ROS

RESEARCH ARTICLES

Acute Hyperbaria and Hyperoxia Effects on Oxidative Stress Kinetic Response: A Simulated Study

A Vezzoli[1], M Gussoni[2], M Montorsi[1,3], S Moretti[1], and S Mrakic-Sposta[1]

[1]Institute of Bioimaging and Molecular Physiology, National Council of Research (CNR), Segrate (Milano), Italy; [2]Institute for Macromolecular Studies, National Council of Research (CNR), Milan, Italy; [3]Department of Human Sciences and Promotion of the Quality of Life, San Raffaele Roma Open University, Milan, Italy

Correspondence: simona.mrakic@ibfm.cnr.it (S.M-S.)

Vezzoli A et al. Reactive Oxygen Species 4(10):290–297, 2017; ©2017 Cell Med Press
http://dx.doi.org/10.20455/ros.2017.845
(Received: March 23, 2017; Revised: May 12, 2017; Accepted: May 12, 2017)

ABSTRACT | This study aims to investigate the effects hyperoxia/hyperbaria on both oxidative stress and antioxidant response. To this purpose two different protocols were performed at rest conditions: (1) fifteen male divers (age of 28.9 ± 5.3 years) simulated immersions at resting conditions in a hyperbaric chamber (HBO) at a depth of 40 m seawater for 30 min; (2) nine healthy male subjects (age of 25.3 ± 1.2 years) breathed pure oxygen for 30 min. Oxidative stress biomarkers in the plasma were monitored before and during the 3 h after the exposure. Total antioxidant capacity significantly decreased immediately at the end of both HBO and pure oxygen exposures (–19% and –16%, respectively; $p < 0.001$), and thereafter increased during the recovery period. Concentrations of protein carbonyls, assessed as a marker of protein oxidation, significantly increased after both HBO (+52%, $p < 0.001$) and pure oxygen (+65%, $p < 0.05$) exposures, but following different kinetic pathways. A significant increase (+21%; $p < 0.0001$) in thiobarbituric acid-reactive substances, assessed as a marker of lipid peroxidation, was found only after HBO, attaining the maximum concentration at about 1 h after the end of exposure. The values of all the examined parameters returned back to the basal levels within 3 h of the recovery phase. HBO exposure probably resulted in a more pronounced ROS formation, and this can be explained by taking into account that ROS leakage by mitochondria is believed to increase in direct proportion to the oxygen pressure rise.

KEYWORDS | Hyperbaric oxygen; Hyperoxia; Oxidative stress; Simulated immersion; Total antioxidant capacity

ABBREVIATIONS | HBO, hyperbaric chamber; HO, hyperoxia; MDA, malondialdehyde; 8-OH-dG, 8-hydroxyl-2′-deoxyguanosine; PC, protein carbonyl; ROS, reactive oxygen species; TABRS, thiobarbituric acid-reactive substances; TAC, total antioxidant capacity

CONTENTS

1. INTRODUCTION

Oxygen is required to sustain life, but too much oxygen is toxic due to excessive production and accumulation of reactive oxygen species (ROS) which are normally generated in human cells during aerobic metabolism [1]. Hyperoxia is a popular model of oxidative stress [2], however, routinely, but prudently, used in clinical (hyperbaric chamber, high-dose O_2 to treat or prevent hypoxemia and tissue hypoxia) and nonclinical (scuba diving) settings.

Scuba diving is characterized by hyperoxia resulting from hyperbaric exposure during diving and oxygen availability due to high pressure. The combination of hyperbaria and physical activity present in scuba diving was reported to lead to an oxidative stress condition [3]. In comparison with dry hyperbaric chambers, the physiological stress of scuba diving is greater because of additional factors such as immersion, exercise, and cold water. Nevertheless, the simple exposure to hyperbaric oxygen (HBO), by increasing oxidative burst, seems to be enough to lead to an increased ROS formation able to produce cellular damage [4], such as lipid peroxidation as well as protein and DNA oxidation [5]. The cellular response to HBO-induced oxidative stress has been mainly investigated in animal models [6], while only a few human studies are reported [7].

HBO leads to an increase of dissolved oxygen in the blood, which is the reason why it has been successfully used as an adjunctive therapy for several pathophysiological conditions, including decompression sickness, acute carbon monoxide intoxication, soft tissue infections, and radiation necrosis [8]. However, the increased pressure could also facilitate free radical production so that tissue damage may occur.

Breathing pure oxygen at a concentration greater than 21%, the normal percentage present in the air at sea level, also increases ROS production, and the severity of oxygen toxicity increases with increasing the inspired pO_2 and the exposure duration. In hyperoxia (HO), multiple signalling pathways determine a pulmonary cellular response: apoptosis, necrosis, or repair. Therefore, understanding the effects of oxygen exposure is important to prevent inadvertent oxidative damage caused by hyperoxia in patients requiring supplemental oxygenation.

The aim of the present study was to test in humans how a short period (30 min) of hyperbaric hyperoxia would influence oxidative stress generation. To evaluate the contribution of different components in the response to hyperbaric exposure, some oxidative stress indices as well as plasma antioxidant levels were separately measured in the blood samples collected during both hyperbaric exposure in a pressure chamber (HBO) and normobaric hyperoxic exposure sessions.

2. MATERIALS AND METHODS

2.1. Subjects and Study Design

The study was carried out following two separate protocols. Protocol 1: hyperbaric chamber (HBO); protocol 2: hyperoxia exposure (HO, see **Figure 1**). The protocol of both experiments was in accordance with the Declaration of Helsinki for research on human subjects and was approved by an institutional Ethical Committee. All subjects were informed on the purpose and demands of the study before giving their written consent to participate.

2.1.1. Protocol 1 (HBO)

Protocol 1 consisted of a simulated diving session in a hyperbaric chamber at the Hyperbaric Medicine and Intensive Care Unit, Nagar Hospital (Pantelleria, Sicily) at least two weeks after the last seawater dive.

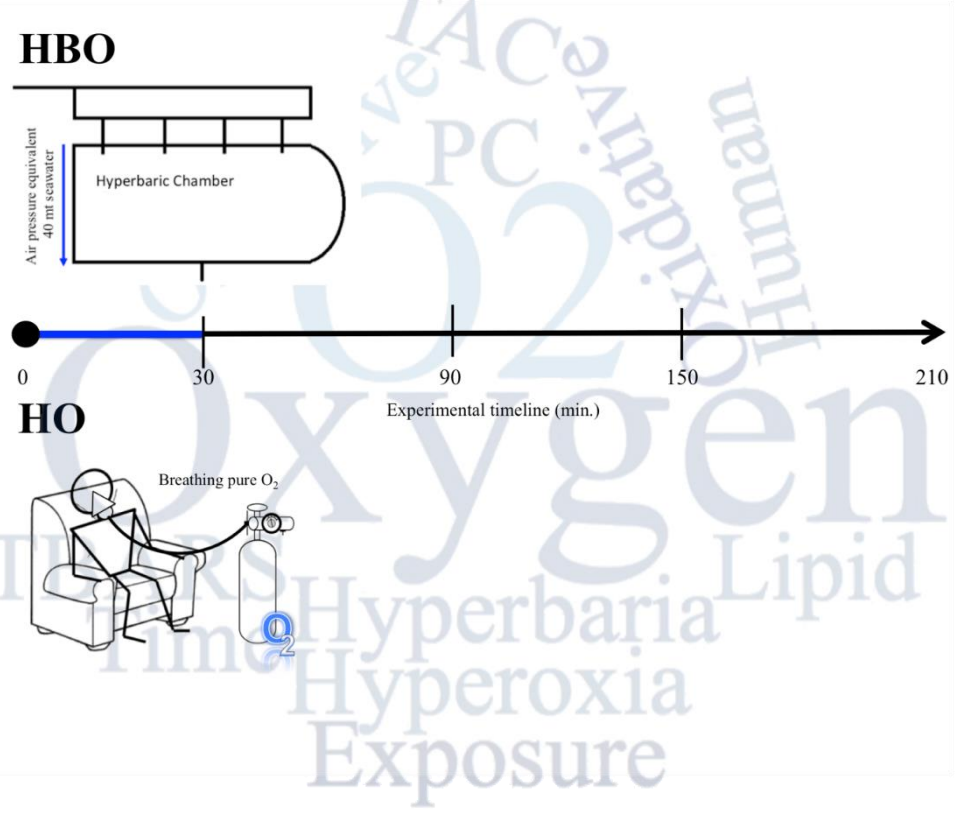


FIGURE 1. Study design: protocol 1, HBO—hyperbaric chamber (upper panel); and protocol 2, HO—exposure to hyperoxia (bottom panel). The black arrow indicates the experimental timeline (min). The blue line represents the simulated immersion (HBO) and pure oxygen breathing (HO) time (30 min).

Fifteen healthy males, aged 28.9 ± 5.3 years (mean ± standard deviation, SD), height 1.75 ± 0.08 m, weight 71.87 ± 11.19 kg, average body mass index (BMI) 23.4 ± 1.7 kg/m^2, volunteered to take part in this study. The subjects were all non-smoker scuba diving professionals. All participants, at the time of the study, had a valid medical certificate for diving. The test was administered in a dry hyperbaric chamber on air compressed to a pressure equivalent to 40 meters depth of seawater. Divers performed the simulated immersion with a descent rate of 8 m/min, 30 min rest at 40 m and ascent rate to decompression depths of 10 m/min for a total time of 40 min in which they breathed atmospheric air. Subjects were exposed to 100% oxygen at a pressure of 2.2 atmospheres absolute (ATA). The chamber air temperature was maintained at 24–26°C.

2.1.2. Protocol 2 (HO)

The protocol was designed to investigate whether hyperoxia alone is able to induce scuba diving-associated changes in oxidative stress. Nine healthy physically active non-smokers male subjects aged 25.3 ± 1.2 years, height 1.79 ± 0.04 m, weight 79.89 ± 9.67 kg, average body mass index (BMI) 24.9 ± 2.8 kg/m^2, volunteered to take part in this study where they were exposed up to 30 min at greater than 99% normobaric oxygen at rest.

2.2. Experimental Procedure

Subjects were instructed to refrain from strenuous exercise and to ingest their habitual diet during the 48 h preceding each experimental session. All of

them did not take any antioxidant dietary supplementation or routine medication for at least one month before the study.

A catheter was inserted into forearm vein and was kept patent by flushing with normal saline. Each subject had 3 ml of blood drawn, collected in heparinized vacutainer tubes, and centrifuged at 1000 *g* for 10 min at 4°C to obtain the plasma. Venous blood samples were drawn at rest before the simulated diving session or hyperoxia exposure (basal sample) and immediately and at 1, 2, and 3 h. The plasma samples were then immediately stored in multiple aliquots at –80°C until assayed.

Urine samples were collected at rest and at 3 h. Aliquots were stored at –80°C until analysed. Samples were thawed only for the analyses, which were performed within two weeks from the collection.

Antioxidant in both hydrophilic and lipophilic plasma compartments are actively involved as a defence system against ROS, which are continuously generated. Because of the difficulty in measuring each antioxidant component separately and interactions among antioxidants, methods have been developed to assess the total antioxidant status of the serum or plasma. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox)-equivalent total antioxidant capacity (TAC) assay, a widely used kit-based commercial method (Cayman Chemical, Ann Arbor, USA) was used and the reaction mixtures were incubated for 3 min at room temperature and then read by an Infinite M200 microplate reader spectrophotometer (Tecam, Austria). A linear calibration curve was built up from pure Trolox-containing solutions.

In the plasma, the major radical damage comes from lipid peroxidation. This process leads to reactive intermediates formation such as malondialdehyde (MDA) and carbonyl compounds. The assessment of thiobarbituric acid-reactive substances (TBARS) levels is a well-established method in order to detect lipid peroxidation. A TBARS assay kit (Cayman Chemical), which allows a rapid photometric detection at 532 nm of the thiobarbituric acid malondialdehyde (TBAMDA) adduct, was used. A linear calibration curve was obtained from pure MDA-containing solutions.

The accumulation of oxidized proteins was determined by measuring the content of reactive carbonyls. A protein carbonyl assay kit (Cayman Chemical) was used to quantify colorimetrically oxidized proteins. The samples were read at 370 nm, as described in detail by the manufacturer. Obtained values were normalized to the total protein concentration in the final pellet (absorbance reading at 280 nm) in order to consider protein loss during the washing steps, as suggested by the kit's user manual.

A multitude of products is generated by the radical attack against DNA. 8-Hydroxyl-2′-deoxyguanosine (8-OH-dG) has been established as a marker for oxidative DNA damage. This compound has been quantified in excreted urine at basal level and 3 h after the exposure sessions. A commercial ELISA kit (Cayman Chemical) for the measurement of 8-OH-dG was utilized.

Urinary concentrations of 8-OH-dG, like any other urinary marker, vary considerably. As such, urinary parameters, including 8-OH-dG are usually estimated based on the amount of creatinine excreted in the urine when the collection of the 24 h urine is not possible. Indeed, in humans, in the absence of renal disease, the excretion rate of creatinine keeps relatively constant. Thus, urinary creatinine levels may be used as a standardization index. A creatinine assay kit (Cayman Chemical) was used to measure creatinine levels in the urine samples. Creatinine concentration was determined using a provided creatinine standard curve.

2.3. Statistical Analysis

Statistical analysis was performed using the GraphPad Prism package (GraphPad Prism 6, GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± SD. Experimental data were analysed using repeated Shapiro–Wilk test and compared by variance analysis, ANOVA repeated measures, with Tukey's multiple comparison test to further check the among-groups significance. A p value < 0.05 was considered statistically significant.

3. RESULTS

No significant differences were observed in the general anthropometric features of the subjects of both the evaluated groups. The basal levels of the examined biomarkers are reported in **Table 1**. No significant differences were observed in TAC, protein carbonyl (PC), and 8-OH-dG concentrations in the subjects recruited for both the experimental proto-

TABLE 1. The concentrations of oxidative damage markers as measured in the plasma (TAC, PC and TBARS) and urine (8-OH-dG) of HBO and HO subjects at rest

Condition	TAC mM	PC nmol/mg protein	TBARS µM	8-OH-dG nmol/mg creatinine
HBO (n = 15)	1.88 ± 0.20	0.73 ± 0.21	9.33 ± 1.64*	4.32 ± 1.68
HO (n = 9)	1.85 ± 0.16	0.58 ± 0.10	5.75 ± 0.97	2.45 ± 0.41

*Note: Results are reported as mean ± SD. *, significant difference ($p < 0.01$).*

cols. On the contrary, significantly higher (+62%; $p < 0.01$) TBARS values were found in the subjects belonging to the HBO group. TAC significantly decreased immediately at the end of both HBO and HO exposures (–19% and –16% respectively; $p < 0.001$), and thereafter increased during the recovery period returning back to the basal level after 3 h (**Figure 2A**).

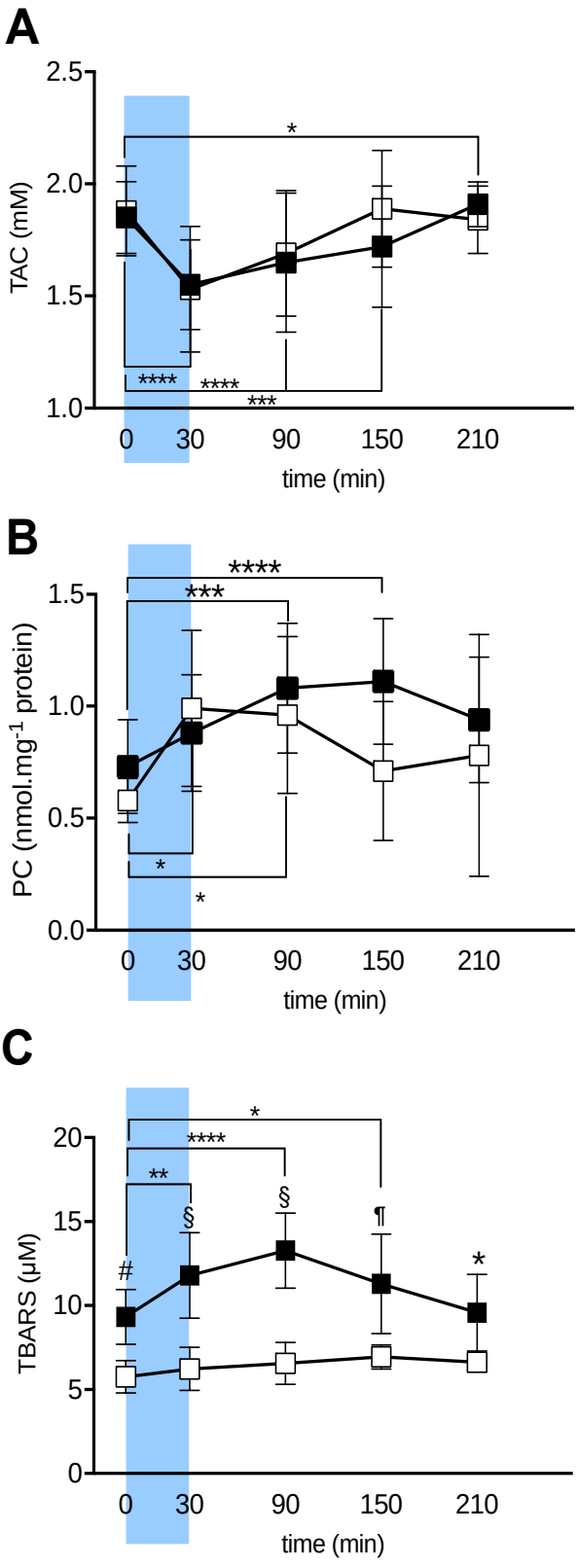
The plasma concentrations of PC, assessed as a marker of protein oxidation (**Figure 2B**), significantly increased after both HBO ($p < 0.001$) and HO ($p < 0.05$) exposures, but following different kinetic patterns, coming back to the basal levels within 3 h of recovery. In the HBO group, PC level reached its maximum 2 h after the end of exposure (+52%), then coming back to the basal level. On the other hand, in the HO group the maximum level was reached earlier, just at the end of exposure (+65%), and the return to the basal value was more rapid as well. A significant increase (+21%; $p < 0.0001$) in TBARS, assessed as a marker of lipid peroxidation (**Figure 2C**), was found only after HBO, attaining the maximum level at about 1 h after the end of exposure.

The 8-OH-dG levels obtained before and at 3 h of recovery are displayed in **Figure 3**. As shown in the figure, HBO exposure led to significantly higher (+110 %; $p<0.001$) oxidative DNA damage levels with respect to the data recorded after HO exposure.

4. DISCUSSION

Oxygen is life essential but, like a drug, it shows a maximum positive biological benefit and accompanying toxic effects. It is widely accepted that oxygen toxicity is primarily a consequence of an excess of ROS production and a subsequent failure of the antioxidant system in neutralizing the induced oxidative stress condition. Multiple oxidative stress biomarkers were monitored in the present study, considering that oxidative damage cannot currently be reliably described by a single parameter. Although the effects of HBO on oxidative stress in the blood were reported in previous studies [3–5, 7], most of them collected only a few samples after the exposure, only partially explaining the discrepancy with respect to most of the literature. In fact, the practice of the vast majority of the relevant studies to collect a single blood sample immediately after the end of the experiment could potentially lead to inaccurate conclusions. Indeed, the results shown in the present study clearly suggest that, taken as a single one, different sampling times after exposure may lead to different conclusions about the induced oxidative stress responses. In fact, non-uniform changes in the marker levels were found: both transient (i.e., TAC) and prolonged (i.e., PC, TBARS) changes were observed. As a matter of facts removal of oxidative damage biomarkers from blood is a time-consuming process. Protein oxidation is known to occur during high doses of HBO, and it may be an earlier and wider spread event than lipid peroxidation. Studies have shown that protein damage by oxygen radicals precedes the onset of lipid peroxidation and occurs independently of membrane lipid damage [9, 10].

HBO induces acute oxygen toxicity similar but not identical to that observed from breathing in normobaric hyperoxia. In normobaric hyperoxia, oxygen pressure is a tightly regulated system in providing an oxygen level just to perform normal metabolic functions, but low enough to minimize potentially dangerous ROS production [11]. During a 30-min oxygen tolerance test at sea level, characterized by 100% oxygen breathing at a partial pressure of 280 kPa (i.e. 2100 mm Hg), protein carbonyls were found to increase as well as total thiol proteins to decrease significantly, when compared to air breathing [12]. Protein degradation, generated by HO, was clearly



increased in the absence of detectable lipid peroxidation products: this was reported to occur at low ROS concentration levels [9]. Increased proteolysis was suggested to occur independently of membrane damage and to be a more sensitive indicator of cell exposure to oxygen radicals with respect to lipid peroxidation [13]. Membrane lipid peroxidation process has been reported as one of the primary events in oxidative cellular damage and shown to be associated with fine structure disturbance and subsequent function loss of biological membranes. In the present study, oxidative biomarkers production was measured when coming back to normobaric conditions. As well known, the return to normobaria and normoxia is accompanied by vascular vasodilatation so that membrane changes might have been induced by HBO exposure, persisting even after returning to normobaric conditions.

During hyperbaric hyperoxia, the body compensates the increased oxygen stimulus with a number of adaptive mechanisms. A vasoconstriction response helps to reduce the amount of oxygen delivered to the brain and tissues [14]. Although this adaptive mechanism is not present in the HO response, it might be suggested as the cause of the higher lipid peroxidation recorded after the HBO session.

Endogenous antioxidant defence systems are enhanced to fight induced oxidative stress. However, sometimes these responses are inadequate and the scales between oxidative stress condition and cellular protection are tipped, as shown in the present study by the TAC level decrease recorded just after the end of both HO and HBO sessions. Plasma TAC increase

FIGURE 2. Time course of (A) total antioxidant capacity (TAC), (B) protein carbonyls (PC), and (C) thiobarbituric acid-reactive substances (TBARS) concentrations in HBO (full squares) and HO (empty squares) groups measured before (0 min), immediately after the experimental test (30 min), and after 1, 2, and 3 h of recovery (90, 150 and 210 min from t_0, respectively). Data are expressed as mean ± SD. Compared to rest, changes over time were significant at: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; and ****, $p < 0.0001$. Differences in changes between groups (HBO versus HO) are indicated by: *, $p < 0.05$; #, $p < 0.01$; §, $p < 0.001$; and ¶, $p < 0.001$.

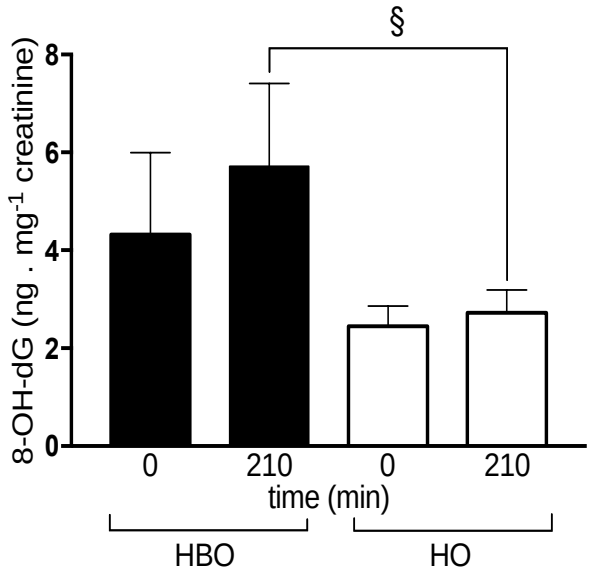


FIGURE 3. 8-Hydroxy-2′-deoxyguanosine (8-OH-dG, ng/mg creatinine) concentrations in the HBO (full bars) and HO (empty bars) groups at 0 and 210 min. Results are expressed as mean ± SD; §, significant difference ($p < 0.001$).

recorded later in the recovery period suggested that HBO and HO exposure activate the body's antioxidant defenses. Mobilization of tissue antioxidant stores into the plasma [15], is probably one of the mechanisms responsible for TAC increase after both HBO and HO exposures. This is a widely accepted phenomenon that would help maintain or even increase plasma antioxidant status in times of need [16].

HBO group showed high levels of DNA damage after exposure, when compared to the subjects of the other examined group. A similar finding after HBO exposure was previously reported [17].

5. LIMITATIONS

The presence of increased TBARS levels found in the subjects belonging to the HBO group was previously observed in patients exposed to consecutive HBO treatment (1 session/day) according to a routine therapy protocol [8]. Indeed, because of their activity (diving), the HBO subjects were exposed to repeated hyperbaric-hyperoxic events. Thereafter physiological exposure to scuba diving led to a permanent oxidative stress condition. The selected subjects' sample and the stressor condition (depth) might limit the generalization of the study. However, dry chamber exposure would not be wise for sedentary or non-trained people because of the associated physiological stress.

Although many markers are available to assess oxidative stress, in general, every assay shows advantages and disadvantages. For example, there are some doubts about the validity of the TBARS assay in detecting lipid peroxidation in vivo, because the assay is not specific to malondialdehyde, and lipid peroxidation is not the exclusive source of malondialdehyde [18]. However, in accordance with previously reported data [8], the plasma accumulation after HBO exposure was so significant that, in the present context, data interpretation did not appear to lead to particular problems.

6. CONCLUSIONS

In conclusion, short (30 min) hyperbaric hyperoxic (at a depth of 40 m) or hyperoxic exposures were enough to induce changes in the antioxidant status and oxidative stress. The combination of hyperbaria and hyperoxia, present in a hyperbaric chamber, led to a condition of oxidative stress greater than that generated by hyperoxia exposure. HBO exposure probably resulted in a more pronounced ROS formation. This can be explained by taking into account that ROS leakage by mitochondria is believed to increase in direct proportion to the oxygen pressure rise [19]

ACKNOWLEDGMENTS

The authors are grateful to all the athletes and subjects who participated in the experimentation. The authors thank Dr Jamil Sadeeh of Hyperbaric Medicine and Intensive Care Unit, Nagar Hospital (Pantelleria, Sicilia), Dr Claudio Marconi, Dr Mauro Marzorati, and Dr Simone Porcelli for the collaboration, and the Diving Center Cala Levante Pantelleria, Trapani for organizing contribution.The authors declare no conflicts of interest for the work described in this manuscript.

REFERENCES

1. Mrakic-Sposta S, Gussoni M, Montorsi M, Porcelli S, Vezzoli A. Assessment of a standardized ROS production profile in humans by electron paramagnetic resonance. *Oxid Med Cell Longev* 2012; 2012:973927. doi: 10.1155/2012/973927.
2. Dean JB, Mulkey DK, Henderson RA, 3rd, Potter SJ, Putnam RW. Hyperoxia, reactive oxygen species, and hyperventilation: oxygen sensitivity of brain stem neurons. *J Appl Physiol (1985)* 2004; 96(2):784–91. doi: 10.1152/japplphysiol.00892.2003.
3. Ferrer MD, Sureda A, Batle JM, Tauler P, Tur JA, Pons A. Scuba diving enhances endogenous antioxidant defenses in lymphocytes and neutrophils. *Free Radic Res* 2007; 41(3):274–81. doi: 10.1080/10715760601080371.
4. Narkowicz CK, Vial JH, McCartney PW. Hyperbaric oxygen therapy increases free radical levels in the blood of humans. *Free Radic Res Commun* 1993; 19(2):71–80.
5. Oter S, Korkmaz A, Topal T, Ozcan O, Sadir S, Ozler M, et al. Correlation between hyperbaric oxygen exposure pressures and oxidative parameters in rat lung, brain, and erythrocytes. *Clin Biochem* 2005; 38(8):706–11. doi: 10.1016/j.clinbiochem.2005.04.005.
6. Tibbles PM, Edelsberg JS. Hyperbaric-oxygen therapy. *N Engl J Med* 1996; 334(25):1642–8. doi: 10.1056/NEJM199606203342506.
7. Harabin AL, Braisted JC, Flynn ET. Response of antioxidant enzymes to intermittent and continuous hyperbaric oxygen. *J Appl Physiol (1985)* 1990; 69(1):328–35.
8. Benedetti S, Lamorgese A, Piersantelli M, Pagliarani S, Benvenuti F, Canestrari F. Oxidative stress and antioxidant status in patients undergoing prolonged exposure to hyperbaric oxygen. *Clin Biochem* 2004; 37(4):312–7. doi: 10.1016/j.clinbiochem.2003.12.001.
9. Davies KJ, Goldberg AL. Oxygen radicals stimulate intracellular proteolysis and lipid peroxidation by independent mechanisms in erythrocytes. *J Biol Chem* 1987; 262(17):8220–6.
10. Davies MJ. Protein oxidation and peroxidation. *Biochem J* 2016; 473(7):805–25. doi: 10.1042/BJ20151227.
11. Erecinska M, Silver IA. Tissue oxygen tension and brain sensitivity to hypoxia. *Respir Physiol* 2001; 128(3):263–76.
12. Kot J, Sicko Z, Wozniak M. Oxidative stress during oxygen tolerance test. *Int Marit Health* 2003; 54(1–4):117–26.
13. Chavko M, Harabin AL. Regional lipid peroxidation and protein oxidation in rat brain after hyperbaric oxygen exposure. *Free Radic Biol Med* 1996; 20(7):973–8.
14. Demchenko IT, Welty-Wolf KE, Allen BW, Piantadosi CA. Similar but not the same: normobaric and hyperbaric pulmonary oxygen toxicity, the role of nitric oxide. *Am J Physiol Lung Cell Mol Physiol* 2007; 293(1):L229–38. doi: 10.1152/ajplung.00450.2006.
15. Fatouros IG, Destouni A, Margonis K, Jamurtas AZ, Vrettou C, Kouretas D, et al. Cell-free plasma DNA as a novel marker of aseptic inflammation severity related to exercise overtraining. *Clin Chem* 2006; 52(9):1820–4. doi: 10.1373/clinchem.2006.070417.
16. Prior RL, Cao G. In vivo total antioxidant capacity: comparison of different analytical methods. *Free Radic Biol Med* 1999; 27(11–12):1173–81.
17. Dennog C, Radermacher P, Barnett YA, Speit G. Antioxidant status in humans after exposure to hyperbaric oxygen. *Mutat Res* 1999; 428(1–2):83–9.
18. Vollaard NB, Shearman JP, Cooper CE. Exercise-induced oxidative stress:myths, realities and physiological relevance. *Sports Med* 2005; 35(12):1045–62. doi: 10.2165/00007256-200535120-00004
19. McCord JM. The evolution of free radicals and oxidative stress. *Am J Med* 2000; 108(8):652–9.