is the duration of exposure in minutes.

The nitrogen partial pressure at the end of pre-oxygenation (first step of Condition B used in our protocol) can be estimated by resolving the above-mentioned equation:

$P_0 = (10 - 0.627) \times 0.79 = 7.4$ meters sea water (msw);

$Pi = (16 - 0.627) \times 0.10 = 1.5$ msw;

$T_{(1/2)} = 5$ and 10 min;

$P_t = 7.4 + [1.5 - 7.4] \times [1 - e^{-0.693 \times 45/5}] = 1.55$ msw (pressure of inert gas in the tissue-compartment with a half-time of 5 minutes at the end of pre-oxygenation);

$P_t = 7.4 + [1.5 - 7.4] \times [1 - e^{-0.693 \times 45/10}] = 1.8$ msw (pressure of inert gas in the tissue-compartment with a half-time of 10 minutes at the end of pre-oxygenation).

These data show how nitrogen wash-out from fast tissues is efficacious using hyperbaric oxygenation.

After compression, the behaviours of two fast tissues (with 5 and 10 min half-times) show the same data in both the group that received pre-oxygenation and the group that didn't receive previous pre-oxygention (Table 6). This result demonstrates that fast tissue saturation, even after pre-oxygenation, becomes equal for both groups, meaning that the efficacy of hyperbaric oxygen pretreatment is due rather to a second mechanism in additional to denitrogenation. This mechanism could refer to the interference of hyperbaric oxygen with the inflammatory cascade.

The role of pretreatment with hyperbaric oxygen in decompression-induced platelet activation

Circulating activated platelets may be involved in prothrombotic and pro-inflammatory processes [7] and associ-

Table 6 Data of tissues nitrogen on-gassing

Pre-oxygenation group	No pre-oxygenation group
$P_t=1.55+[31.1-1.55] \times [1-e^{-0.693 \times 25/5}]=30.1(msw)^a$	$P_t=7.4+[31.1-7.4] \times [1-e^{-0.693 \times 25/5}]=30.3(msw)^b$
$P_t=1.8+[31.1-1.8] \times [1-e^{-0.693 \times 25/10}]=25.5(msw)^a$	$P_t=7.4+[31.1-7.4] \times [1-e^{-0.693 \times 25/10}]=26.9(msw)^b$

[a] Pressure of inert gas in fast tissues (5 and 10 min half-times) at the end of bottom time (Condition B)

[b] Pressure of inert gas in fast tissues (5 and 10 min half-times) at the end of bottom time (Condition A)

ated with many common clinical disorders such as coronary artery disease [20]. Several studies have demonstrated that drastic pressure changes, such as decompression, induce platelet activation [1, 21]. It is not fully understood what causes platelet activation during decompression; nitrogen microbubbles have been hypothesized to be an important factor in activating platelets [22, 19]. Theoretically, reduction of nitrogen partial pressure reduces platelet activation; this hypothesis is supported by the fact that breathing nitrox rather than air reduced the level of decompression-induced platelet activation [21]. An animal study showed that acute exposure to hyperbaric oxygen at 2.4 atm absolute oxygen for 90 min significantly reduced the maximal rate of ADP- and collagen-induced platelet aggregation [23]. The present study shows for the first time that hyperbaric oxygen, when administered immediately before simulated diving, significantly reduces decompression-induced platelet activation.

It is unclear whether decompression-induced platelet activation poses any danger to healthy divers. However, enhanced platelet activation has been likened to the development of most cases of ischaemic stroke [24]. Platelet activation is also observed in patients with atherothrombotic lesions, arrhythmias [23] and renal insufficiency [25]). Considering that platelet activation is also associated with microbubble formation during decompression [21, 26], reduction of platelet activation may be beneficial to reduce the risk of decompression sickness.

In summary, pretreatment with hyperbaric oxygen (1.6 ATA for 45 min with 100% oxygen) immediately before simulating diving significantly reduced decompression-induced bubble formation and platelet activation. Pretreatment with hyperbaric oxygen may be beneficial in reducing the risk of decompression sickness.

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