ACTA PHYSIOLOGICA

Acta Physiol 2011, **202**, 69–78

Effect of pre-breathing oxygen at different depth on oxidative status and calcium concentration in lymphocytes of scuba divers

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Received 19 August 2010,
revision requested 2 November 2010,
revision received 28 November 2010,
accepted 16 December 2010
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Abstract

Aim: In-water pre-breathing oxygen at various depths reduces decompression-induced bubble formation and platelet activation, but it could induce side effects such as oxidative stress. The aim of this study was to investigate the effect of in-water pre-breathing oxygen, at different depths, on the oxidative status and intracellular calcium ($[Ca^{2+}]i$) of peripheral blood lymphocytes isolated from six divers. They participated in a 4-diving protocol. Two week recovery time was allowed between successive dives. Before diving, all divers, for 20 min, breathed normally at sea level (dive 1), 100% oxygen at sea level (dive 2), 100% oxygen at 6 msw (dive 3), 100% oxygen at 12 msw (dive 4). Then they dived to 30 msw for 20 min with air tank.

Methods: Blood samples were collected before and after each dive. Hydrogen peroxide (H_2O_2) levels, catalase (CAT) activity, mRNA expression of CAT, glutathione peroxidase (GPx) and superoxide dismutase (SOD), and the $[Ca^{2+}]i$ in lymphocytes were measured.

Results: The dives slightly decreased lymphocyte number and significantly reduced lymphocyte H_2O_2 levels. CAT activity was higher after scuba diving and, dive 3 enhanced mRNA gene expression of CAT, GPx and SOD. The $[Ca^{2+}]i$ was higher after dive 1 and 2 than pre-diving, while was maintained at pre-diving value after dive 3 and 4.

Conclusion: Our results suggest that pre-breathing oxygen, in particular at 12 msw, may enhance lymphocyte antioxidant activity and reduce reactive oxygen species levels. Pre-breathing oxygen in water may also preserve calcium homeostasis, suggesting a protective role in the physiological lymphocyte cell functions.

Keywords decompression sickness, hyperbaric oxygen, intracellular calcium, lymphocytes, oxidative stress, scuba diving.

Introduction

Oxygen inhalation therapy has been effectively used to manage many clinical conditions (Howard 2009) including decompression sickness (DCS) (Vann *et al.* 2009). Oxygen inhalation therapy can be administered at normal or at higher than atmosphere pressure, typically 2–3 ATA (202–303 kPa) with 100% of oxygen [Undersea and Hyperbaric Medical Society (UHMS), H.O.T.C. 2009]. Beneficial effect of breathing normobaric or hyperbaric oxygen (NBO or HBO respectively) prior to scuba diving has been observed to reduce bubble formation and alleviated platelet activation (Castagna *et al.* 2009, Bosco *et al.* 2010). However,

exposure to HBO and hyperoxia may increase formation of reactive oxygen species (ROS), which is in direct proportion to the increased oxygen tension. An overly produced ROS is detrimental because it significantly damages cell structures such as lipids, proteins and nucleic acids resulting in relevant alteration of health status (Wells *et al.* 2009). On the other hand, slightly increased ROS may be beneficial because ROS may also act as secondary messengers in the intracellular signal transduction as well as in a variety of cell processes in different biological systems (Droge 2002, Thom 2009).

There are few studies investigating the effect of pre-breathing oxygen on oxidative status in humans. Eken *et al.* reported that HBO treatment did not cause significant changes on erythrocyte antioxidant capacity and lipid peroxidation (Eken *et al.* 2005). On the contrary, other studies showed that HBO treatment induced the increase of the antioxidant defences in blood cells counteracting possible ROS-induced macromolecular damage (Dennog *et al.* 1999, Larbi *et al.* 2007). In addition, Ferrer and his colleagues have shown that HBO treatment, in simulated hyperbaric environment, increased lymphocyte capabilities to produce ROS, but also enhanced the antioxidant defence to avoid oxidative cell damage (Ferrer *et al.* 2007). These results support the hypothesis, reported above, that ROS can also act as secondary messengers in the intracellular signal transduction. Among ROS, hydrogen peroxide (H_2O_2) best fulfils the requirements of being a second messenger. Its enzymatic production and degradation, along with the requirements for the oxidation of thiols by H_2O_2, provide the specificity for time and place that are required in signalling. The ROS signalling pathways have been linked to cytoplasmic Ca^{2+} variations (Fanò *et al.* 2001, Camello-Almaraz *et al.* 2006, Feissner *et al.* 2009, Morabito *et al.* 2010). In fact ROS, such as H_2O_2, may induce transient variation in intracellular calcium concentration ($[Ca^{2+}]i$), consequently, influencing many processes including immune response (Redondo *et al.* 2004b, Dunstan *et al.* 2006, Arranz *et al.* 2009). The ROS-dependent $[Ca^{2+}]i$ variations, have been attributed to the ion mobilization from intracellular stores (Wang *et al.* 1999, Davidson & Duchen 2006) or/and to the influx of extracellular Ca^{2+} through plasma membrane Ca^{2+} channels (Bejarano *et al.* 2007, Belia *et al.* 2009). This processes could induce the calcium waves and, the frequency and time development of the waves may modulate the cell functional status (Zhu *et al.* 2008). We previously showed that pre-breathing either NBO or HBO significantly reduced the decompression-induced air bubbles and alleviated the platelet activation in scuba divers (Bosco *et al.* 2010). However, the effect of pre-breathing oxygen at different depths on the ROS production and antioxidant system function in blood cells has not yet been investigated.

Considering these data, the main goal of this study was to investigate the effect of in-water pre-breathing oxygen, at different depths, on the oxidative status and intracellular calcium of peripheral blood lymphocytes isolated from six divers.

For this reason the experimental plan was designed to perform a 4-diving protocol in different conditions in order to assay H_2O_2 production and antioxidant enzymes in lymphocytes immediately following the diving. The effect of pre-breathing oxygen on $[Ca^{2+}]i$ was also examined to explore possible linkage between calcium homeostasis and ROS.

Material and methods

Study subjects

Six healthy, male, well-trained recreational divers were enrolled in the study and their anthropometric characteristics are summarized in Table 1. Written consent was obtained from each participant, and the study was designed in accordance with the recommendations of the Declaration of Helsinki and approved by the Ethics Committee of the 'G. d'Annunzio' University of Chieti-Pescara, Italy. All divers did not consume medications and fly 48 h prior to each immersion.

Experimental protocol

The experiment was conducted in open sea located in Tremiti Island, Italy, with a water temperature of

Table 1 Anthropometric characteristics of the subjects

Subjects	Age	Weight (kg)	Height (cm)	BMI
1	41	69.3	175	26.3
2	48	86.3	176	27.86
3	21	74.6	188	21.11
4	33	71.2	171	24.35
5	51	106.7	176	34.45
6	35	101.8	177	32.49
Mean ± SD	38.2 ± 11.0	85.0 ± 16.1	177 ± 6	27.1 ± 5.4

BMI, body mass index.

20 ± 5 °C. The depth of dive was set for 30 msw (4 ATA) with 20 min of bottom time. All divers breathed compressed air (N_2O_2) from self contained underwater breathing apparatus (scuba) breathing gear during their bottom time.

The diving protocol is depicted in Figure 1.

The study protocol included 4 diving exposures with a 2 week recovery time prior to each successive dive. On dive 1, before diving, all subjects were asked to breathe air for 20 min on the surface of the sea (Air). On dive 2, before diving, all divers were asked to breathe 100% oxygen for 20 min on the surface of the sea (NBO). On dive 3, before diving, all divers were asked to breathe 100% oxygen for 20 min while submerged 6 m below the surface of the sea (HBO 1.6 ATA). On dive 4, before diving, all divers were asked to breathe 100% oxygen for 20 min while submerged 12 m below the surface of the sea (HBO 2.2 ATA). O_2 was provided via a pre-filled tank. After the pre-treatment, all divers dived to 30 m and stayed for 20 min breathing from scuba breathing gear. The ascent rate was set at 10 m min^{-1} with a decompression stop at 5 m for 3 min according to US Navy Manual Diving Table. All subjects were asked to perform the same mild workload at the bottom on a bike (underwater bicycle by OKEO, Genova, Italy) with a pedalling rate of 25 rpm to ensure no difference of ventilation and gas exchange in all dives guided by Borg CR-10 scale at intensity 3 level (Borg 1982). The ascent rate was set at 10 m min^{-1} with a decompression stop at 5 m for 3 min. As soon as the divers surfaced, the venous blood samples were collected.

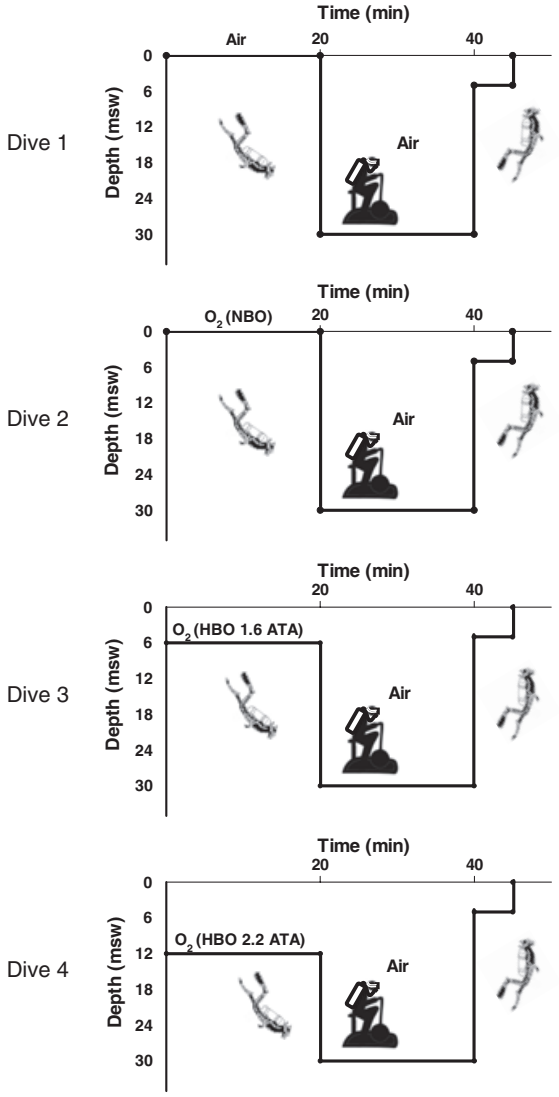

Figure 1 The study protocol. The divers pre-breathed air or oxygen at sea level (0 msw), (dive 1 and 2 respectively), or oxygen at 6 msw (dive 3) or 12 msw (dive 4) for 20 min before diving. Then they dived to 30 msw for 20 min with air. Blood samples were collected before and immediately after surfacing. NBO, normobaric oxygen; HBO, hyperbaric oxygen.

Reagents and materials

All media, sera, antibiotics and culture solutions were, if not otherwise indicated, purchased from Gibco BRL (Paisley, Scotland, UK). All sterile culture plastics were obtained from Falcon (Plymouth, UK). All other reagents were analytical grade.

Isolation of lymphocytes

Blood samples were collected in sodium-heparinised vacutainers. The lymphocytes were separated under sterile conditions on a Ficoll-Histopaque 1077 (Sigma, Milan, Italy) gradient using the method described by Belia *et al.* (Belia *et al.* 2009). Aliquots of heparinised whole blood, diluted with an equal volume of Dulbecco's phosphate buffered saline (PBS) were gently applied to an equal volume of Ficoll-Histopaque 1077 in centrifuge tubes. Samples were centrifuged at 400× ***g*** for 30 min, and the resultant interface (buffy coat) was carefully aspirated from the gradient and washed twice in Dulbecco's PBS. The cell pellet was re-suspended in RPMI 1640 medium supplemented with 10% (v/v) foetal bovine serum, 2% (w/v) L-glutamine, and 1% (w/v) penicillin/streptomycin. Monocytes were removed from the mononuclear fraction by adherence to Petri dishes during overnight incubation at 37 °C. Purified lymphocytes were re-suspended in complete RPMI 1640 medium at a density of 1–2 × 10^6 cells mL^{-1} and used in experimental analyses within 2 days after isolation. Cell viability and number was determined by Trypan blue dye Exclusion assay.

H_2O_2 levels

Hydrogen peroxide levels in lymphocytes were assayed using a colorimetric method involving the oxidation of

iodide in the presence of ammonium molybdate, with photometric analysis of the resulting blue starch–iodine complex at 570 nm (M'Bemba-Meka *et al.* 2005). Briefly, blood lymphocytes were treated with 38.5 mM HCl, 80 mM potassium iodide, 80 mM ammonium molybdate in H_2SO_4, and 0.38% (w/v) starch. Twenty minutes after adding potassium iodide, sample absorbance was measured at 570 nm using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA). The H_2O_2 concentration was estimated using a standard curve. Results are expressed as ng H_2O_2 per 1.5×10^5 cells. For each experimental condition and for each sample derived from any subject, at least five analyses were performed.

Catalase activity

Catalase (CAT) activity was measured in samples derived from sonicated lymphocytes, suspended in 20 mM sodium–phosphate buffer, pH 7.0, along with 1 μg mL^{-1} pepstatin, 1 μg mL^{-1} leupeptin and 100 μM phenylmethylsulfonyl fluoride as proteases inhibitors. Enzyme assay was carried out by the spectrophotometric method as previously described (Fanò *et al.* 2001). Briefly, the decrease in absorbance due to H_2O_2 consumption ($\epsilon = -0.04$ mM^{-1} cm^{-1}) was determined and measured at 240 nm. The final reaction volume of 1 mL contained 100 mM sodium–phosphate buffer (pH 7.0), 12 μM H_2O_2, and 100 μg of sample. Protein concentrations were determined using a Protein Assay Kit (Bio-Rad Laboratories Srl, Milan, Italy) using bovine serum albumin as a standard. For each experimental condition and for each sample derived from any subject, at least three analyses were performed.

mRNA gene expression

Total mRNA from isolated lymphocytes was purified using the standard Tri Reagent protocol (Sigma). For cDNA synthesis, 1 μg of total RNA was directly processed with the High-Capacity cDNA Archive Kit (P/N 4368814, Applied Biosystems, Foster City, CA, USA). The reactions were incubated in a GeneAmp PCR System 9700 (Applied Biosystems) at 25 °C for 10 min, 37 °C for 2 h, 85 °C for 5 s and then at 4 °C. The sample-specific PCR mix is loaded into a Micro Fluidic Card. The Micro Fluidic Card is run on the 7900HT system, where AmpliTaq Gold® DNA polymerase amplifies the cDNA synthesized from the original total RNA sample. Real-time PCR was performed for the relative quantitation of SOD1 (Hs00916176_m1, Applied Biosystems), GPX1 (Hs00829989_gH, Applied Biosystems) and CAT (Hs00156308_m1, Applied Biosystems) gene expression vs. GAPDH (Hs99999905_m1. Applied Biosystems), by using TaqMan™ technology (Applied Biosystems) on the ABI Prism 9700HT Sequence Detection System Instrument (Applied Biosystems), connected to Sequence Detector Software (SDS version 2.0, Applied Biosystems) for collection and analysis of data. PCR was performed at 50 °C for 2 min and at 94.5 °C for 10 min and then run for 45 cycles at 97 °C for 30 s and at 59.7 °C for 1 min. The relative quantification of target gene expression was evaluated with data from SDS software using the arithmetical formula 2^{-DDCt}, according to the comparative Ct method, representing the amount of target, normalized to the GAPDH endogenous control.

Measurement of $[Ca^{2+}]i$

The intracellular calcium concentration ($[Ca^{2+}]i$) was measured using the dye Fura-2/AM (Molecular Probes), and an inverted Olympus microscope connected to a high-speed wavelength switcher (Polychrome II; Till Photonics, Germany) equipped with a 75 W stabilized Xenon lamp (Ushio, Japan) and a cooled charge-coupled device (CCD) camera (C6790 model; Hamamatsu Photonics, Hamamatsu, Japan). Isolated lymphocytes (1.0×10^5 cells mL^{-1}) were loaded in suspension with 5 μM Fura-2/AM for 30 min at 37 °C in NES supplemented with 1% (w/v) bovine serum albumin. Cells were centrifuged at 400 × g for 10 min and washed twice to remove extracellular dye. Next, cells were re-suspended in fresh NES, transferred to special-optics 96-well plates (Corning-Costar, distributed by Sigma-Aldrich, Milan, Italy) coated with poly-L-lysine, and maintained for 10 min at room temperature to allow adhesion before image acquisition. Fura-2/AM-loaded lymphocytes were sequentially and repetitively excited at 340 and 380 nm; fluorescence images were acquired with a CCD camera and stored on an interfaced computer. The acquisition time was one image ratio per second. The image ratio calculations were carried out pixel-by-pixel on a pair of corresponding 340 and 380 nm image files. The temporal plots (mean value of the fluorescence signal in a selected cellular area) were calculated from the image ratios. $[Ca^{2+}]i$ in a single cellular field, recorded by a $[Ca^{2+}]$ calibration plot of the 340/380 ratio, was calculated using a Calcium Calibration Kit for video imaging (Molecular Probes, Eugene, OR, USA). For each experimental condition, and for each sample derived from any subject, at least five different wells were analyzed.

Data processing and statistical analysis

The data presented in this study were expressed as mean values ± SEM or SD, as indicated, with the exception of the RT-PCR results. The mean values were calculated considering all the repetitions (in triplicate or quintu-

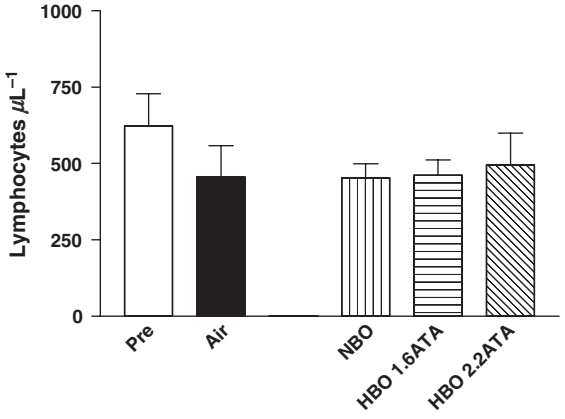

Figure 2 Effect of in-water pre-breathing oxygen at different depths on the number of *in vitro* isolated lymphocytes. The data are means ± SEM. NBO, normobaric oxygen; HBO, hyperbaric oxygen.

plicate) of the sample from each of the six divers in each experimental condition. Statistical analyses, where indicated, were performed using GRAPHPAD PRISM 4 software (GRAPHPAD Software, San Diego, CA). Comparisons between groups were made using *t*-tests and $P < 0.05$ was considered statistically significant.

Results

The effect of in-water pre-breathing oxygen at different depths on in vitro *isolated lymphocyte number*

The first result emerged from *in vitro* lymphocyte isolation. As shown in Figure 2, there was a trend in reduction of *in vitro* isolated lymphocytes after all scuba dives than in pre-diving conditions.

The effect of in-water pre-breathing oxygen at different depths on lymphocyte oxidative status

Exposure to NBO or HBO supplementation induced an increase in the PO_2 which in turn could lead to increase the formation of ROS with a consequent activation of antioxidant defences also in lymphocytes (Ferrer *et al.* 2007). For this reason, in isolated lymphocytes we tested the intracellular levels of H_2O_2, the activity and/or gene expression of antioxidant enzymes to give a picture of the cell oxidative status in our experimental conditions.

The levels of H_2O_2 in lymphocytes were significantly lower immediately after all scuba dives in respect to pre-immersion (Pre) (Fig. 3a). There was no significant difference in the levels of H_2O_2 among pre-breathing with air (Air), oxygen at surface (NBO) or at 6 msw (HBO 1.6 ATA). The levels of H_2O_2 in lymphocytes were the lowest after pre-breathing oxygen at 12 msw (HBO 2.2 ATA).

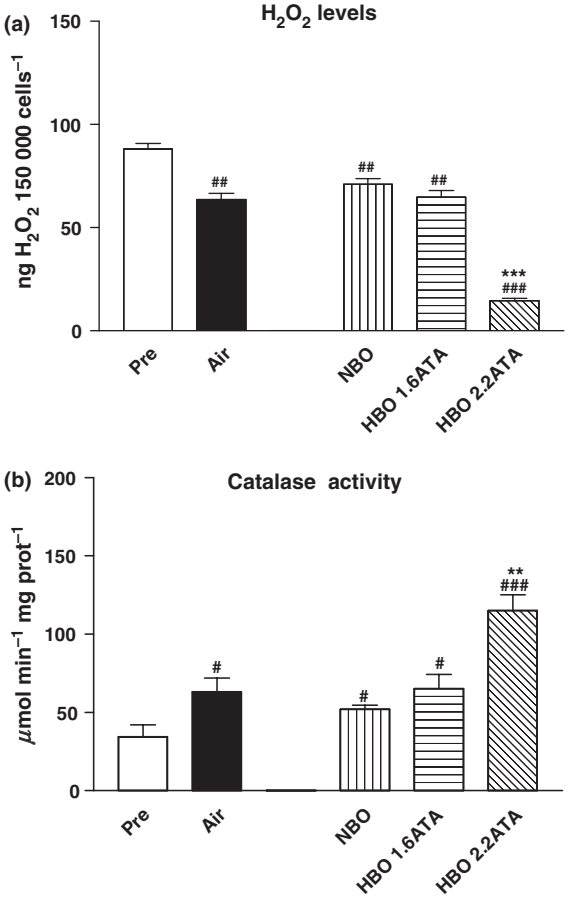

Figure 3 Effect of in-water pre-breathing oxygen at different depths on the oxidative status of lymphocytes. Panel (a): H_2O_2 levels. Pre is referred to pre-dive condition, air to pre-breathing air, NBO to pre-breathing oxygen at sea level (1 ATA), HBO 1.6 ATA to pre-breathing oxygen at 6 msw and HBO 2.2 ATA to pre-breathing oxygen at 12 msw. The data are means ± SEM. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. Pre; $^{***}P < 0.001$ vs. Air, NBO and HBO 1.6 ATA. Panel (b): Catalase activity in lymphocytes. The data are means ± SEM. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ vs. Pre; $^{**}P < 0.01$ vs. Air, NBO or HBO 1.6 ATA. NBO, normobaric oxygen; HBO, hyperbaric oxygen.

The activity of CAT, one of the main H_2O_2 detoxifying intracellular enzyme, in lymphocytes was significantly higher immediately after all scuba dives in comparison with pre-dive value (Fig. 3b). There was no significant difference in CAT activity among pre-breathing air, NBO or HBO (1.6 ATA). The activity of CAT was the highest after HBO at 12 msw (2.2 ATA) (Fig. 3b).

In isolated lymphocytes, we also tested the gene expression of the different cellular antioxidant enzymes directly or indirectly related to scavenging of H_2O_2. Figure 4 shows the relative quantification of gene expression of CAT, glutathione peroxidase (GPx1)

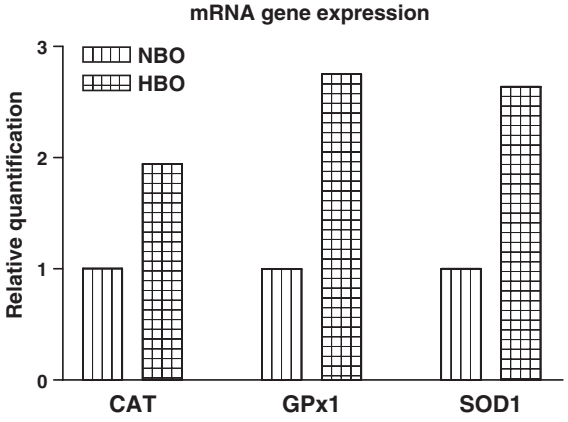

Figure 4 Effect of in-water pre-breathing oxygen at different depths on mRNA gene expression of antioxidant enzymes. The graph shows the relative quantification of CAT, GP × 1 and SOD1 gene expression on isolated lymphocytes in pre-breathing oxygen at sea level (NBO) or 6 msw (HBO). Data are representative of three experiments. NBO, normobaric oxygen; HBO, hyperbaric oxygen; CAT, catalase; GPx, glutathione peroxidase; SOD, superoxide dismutase.

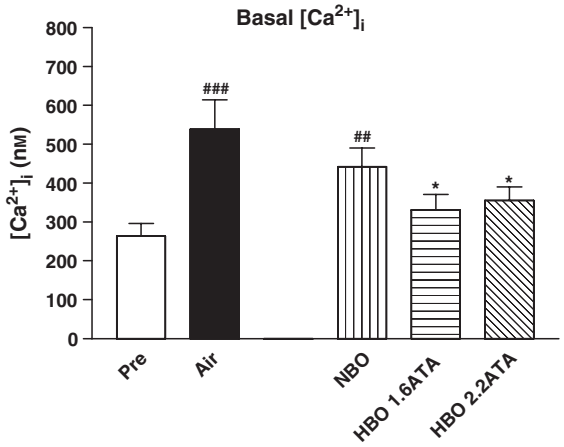

Figure 5 Basal intracellular Ca^{2+} levels in isolated lymphocytes. The graph shows the basal $[Ca^{2+}]$i values (mean ± SEM) in the tested cells from different conditions. Pre, n = 165 (where n is the number of tested cells from six scuba divers); Air, n = 154; NBO, n = 232; HBO 1.6 ATA, n = 131; HBO 2.2 ATA, n = 121. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. Pre; $^{*}P < 0.05$ vs. Air. NBO, normobaric oxygen; HBO, hyperbaric oxygen.

and superoxide dismutase (SOD1) from isolated lymphocytes. HBO (6 msw, 1.6 ATA) condition enhanced enzymes' gene expression in respect to NBO protocol (Fig. 4)

In-water pre-breathing oxygen at different depths on $[Ca^{2+}]i$ in lymphocytes

In many biological systems, ROS have been linked to the regulation of intracellular Ca^{2+} signalling in lymphocytes (Singh *et al.* 2005, Giambelluca & Gende 2008, Belia *et al.* 2009). To determine whether $[Ca^{2+}]$i was altered in lymphocytes from divers after different scuba diving protocol, Ca^{2+} concentration was measured. Video-imaging experiments on single cell revealed that pre-breathing air and oxygen at the surface (NBO) caused significantly higher mean basal $[Ca^{2+}]$i than pre-dive (Pre) value as shown in Figure 5. Pre-breathing oxygen at 6 msw and 12 msw (HBO 1.6 ATA and 2.2 ATA respectively) effectively prevented scuba diving-induced increase in $[Ca^{2+}]$i (Fig. 5). Cell activity was characterized not only by the basal intracellular calcium concentration, but also by calcium mobilization that was also revealed by intracellular ionic variations. Quantitative analyses of video-imaging time lapse experiments showed that the spontaneous intracellular Ca^{2+} oscillations were recorded in about 37 ± 9% of lymphocyte population before diving (Fig. 6). This value significantly decreased after air-pre-breathing scuba diving. The spontaneous Ca^{2+} variations in lymphocytes after NBO and HBO dives remained higher in comparison to air-breathing condition and unchanged in comparison to pre-diving condition (Fig. 6).

We tested also the cell susceptibility to sodium nitroprusside (SNP), a nitric oxide (NO) donor also considered as an oxidant. The addition of SNP, induced an increase in calcium concentration in lymphocytes (14 ± 4% of cell population) before diving (Fig. 7). The percentage of cell sensitive to SNP increased to 40 ± 8% immediately after scuba diving with air-pre-breathing (Fig. 7). Pre-breathing with NBO or HBO showed a SNP-responsive cell percentage similar to that observed in pre-dive lymphocytes (Fig. 7).

Discussion

The effects of in-water pre-breathing oxygen at different depths on oxidative stress and calcium concentration in lymphocytes from scuba divers were investigated in the present study. The main findings are that (i) there was a trend in reduction of isolated lymphocytes after the dives; (ii) the dives significantly enhanced CAT activity and decreased the levels of H_2O_2, in isolated lymphocytes; (iii) the relative quantification of mRNA gene expression of antioxidant enzymes (CAT, GPx1, SOD1) was enhanced in isolated lymphocytes after pre-breathing oxygen at 6 msw than on the surface; (iv) pre-breathing air or NBO significantly increased the basal calcium concentration and pre-breathing HBO effectively prevented this increase; (v) spontaneous intracellular Ca^{2+} oscillations decreased in lymphocytes after scuba diving and remained at the pre-dive value after

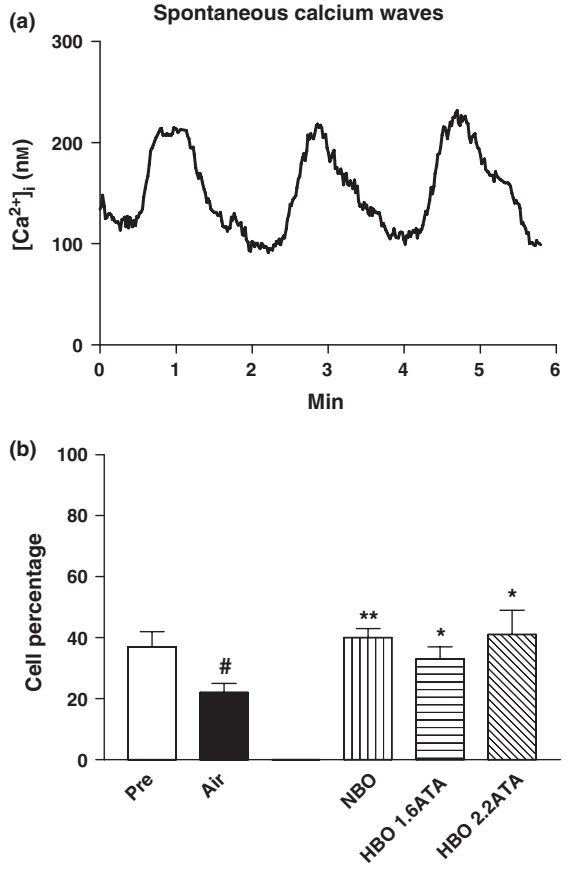

Figure 6 Spontaneous calcium waves in isolated lymphocytes. In panel (a) the trace depicts the spontaneous activity of a representative single cell. Panel (b) reports cell percentages (means ± SEM) showing calcium waves in the tested cells from different conditions. Pre, n = 165 (where n is the number of tested cells from six scuba divers); Air, n = 154; NBO, n = 232; HBO 1.6 ATA, n = 131; HBO 2.2 ATA, n = 121. $^{\#}P < 0.05$ vs. Pre-dive; $^{*}P < 0.05$ and $^{**}P < 0.01$ vs. Air. NBO, normobaric oxygen; HBO, hyperbaric oxygen.

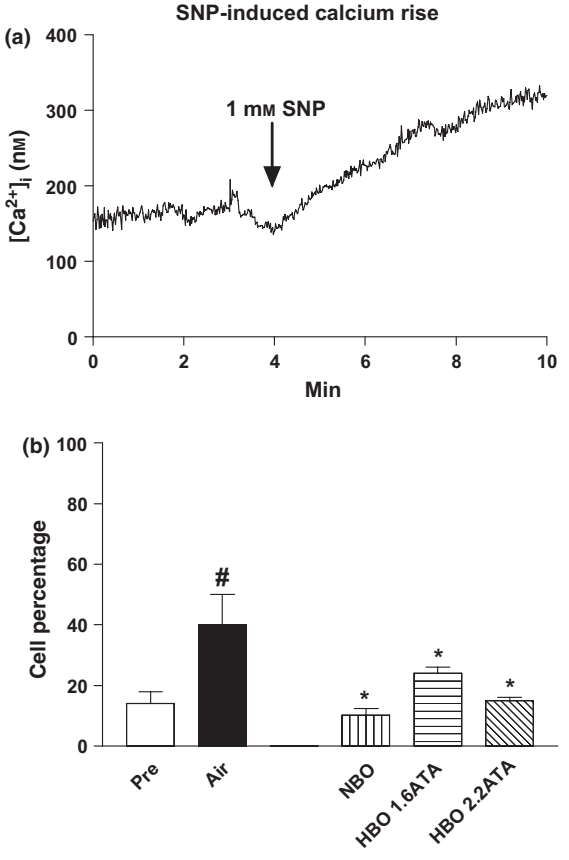

Figure 7 Cell susceptibility to sodium nitroprusside (SNP). In panel (a) the trace depicts a representative SNP calcium rise in a single cell. Panel (b) reports cell percentages (means ± SEM) showing SNP-induced calcium rise. The data are mean ± SEM in the tested cells from different conditions. Pre, n = 155 (where n is the number of tested cells from six scuba divers); Air, n = 133; NBO, n = 135; HBO 1.6 ATA, n = 137; HBO 2.2 ATA, n = 117. $^{\#}P < 0.05$ vs. Pre; $^{*}P < 0.05$ vs. Air. NBO, normobaric oxygen; HBO, hyperbaric oxygen.

pre-breathing either NBO or HBO, and (vi) air-pre-breathing significantly increased lymphocyte percentage sensitive to nitroprusside-induced calcium rise, while pre-breathing NBO and HBO showed a SNP-responsive cell percentage similar to that observed in pre-dive lymphocytes.

Lymphocytes were chosen in the present study because they offer several advantages. For cellular and molecular study, lymphocytes are easy for collection, have well characterized Ca^{2+} signalling pathways and they were considered a biosensor of physio-pathological conditions (Belia *et al.* 2009, Blandini *et al.* 2009, Mariggiò *et al.* 2010).

In addition, exercise appears to affect the quantity and function of circulating immune cells, including lymphocytes and a leukocytosis condition have been observed during or following exercise, depending on the intensity and duration of the efforts (McCarthy & Dale 1988, Natale *et al.* 2003, Tauler *et al.* 2004).

Few studies investigated the effect of a specific physical activity, as the scuba diving, on lymphocyte number. Ferrer *et al.* recently reported that there was no significant change in lymphocyte number immediately after scuba diving at 40 msw (5 ATA) for 10 min. They also observed that the number of lymphocytes did not change when detected 30 min after dry HBO exposure at 2.2 ATA for 60 min, suggesting that hyperbaric condition alone may not affect the lymphocyte number (Ferrer *et al.* 2007).

In the present study, the scuba divers dived to 30 msw (4 ATA) for 20 min. During the bottom stay, all divers were performing the same mild workload on a bike. There was no significant change in lymphocyte number after diving. Our data conform Ferrer *et al.*'s study and

further showed that, pre-breathing oxygen at 6 msw (1.6 ATA) and 12 msw (2.2 ATA) did not significantly affect the number of isolated lymphocytes that could be considered indirectly related to the circulating ones.

This effect could be because of the regular mild workload to which the divers were subjected considering that water immersion induced haemodynamic, neuroendocrine, and autonomic activity changes (Pendergast & Lundgren 2009) responsible for possible mechanisms underlying exercise-induced changes in lymphocyte number.

Physical activities increase oxygen consumption and, consequently, the production of ROS at a rate determined by the intensity, frequency, and duration of the exercise protocols (Dennog *et al.* 1999). ROS are generated as natural products of metabolism and they include superoxide, H_2O_2, hypochlorous acid and hydroxyl radical (Turrens 2003). Increased free radical formation during exercise is often coupled with an increased antioxidant defences to reduce oxidative stress. The antioxidant enzyme activity of tissue affected by exercise represents the primary endogenous defence against ROS and involves the cooperative action of the three main intracellular antioxidant enzymes such as SOD, CAT and GPx (Homi *et al.* 2002, Bloomer & Goldfarb 2004, Fulle *et al.* 2004). However, oxidative stress may occur while an imbalance between pro-oxidants and antioxidants occurs when oxidant production overwhelms the cellular antioxidant capacity (Bloomer & Goldfarb 2004). Though, scuba diving did not affect the number of lymphocytes, it may affect the function of lymphocytes. Ferrer *et al.* (Ferrer *et al.* 2007), analyzed lymphocyte oxidative status after immersions at a depth of 40 msw for 10 min or at rest condition in a hyperbaric chamber at the same barometric value. They found that, immediately after diving, there was no change in H_2O_2 production in lymphocytes and GPx and CAT activities increased. After HBO treatment only an increased GPx activity was observed. In the present study, a significantly decreased levels of H_2O_2 in lymphocytes were observed after scuba diving. These could be due, at least in part, to the different experimental conditions. The concentration of H_2O_2 detected *in vivo* depends on its formation and degradation. Decreased H_2O_2 levels, after scuba diving, could be due to an unbalance of its metabolic system. It is well known that regular exercise enhances antioxidant activity (Wang & Huang 2005). However, Sureda *et al.* recently reported that only high intensive exercise induces lymphocyte oxidative damage, on the other hand, low to moderate intensity exercise is beneficial because it enhances intracellular antioxidant activity and therefore, counteracts ROS production (Sureda *et al.* 2009). We speculate that decreased H_2O_2 levels observed in this study after scuba diving could be due to significantly enhanced CAT activity. In addition, the antioxidant enzyme activation was also strictly supported by the increased mRNA gene expression of CAT, SOD1 and GPx1 after HBO condition.

Our study suggests that pre-breathing oxygen, hyperbaric being more effective than normobaric, enhances antioxidant enzymes, which may be, at least in part, responsible for the reduced levels of H_2O_2 during scuba diving.

ROS have been hypothesized to be either harmful or beneficial to living systems depending on their quantity (Valko *et al.* 2007). Low to moderate concentrations of ROS may be considered second messengers involved in cellular response to physiological stimuli and pathological ravages. Intracellularly, there is a complex cross-talking between ROS and other systems. In particular intracellular calcium handling is also regulated by the redox status of cellular thiols which can modulate intracellular calcium distribution (Belia *et al.* 2009).

HBO has also been reported to modify intracellular Ca^{2+} concentration of endothelial and smooth muscle cells (Liu *et al.* 1999). Our study shows that scuba diving provoked lymphocyte activation demonstrated by the induction of a significantly modified intracellular Ca^{2+} mobilization with the increased basal intracellular Ca^{2+} concentration and the decreased spontaneous cell activity. Activated lymphocytes presented a higher susceptibility to a stressor stimulus like SNP which induces NO release involving the modulation of intracellular calcium mobilization as in other models (Nagy *et al.* 2003, Sharma *et al.* 2003, Otton *et al.* 2007). In the periphery, NO is primarily the endothelially derived mediator of vasodilation and a regulator and effector of the immune response. A possible crosslink exists between intracellular calcium mobilization and NO release. Sharma and colleagues (Sharma *et al.* 2003) showed that in coronary blood samples, cardiac ischaemia and further reperfusion resulted in significant higher NO levels and intracellular calcium concentration in lymphocytes, consequently, it leads to a release of an array of pro-inflammatory mediators. Under our experimental conditions, although we did not measure NO, SNP-induced increase in $[Ca^{2+}]i$ provides indirect evidence to support the existence of a link between NO production and calcium mobilization.

From our results, we can speculate that pre-breathing NBO and HBO reduced scuba diving-induced increase in $[Ca^{2+}]i$ and alleviate SNP-induced susceptibility to oxidative stress in lymphocytes. These data suggest that oxygen inhalational therapy may, via modifying intracellular signal system/s, influence the antioxidant response in scuba divers.

Sudden and rapid changes of barometric pressure during decompression induce different potentially dangerous effects in the blood such as air bubble formation,

leucocyte and complement activation, and platelet aggregation (DeGorordo *et al.* 2003, Wang *et al.* 2003, Bosco *et al.* 2010). A cross-talking may exist between blood white cells and platelets. Under pathophysiological conditions, such as thrombosis, inflammation, immune response, atherosclerosis, platelets influence lymphocyte functions via direct cell-cell contact and/or diffusible factors (Li 2008). It has been demonstrated that platelet aggregation is also dependent on their oxidative status. Platelet aggregation can also be induced by a burst of H_2O_2 production that in turn provokes a rise in intracellular calcium and actin reorganization (Redondo *et al.* 2004a,b). Our study suggests that reduction in H_2O_2 levels in lymphocytes, following pre-breathing oxygen, could be one of the intercellular mechanism/s able to counteract platelet activation.

In summary, pre-breathing either NBO or HBO before scuba diving may be beneficial in open sea scuba divers. Pre-breathing HBO seems more effective than NBO in enhancing antioxidant defences as evidenced by: (i) the increased antioxidant CAT enzyme activity; (ii) the enhanced mRNA gene expressions of CAT, SOD1 and GPx1; and (iii) the prevention in the increase of intracellular Ca^{2+} concentration, consequently, decreasing oxidative stress. In addition, the present study suggests that pre-breathing oxygen at the various depth of the sea water may be beneficial in reducing the development of DCS.

We wish to thank the scuba divers who enthusiastically participated in this study and the Company OKEO aqua fitness-Padova (Italy) and scuba agency NASE (Italy) for technical support. This study was supported by the Young Project 2007 sponsored by the Dept of Basic and Applied Medical Sciences, University G.d'Annunzio of Chieti-Pescara. The authors also wish to thank Dr. Danielle Masursky for her English editorial assistance.

Conflict of interest

All Authors have no conflicts of interest or financial ties to disclose.

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